Dietary flavonoids and their colonic catabolites as cytoprotective antioxidants against oxidative stress

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SAYAKA NAKASHIMA

Graduate School of Environmental and Life Science

(Doctor Course)

OKAYAMA UNIVERSITY, JAPAN

CONTENTS

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ABBREVIATION

AhR, aryl hydrocarbon receptor

ARNT, aryl hydrocarbon receptor nuclear translocator

AU, aureusidin

BNPP, bis(4-nitrophenyl)phosphate

CDNB, 1-chloro-2,4-dinitrobenzene

CuAAC, copper(I)-catalyzed azide alkyne cycloaddition

CYP1A1, cytochrome P450 1A1

DBE, DOPAC butanol ester

DMSO, dimethyl sulfoxide

DOPAC, 3,4-dihydroxyphenylacetic acid

DPE, DOPAC propargyl ester

DPPH, diphenyl-2-picrylhydrazyl

DTNB, 5,5′- dithiobis (2-nitrobenzoic acid)

FOX, ferrous ion oxidation-xylenol orange

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GCLC, glutamylcysteine ligase, catalytic subunit

GSH, glutathione

GST, glutathione *S*-transferase

HO-1, heme oxigenase-1

HPA, hippuric acid

HRP, horseradish peroxidase

NBT, nitroblue tetrazolium

NQO1, NAD(P)H:quinone oxidoreductase 1

OPAC, 3-hydroxyphenylacetic acid

PBS, phosphate buffered saline

PCA, protocatechuic acid

PTSA, *p*-toluensulfonic acid monohydrate

Q3G, 3-O-β-glucoside

Q4′G, 4′-O-β-glucoside

ROS, reactive oxygen species

RT-PCR, reverse transcription-polymerase chain reaction

SOD, Superoxide dismutase

TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine

TLC, thin-layer chromatography

XA, xanthine

xCT, cystine/glutamate antiporter

XOD, XA oxidase

XRE, xenobiotic response element

α-MEM, α-minimum essential medium

Abstract

Flavonoids, widely distributed secondary catabolites in plants, have attracted much attention for their preventive effects against the oxidative stress-related diseases, including cardiovascular disease, cancer and neurodegenerative disease. Flavonoids have potent free radical scavenging activities due to their hydroxyl groups attached to rings. However, recent studies have reported that bioavailability of dietary flavonoids are very low as most flavonoids usually occur in the plant-based foods and drinks as glycoside forms, and thus they are converted into conjugated metabolites or ring fission catabolites before or after entering circulation. The circulating concentration of free flavonoid aglycons is considerably lower than that of other antioxidants such us glutathione, vitamin C and E, and carotenoids, suggesting that free radical scavenging activities of flavonoids *per se* do not completely account for their health benefits. Therefore, it has been considered that the *in vivo* biological actions of flavonoids are mainly mediated by their regulation of signaling pathways via protein modification. Also, the absorption and metabolism of flavonoids should be taken into account to evaluate their effectiveness. In this study, I investigated the cytoprotective effects of the microbiota-derived catabolites of quercetin glycosides as well as aurone on the oxidative stress-induced cytotoxicity and their underlying molecular mechanisms.

In chapter 2, I compared direct and indirect antioxidative activities of major phenolic catabolites including 3,4-dihydroxyphenylacetic acid (DOPAC), 3-hydroxyphenylacetic acid (OPAC), 3,4-dihydroxybenzoic acid (protocatechuic acid, PCA) and hippuric acid (HPA), which are produced from quercetin glycosides by gut microbiota. Both catechol moiety containing compounds, DOPAC and PCA exhibited DPPH radical scavenging and superoxide dismutase-like activities, whereas OPAC and HPA did not. DOPAC also enhanced the gene expressions of several phase 2 drug-metabolizing enzymes more potently than the other phenolic acid catabolites. DOPAC significantly inhibited the hydrogen peroxide-induced cytotoxicity in mouse hepatoma Hepa1c1c7

cells with the enhancement of the total glutathione *S*-transferase activity. These results suggested that DOPAC is partly responsible for the health promoting effects of dietary quercetin.

In chapter 3, target proteins of DOPAC were investigated to elucidate the mechanism where DOPAC induces the phase 2 drag-metabolizing enzyme expression. I synthesized DOPAC propargyl ester (DPE) by fisher esterification of DOPAC and 2-propyn-1-ol as a novel tag-free DOPAC probe which is designed to detect the targeted proteins using the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC), a representative click reaction. Various cellar proteins labeled with DPE were detected by the combination of the azide-labeled biotin and horseradish peroxidase (HRP)-streptavidin. Furthermore, a pull down assay successfully identified Keap1 and aryl hydrocarbon receptor (AhR) as the target proteins. These findings indicated that DOPAC might induce the phase 2 enzyme expression via direct modification of Keap1 and AhR.

In chapter 4, I examined the cytoprotective effects of aureusidin (AU) as a representative aurone. Aurones are a class of flavonoids which confer bright yellow color to ornamental and edible flowers such as snapdragon and cosmos, and have a unique 5-membered C-ring. I first synthesized AU using phloroglucinol as a starting material in 4 steps to give AU in 1.3% overall yield. The pretreatment of AU significantly suppressed the hydrogen peroxide-induced cell death in Hepa1c1c7 cells. RT-PCR experiments revealed that AU enhanced the gene expressions of phase 2 enzymes, heme oxygenase-1 and NAD(P)H: quinone oxidoreductase 1. In addition, AU induced increased gene expression level of CYP1A1, which is regulated by AhR signaling pathway. These results suggested that AU shows cytoprotective effects against oxidative stress by the enhancement of phase 2 enzyme expression, possibly through the activation of AhR signaling pathway as well as Keap1/Nrf2 signaling pathway.

In the present study, I provided new insights into flavonoid research as follows: (i) DOPAC, one of a catabolites of quercetin glycosides produced by colon microbiota might contribute to health

promoting effects of dietary quercetin through direct modification of Keap1 and AhR and consequent induction of phase 2 enzyme expression (chapter 2 and 3) and (ii) AU might protect cells from oxidative stress through phase 2 enzyme induction via not only activation of Keap1/Nrf2 pathway, but also AhR pathway (chapter 4).

CHAPTER 1

General Introduction

1.1 Functional foods

From ancient time, people have empirically recognized that foods provide preventive and therapeutic benefits for their health even without scientific evidence (Weststrate *et al*., 2002). Hippocrates, a Greek physician of the Age of Pericles, who is often called the father of medicine, coined the phrase “let food be thy medicine and medicine be thy food” 2500 years ago (Hasler, 2012) and ancient Chinese had the concept that "medicine and food share a common origin" (Arai, 2002).

In 1984, a Japanese *ad hoc* research group launched a systematic and massive national project to explore the interface between medical and food sciences under the sponsorship of the Ministry of Education, Science and Culture. As a result of the project, three functions of food were proposed as follows: The primary is a nutritional function as a source of energy, which is essential for an individual to survive; the secondary is palatability, which appeal to the sense of taste and smell; the tertiary is the modulating function of the physiological systems such as endocrine system, immune system and nervous system (shimizu, 2003). The research defined “functional food” as a food that has a tertiary function, and in 1993 the term “functional food” first emerged in *Nature* news with the headline ‘Japan explores the boundary between food and medicine’ (Swinbanks *et al*., 1993; Arai, *et al*., 2002).

1.2 Flavonoids

Flavonoids, which are a class of plant and fungus secondary metabolites, are first discovered from citrus rind by the Hungarian biochemist Rusznyák and Szent-Györgyi in the 1930s (Rusznyák *et al.*, 1936). At that time, they were named “vitamin P” because they had abilities to improve capillary

fragility and permeability (P for permeability), but later vitamin P was renamed (bio)flavonoids. From now on, more than 5000 flavonoids have been identified and the number has still been increasing (Beecher, 2003)

In 1990s, flavonoids attracted greater attention from researchers with the observation of the phenomenon known as French Paradox: the very low incidence of and mortality rates from cardio-vascular disease in France despite the fact that saturated fat intakes, serum cholesterol, blood pressure and prevalence of smoking are no lower there than other countries, which attributed to the regular drinking of red wine containing variety of flavonoids. (Renaud *et al.*,1992; Robak *et al*., 1996).

1.2.1 Chemical structure and classification of flavonoids

Flavonoids generally have the C_6-C_3-C_6 basic skeleton, in which two C_6 units (ring A and ring B) are connected each other through three carbon bridge that form ring C. Based on the hydroxylation pattern and variations in the ring C, flavonoids can be classified into different subclasses, such as flavonols, flavones, flavanones, flavononols, flavan-3-ols, anthocyanidines, isoflavones and aurones. Only aurones have five-membered ring C instead of six-membered ring C as most flavonoids have. In most cases, flavonoids except for flavan-3-ols occur in natural as sugar-conjugated form. The glycosidic linkage is normally located at C-3 and less frequently at the C-5, C-7, and C-3´ and C-4´ positions (Herrmann, 1988).

1.2.2 Dietary sources of flavonoids

The major dietary sources of flavonoids are foods and beverages of plant origin such as vegetables, fruits, tea, cocoa, coffee, and wine. Each subclass of flavonoids has unique major dietary sources. For example, flavonols are abundant in onions, broccoli, apples; oranges, lemons, and grapefruits are rich in flavanones; flavan-3-ols are high in tea leaf; soybeans and products are typical source of isoflavons (Banjarnahor *et al*., 2014). As the flavonoids content in foods vary considerably from 10

to 10^4 mg kg^{-1} of fresh weight (Passamonti *et al*., 2009), total intakes of flavonoids are different depending on dietary habits, region, and age. Reportedly, the highest intake of daily flavonoids is in Iran (1650 mg/day), followed by UK (>1000 mg/day). The high consumption is thought to be associated with tea drinking habits (Escobar−Cévoli *et al*., 2017).

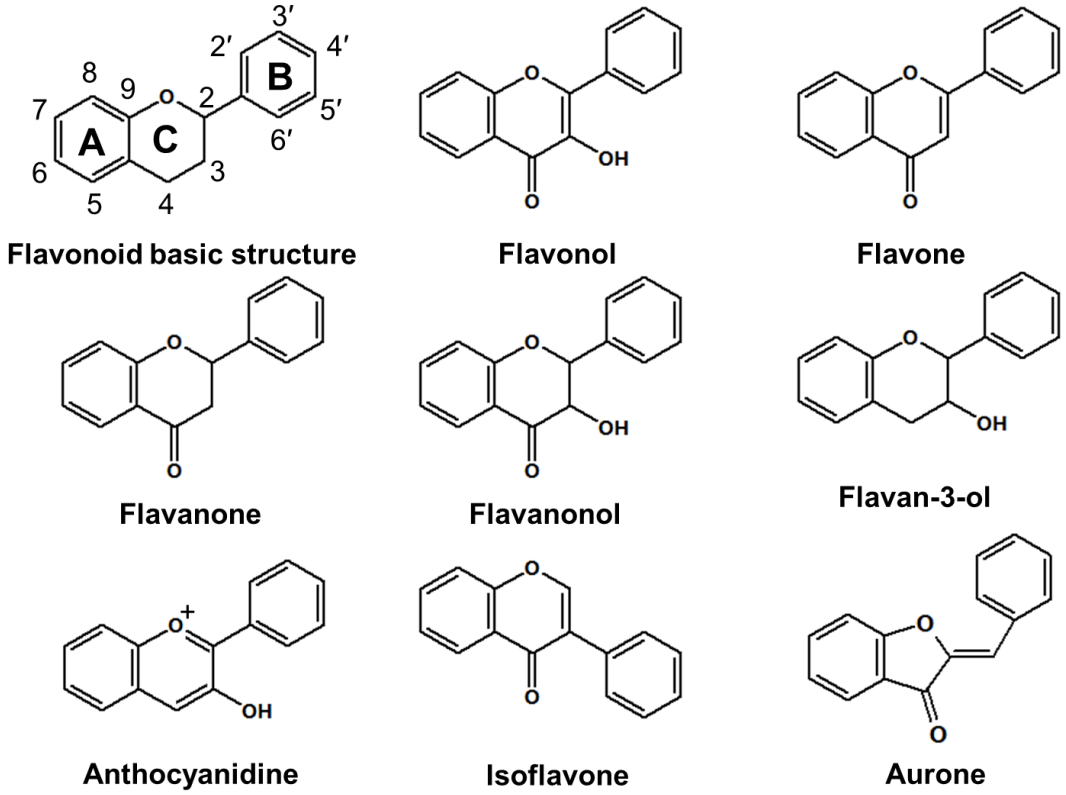

Fig. 1.1. Basic structures of flavonoid subclasses.

1.2.3 Metabolism and absorption of flavonoids

Most flavonoids, except for catechins, present in diet bound to sugar as glycosides, but a growing number of studies reported that the forms detected in circulation is not flavonoid glycosides, but conjugated metabolites or catabolites, because they are subjected to deglycosylation and modification before and/or after absorption. Flavonoid glycosides are considered not to enter cells by passive diffusion due to their high molecular weight and hydrophilicity. Some flavonoid glycosides

are deglycosylated into their aglycones by lactase phlorizin hydrolase (LPH), a β-glucosidase which is located on the outside brush border membrane of the small intestine (Day *et al*., 2000), and then pass through the enterocyte membrane. Sodium glucose cotransporter 1 (SGLT1) has a role in absorption of flavonoid glucosides in the small intestine. Walgren *et al*., demonstrated that quercetin 4′-glucoside is transported by SGLT1 in human Caco-2 cells and the SGLT1-transfected rodent G6D3 cells. In enterocytes, cytosolic β-glucosidase also catalyzes deglycosylation of flavonoid glucosides (Day *et al*., 1998). After absorbed in small intestine, flavonoid aglycons are transformed into conjugated metabolites such as methyl, glucuronide and sulfate derivatives by phase 2 enzymes in the enterocytes and hepatocytes and then finally enter the circulation (Murota *et al*., 2018). It is estimated only about 10% of total intake of flavonoids are absorbed in small intestine and remaining 90% of flavonoid glycosides are reach large intestine, where they are subjected to deglicosylation and ring fission, consequently converted into low molecular weight phenolic compounds by colonic microbiota and then absorbed by passive diffusion (Cardona *et al*., 2013).

1.2.4 Biological activities and health benefits of flavonoids

In recent years, researchers have focused on the beneficial effects of flavonoids on human health. The best-known feature common to every subclass of flavonoids is antioxidative activities. Flavonoids exert antioxidative effects *in vivo* as well as *in vitro* via the following mechanism: (1) direct free radical scavenging activity, (2) activation of cellular antioxidant enzymes, (3) metal chelation, (4) inhibition of enzymes associating with free radical generation (Procházková *et al*., 2011; Banjarnahor *et al*., 2014). In addition to antioxidative property, they have broad spectrum of biological activities, such as anti-inflammatory (Rathee *et al*., 2009; Serafini *et al*., 2010), anti-cancer (So *et al*., 1997; Batra *et al*., 2013), and anti-diabetic effects (Vinayagam *et al*., 2015). An increasing number of epidemiological studies have shown the association between a high intake

of foods rich in flavonoids and lower risk of chronic diseases including cardiovascular disease (Zern *et al*., 2005), asthma (Tanaka *et al*., 2013), and cancer (Batra *et al*., 2013).

1.3 Oxidative stress and disease

Oxidative stress is a state that the generation of reactive oxygen species (ROS) in cells overwhelms antioxidant defense systems (Hallwell, 1994; Betterridge, 2000), which caused thorough abnormally high production of ROS or deficiencies in the antioxidant defenses. Prolonged oxidative stress is associated with development and progression of many diseases including cardiovascular diseases (Dhalla *et al*., 2000; Gracia *et al*., 2017), neurodegenerative disease (Barnham et al., 2004; Niedzielska *et al*., 2014), diabetic (De Cristofaro *et al*., 2003; Giacco *et al*., 2010), allergic disorder (Bowler *et al*., 2002), and cancer (Khansari *et al*., 2009; Sosa *et al*., 2013).

1.3.1 Reactive oxygen species

A free radical can be defined as any chemical species with one or more unpaired electrons in an atomic orbital. The possession of an unpaired electron endows a free radical with common characteristics of being more reactive and unstable than the corresponding non-radical although the actual chemical reactivity varies depending on the type of species (Riley, 1994; Lobo *et al*., 2010). Because of their instability, free radicals attempt to donate an electron to or take electrons away from other molecules to be more stable state. Free radicals that contain oxygen and react with biological molecules such as proteins, lipids, carbohydrates, and nucleic acids are generally called reactive oxygen species (ROS) (Bhattacharya, 2015). The general term ROS often includes not only the radicals $^{\bullet}OH$, $RO2^{\cdot}$, $NO^{\bullet}$ and $O_2^{\bullet -}$, but also the non-radicals HOCl, $^1O_2$, $ONOO^-$, O_3, and H_2O_2 (Aruoma, 1994). Free radicals and ROS are generated from normal essential metabolic processes in the human body or from exogenous sources such as exposure to UV light, ozone, cigarette smoke, air pollutants, and industrial chemicals. Endogenous free radical formation results from both

enzymatic and non-enzymatic reactions. Enzymatic reactions, which are cause of free radicals, include those involved in the respiratory chain, phagocytosis, prostaglandin synthesis, and the cytochrome P-450 system. Free radicals can also occur non-enzymatically as a result of reactions of oxygen with organic compounds as well as those initiated by ionizing reactions (Baguchi *et al*., 1998; Liu *et al*., 1999).

1.4 Cytoprotective system against xenobiotics and oxidative stress

To protect cell from damaging effect of xenobiotics, cells equipped with detoxification mechanism, which is comprised of phase 1, 2 and 3. In the phase 1 reactions, xenobiotics subjected to oxidation (catalyzed by cytochrome P450), hydrolysis or reduction to be converted into more polar metabolites (Oesch *et al*., 2004). Phase 2 reactions are conjugation reaction coupling metabolites with sulfates, glucuronides, glutathiones or amino acids, resulting in the formation of less active and toxic substances (Xu *et al*., 2005). Finally, in phase 3 the conjugated xenobiotics are exported into urine and bile through efflux transporters, such as multidrug resistance-associated protein and P-glycoprotein (Brinkmann *et al*., 2001; Kerb *et al*., 2001).

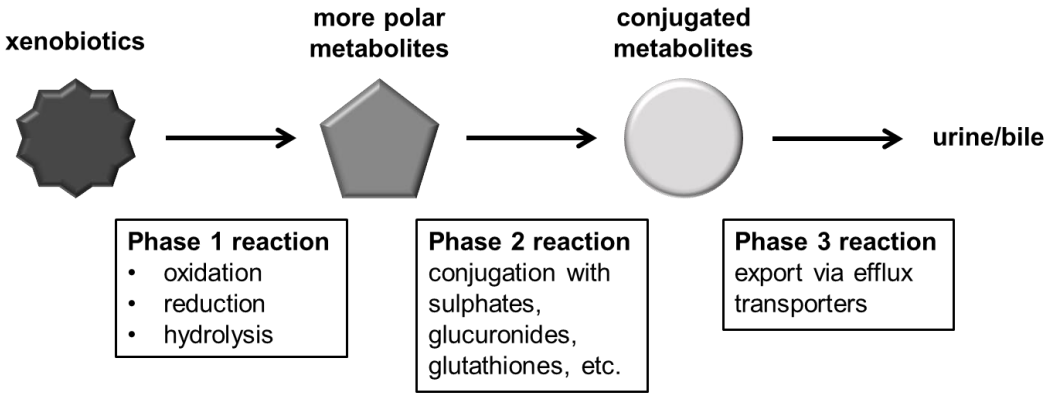

Fig. 1.2. Schematic representation of drug detoxification pathway.

1.4.1 Aryl hydrocarbon receptor signaling pathway

Aryl hydrocarbon receptor (AhR) is the ligand-activated transcription factor that regulates gene expression of drug metabolizing enzyme mainly involved in phase 1 reaction. In its inactivated state, AhR presents in the cytoplasm forming a complex with heat shock protein 90 homodimer, the hepatitis B virus X-associated protein and p23 (Petrulis *et al*., 2002). Once ligand binding, AhR complex translocates into the nucleus. In the nucleus, AhR is dissociated from its chaperon proteins, and then forms a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) (Tsuji *et al*., 2014). This AhR/ARNT heterodimer binds to xenobiotic response element (XRE) and induces expressions of XRE-regulated gene including the cytochrome P450 family member CYP1A1, -1A2 and. -1B1, and uridine diphosphate glycosyltransferase 1 family, polypeptide A1 (UGT1A1) (Ramadoss *et al*., 2005). AhR is activated by various kinds of exogenous and endogenous ligands. The best-known AhR ligands are environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Fernandez-Salguero *et al*., 1996) and polychlorinated biphenyls (PCBs) (Kafafi *et al*., 1993). Some dietary phytochemicals also reported as AhR ligands, for example, indole-3-carbinol found in cruciferous vegetables (Bjeldanes *et al*., 1991) and some flavonoids, although some of them act as an antagonist (Zhang *et al*., 2003).

1.4.2 Keap1-Nrf2 signaling pathway

The Keap1-Nrf2 signaling pathway plays a major role in cytoprotective responses to oxidative stress and electrophiles by regulating phase 2 drug-metabolizing enzyme expressions (Itoh *et al*., 1997; Kansanen *et al*., 2011). Under normal condition, Nrf2 is bound to two molecules of Keap1 in the cytoplasm and poly-ubiquitinylated by the Cul3 system, thereby degraded by the 26S proteasome (Kobayashi *et al*., 2004; Tong *et al*., 2006). Under oxidative and electrophilic stressed condition, Keap1 activity is repressed by covalent modification of cysteine residues, Cys151, 273 and 288 are considered to be critical for the function of Keap1, resulting stabilization of Nrf2 (Zhang *et al*.,

2003). The *de novo* synthesized Nrf2 escape from Keap1 trapping and translocate into the nucleus (Kobayashi *et al*., 2006). Nrf2 then forms heterodimer with small Maf proteins and bind to antioxidant response element (ARE) for drug-metabolizing (Holtzclaw *et al*., 2004; Kobayashi *et al.*, 2005). A series of genes regulated by Keap1-Nrf2-ARE pathway in transcription level are referred to phases 2 drug-metabolizing enzymes, which contains enzymes protect cells against oxidative and electrophile damages. Therefore, activators of Keap1-Nrf2 pathway are regarded as "indirect antioxidants" (Gao *et al*., 2001; Gao *et al*., 2004). Representative antioxidative phase 2 enzymes are as described in table 1.1.

1.4.3 Interaction between Keap1/Nrf2 pathway and AhR pathway

There is a growing number of evidence for cross-talk between Keap1/Nrf2 pathway and AhR pathway. Miao *et al*. (2005) found that TCDD, a typical ligand of AhR, induces Nrf2 expression, demonstrating that the Nrf2 promoter region contains XRE elements. NQO1 gene contains both ARE and XRE in its promoter region and its induction requires both Nrf2 and AhR (Yeager *et al*., 2009). On the other hand, glutathione S-transferase Mu, Pi, and Theta genes do not contain XRE but ARE, but these genes induced by TCDD in an Nrf2 dependent manner (Yeager *et al*., 2009). Shin *et al*. (2007) found that Nrf2 directly binds ARE in the proximal promoter of AhR and activates AhR gene expression. The possibility of direct interaction of AhR and Nrf2 is proposed, but evidence to prove that has not been presented to date.

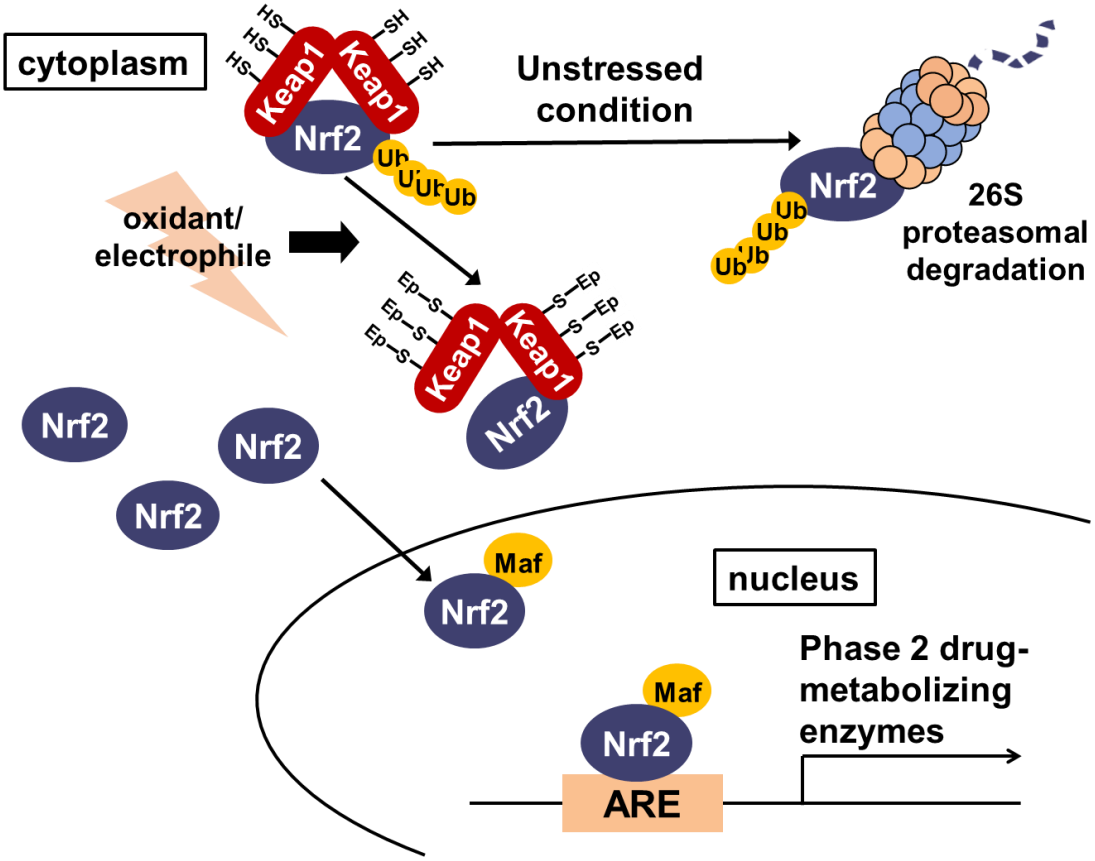

Fig. 1.3. Schematic representation of Keap1/Nrf2 signaling pathway.

Enzymes	Functions	
heme oxigenase-1 (HO-1)	heme catabolism and production of low molecular antioxidants (bilirubin and CO)	Maines, 1988
cystine/glutamate antiporter (xCT)	Transport of cystine into cells in exchange for glutamate	Shih *et al*., 2006.
glutamylcysteine ligase (GCL)	ligation of glutamate and cysteine to form γ-glutamyl-cysteine	Chen *et al*., 2005
glutathione peroxidase (GPx)	reduction of hydrogen peroxide	Cohen *et al*., 1963
glutathione reductase (GR)	reduction of GSSG to GSH	Carlberg *et al*., 1985
glutathione *S*-transferase (GST)	conjugation of electrophiles and reactive metabolites with GSH	Hayes *et al*., 2005
NAD(P)H:quinone oxidoreductase 1 (NQO1)	two-electron reduction of quinones to corresponding hydroquinones	Dinkova-Kostova *et al.,* 2010
superoxide dismutase (SOD)	dismutation of superoxide into hydrogen peroxide and oxygen	Fridovich, 1997

Table 1.1. Phase 2 antioxidative enzymes and their functions.

1.5 Click chemistry

Since Dr. Barry Sharpless' group first introduced the concept of "Click chemistry" (Kolb *et al.*, 2001), the reactions of this category have been used in a variety of fields such as biosciences (Best, 2009), drug discovery (Zeng *et al.*, 2013) and material science (Evans, 2007). In general, the characteristics of click reactions are described as follows:

- giving rapidly desired products with high selectivity, specificity, and yield
- coupling two molecular building blocks
- generating only inoffensive byproducts that can be removed by nonchromatographic methods
- proceeding with ease under mild condition

1.5.1 The Copper (I)-catalyzed azide alkyne cycloaddition

the Copper (I)-catalyzed azide alkyne cycloaddition (CuAAC) is the most popular click reaction, which is the formation of 1,4-disubstituted 1,2,3-triazoles. This reaction is based on Huisgen 1,3-dipolar cycloaddition, but combination with Cu catalysis increases the reaction rate up to 10^7 times. This cycloaddition fulfills all requirements as click reaction. Moreover, azides and terminal alkynes are easily installed and highly stable (Hein *et al.*, 2008).

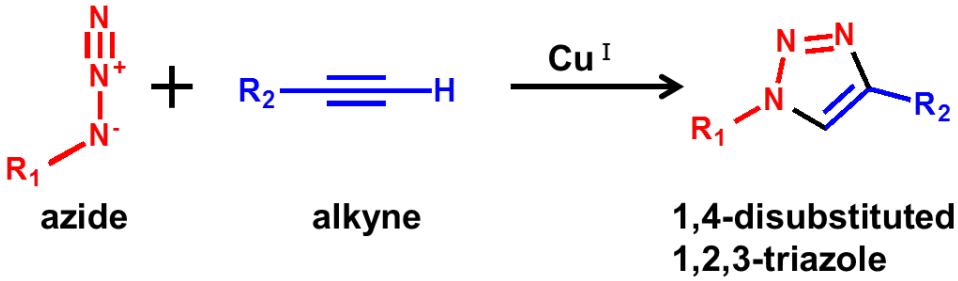

Fig. 1.4. The Copper (I) -catalyzed azide alkyne cycloaddition.

1.6 Study outline

In this study, I evaluated cytoprotective effects of gut microbiota catabolites of quercetin glycosides and aureusidin (AU) on oxidative stress and investigated the underlying mechanism. Antioxidative properties of the substances are assessed mainly by determination of effect on hydrogen peroxide-induced cell death and phase 2 drug-metabolizing enzyme gene expression-inducing activities in Hepa1c1c7 cells.

I found that DOPAC has the most potent direct and indirect antioxidative abilities in the major colonic catabolites of quercetin glycosides. Investigation of the target proteins of provides using click chemistry probe detected Keap1 and AhR as the primary targets. These results suggest that DOPAC enhances cellular antioxidative activities through activation of phase 2 enzyme expression by direct modification of Keap1 and AhR.

AU was synthesized in four steps from phloroglucinol and the overall yield was 1.3%. AU showed significant cytoprotective effect against hydrogen peroxide-induced cytotoxicity and enhancement of gene expressions of phase 1 and 2 enzymes. These results indicated that AU acts as cytoprotective antioxidants via induction of phase 2 enzymes through not only Keap1-Nrf2 pathways but also AhR pathways.

In conclusion, the present study revealed cellular antioxidant mechanism of dietary flavonoids and colorectal catabolites, providing further scientific evidence supporting health-promoting effects of flavonoid intake.

CHAPTER 2

3,4-Dihydroxyphenylacetic acid, a catabolite of dietary quercetin glycosides produced by gut microbiota have cytoprotective effects against oxidative stress

2.1 Introduction

Quercetin is one of the most well studied flavonoids and widely distributed in plants. We consume quercetin from various plant-based foods and beverages including vegetables, fruits and tea on a daily basis. The average daily intake of quercetin is estimated 10-50 mg, which varies depending on the food habits and the region (Formica *et al*., 1995; Scalbert *et al*., 2000). Nishimuro *et al*. reported that the average daily quercetin consumption of Japanese living in Hokkaido was 16.2 mg and the major sources were onions and green tea (Nishimuro *et al*., 2015). On the other hands, for U.S. adults, the intake of quercetin in approximately 10 mg/day (Sampson *et al*., 2002).

In recent years, quercetin has attracted much attention for the beneficial effects on our health. The health promoting effects of quercetin was reported not only by *in vitro* studies, but also by epidemiological studies. A prospective cohort study on associations between flavonoids intake and a risk of cardiovascular disease showed that increased consumption of flavonols including quercetin and kaempferol reduced cardiovascular disease mortality (Peterson *et al*., 2012). Another cohort study reported that the person with higher quercetin intakes had lower risk of mortality from ischemic heart disease and incidence of asthma and type 2 diabetes (Kenkt *et al*., 2002).

Quercetin is generally present in our diet as a glycoside form, not an aglycone form. More than 170 different quercetin glycosides have been identified, the major glycoside forms are 3-*O*-β-glucoside (Q3G, isoquercetrin) occurring in a variety of fruits and vegetables and 4′-*O*-β-glucoside (Q4′G) accounting for considerable portion of total amount of flavonoids in onion (Tsushida *et al*., 1995; Yang *et al*., 2001). It is widely accepted that intact quercetin glycosides are hardly absorbed into the

body due to their high hydrophilicity. Only 5-10% of ingested quercetin glycosides are absorbed in the small colon through sodium glucose cotransporter 1 (SGLT1) or undergo passive absorption after deglycosylated by lactase phlorizin hydrolase (LPH) which located in the brush border of the small intestine (Day *et al*., 1998; Walgren *et al*.,2000). 90-95% of quercetin glycosides pass through the small intestine as intact forms or conjugated forms, and reach at the large intestine where an enormous number of microorganisms inhabit and form a complex community known as the intestinal microbiome (Duda-Chodak *et al*., 2015). Food compounds including flavonoids are subjected to various enzymatic conjugations of the gut microbiota, which is believed to have effect on availability of them. Quercetin glycosides also can be converted enzymatically into several phenolic acids. Mullen *et al*., investigated the fate of ingested Q4′G using rats and identified DOPAC and OPAC as major catabolites and protocatechuic acid PCA as a minor catabolite formed in the colon, and detected OPAC and HPA in faces and urin (*Mullen et al*., 2008). Moreover, studies reported that *Eubacterium ramulus*, *Bacteroides fragilis* and *Clostridium perfringen*s which are human colorectal bacteria have the capability to degrade quercetin to DOPAC (Schneider *et al*., 1999; Peng *et al*., 2014)

As mentioned above, most of ingested quercetin glycosides are not absorbed into the circulation as intact forms and converted into several phenolic acids by microbiota in the large intestine which can enter into enterocytes. Therefore, phenolic catabolites have the potential to account for the health promoting effects of dietary quercetin. In the present study, I compared the effects of the major phenolic acid catabolites of dietary quercetin glycosides, including DOPAC, OPAC, PCA and HPA on DPPH radical, superoxide and detoxification enzyme level *in vitro*. DOPAC showed most potent direct and indirect antioxidative properties, suggesting that DOPAC is a predominant antioxidative catabolite of quercetin glycosides formed by the colonic microbiota in the large colon.

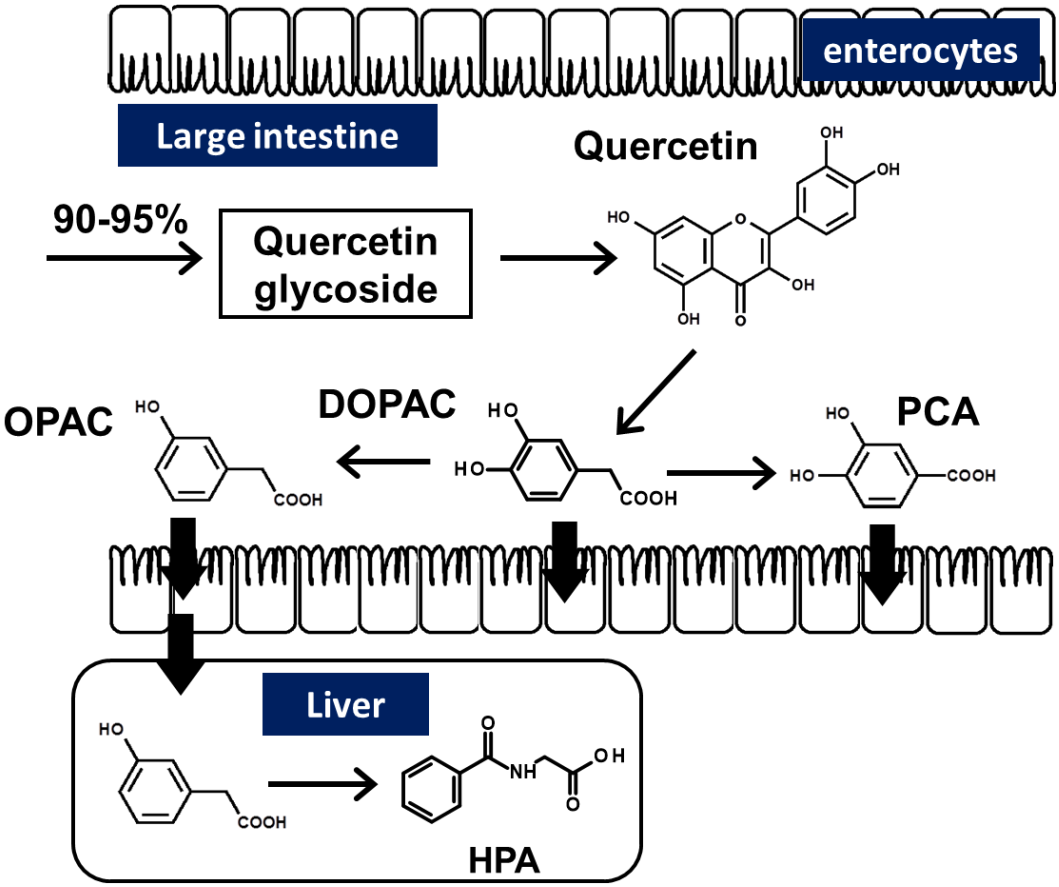

Fig. 2.1. Schematic representation of quercetin glycoside metabolism and absorption in the large intestine.

2.2 Materials and methods

2.2.1 Materials

Quercetin, DOPAC, OPAC, PCA and HPA were purchased from Sigma (St. Louis, MO, USA). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Tokyo Chemical Industry (Tokyo, Japan). α-Minimum essential medium (α-MEM), and Trizol reagent were purchased from Life Technologies (Carlsbad, CA, USA) Fatal bovine serum was purchased from Nichirei corporation (Tokyo, Japan). SOD assay kit was purchased from DOJINDO LABORATORIES (Kumamoto, Japan) Hydrogen peroxide was purchased from nacalai tesque (Kyoto, Japan). All other chemicals were obtained from Wako Pure Chemicals Industries (Osaka, Japan).

2.2.2 Cell culture and treatment

The mouse hepatoma cell line Hepa1c1c7, obtained from the American Type Culture Collection, was grown and maintained at 37 ℃ in an atmosphere of 95% air and 5% CO_2 in α-MEM 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 dimethyl sulfoxide (DMSO) vehicle (final 0.1%, v/v). RL34 cells were obtained from the Health Science Research Resources Bank, Osaka, Japan (Yamada *et al.*, 1987). The cells were grown and maintained at 37 ℃ in an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium 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 (final 0.1%, v/v).

2.2.3 DPPH radical scavenging assay

A test compound (ethanol solution, 2 ml) mixed with a 100mM Tris-HCl buffer (pH 7.4, 2 ml) was

added to 0.5 mM DPPH in ethanol (1ml), and the mixture was shaken vigorously and kept for 20 min at room temperature in the dark. The DPPH radical scavenging activity is expressed as the ratio of the relative decrease in the absorbance of the test sample mixture at 517 nm to that of the 1 mM Trolox solution. The experiment was done in triplicate. DPPH radical scavenging activity (%)={(ethanol alone) − (test compound)} / {(ethanol alone) − (Trolox)} × 100.

2.2.4 Superoxide scavenging assay in the xanthine/xanthine oxidase system

SOD-like activity was measured by a xanthine (XA)/XA oxidase (XOD) system using a SOD Test Wako kit accordingly to the manufacturer's instruction. In this system, SOD-like superoxide scavenging activity was estimated by measuring the nitroblue tetrazolium (NBT) reduction.

2.2.5 Determination of hydrogen peroxide formation

Hydrogen peroxide amount was determined by the ferrous ion oxidation-xylenol orange (FOX) method. The FOX reagent-A contained 25 mM H_2SO_4, 100 mM sorbitol, 250 μM $Fe(NH_4)_2(SO_4)_2$, and 125 μM xylenol orange. The FOX-B contained 25 mM H_2SO_4, 100 mM sorbitol, and 250 μM $Fe(NH_4)_2(SO_4)_2$. This assay method was applicable to determine hydrogen peroxide concentrations as low as 0.2 μM. Briefly, the collected sample (45 μl) was mixed with 5 μl of methanol and incubated at room temperature for 30 min. The FOX-A reagent (0.45 ml) or FOX-B reagent for reference was added and incubated for 30min. The solutions were then centrifuged at 15,000g for 10min at room temperature and the absorbance at 560 nm was measured. The concentration of hydrogen peroxide was calculated from standard curve.

2.2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were washed with ice-cold phosphate buffered saline (PBS) (−). Total cellular RNA was

isolated using Trizol reagent according to the manufacturer's recommendations. RNA was quantified by measuring absorbance at 260 nm. Total RNA (5 μg) was reverse transcribed with Oligo dT to cDNA using ReverTra Ace (TOYOBO, Osaka, Japan). PCR amplification was then performed with BIOTAQ DNA Polymerase (BIOLINE, Toronto, Canada) and specific primers. Primers used in PCR amplification are as follows: mNQO1, (F) 5′-TCgAAgAACTTTCAgTATCC-3′ and (R) 5′-TgAAgAgAgTACATggAgCC-3′ (20 cycles, product size 290 bp); mGCLC, (F) 5′-ggCgATgTTCTTgAgACTCTgC-3′ and (R) 5′-TTCCTTCgA TCATgTAACTCCCATA-3′ (26 cycles, product size 99 bp); mxCT, (F) 5′- CCTggCATTTggACgCTACAT-3′ and (R) 5′-TgAgAATTgCTgTgAgCTTgCA- 3′ (25 cycles, product size 182 bp); mHO-1, (F) 5′-gTgATggAgCg TCCACAgC-3′ and (R) 5′-TggTggCCTCCTTCAAgg-3′ (26 cycles, product size 66 bp); mCYP1A1, (F) 5′-CCTCTTTggAgCTgggTTTg-3′ and (R) 5′-TgCTgTgggggATggTgAAg-3′ (24 cycles, product size 229 bp). The PCR products were separated on an 3% agarose gel, stained with ethidium bromide, and visualized under UV light using Luminescent Image Analyzer LAS-3000 (FUJIFILM Corporation, Tokyo, Japan). The relative density of the bands was analyzes using the Image J Software.

2.2.7 Glutathione *S*-transferase (GST) activity assay

After treatment, the cells were lysed with RIPA buffer (10 mM Tris- HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 2.41 μM deoxycholic acid sodium salt, 2mM phenylmethyl sulfonyl fluoride). Total GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate according to the method of Habig and Jakoby (1981).

2.2.8 Glutathione titration

Glutathione (GSH) contents were determined using 5,5′- dithiobis (2-nitrobenzoic acid) (DTNB) and glutathione reductase according to the method of Baker *et al*. (1990).

2.2.9 Cell viability determination with MTT assay

Hepa1c1c7 cells were suspended at a density of 5×10^4 cells per well in a 96-well plate. After overnight preculture, the cells were incubated with DOAPC (50 or 100 μM), quercetin (10 or 50 μM), or hydrogen peroxide (20 or 40 μM) for 30 min or 24 h, followed by additional incubation with hydrogen peroxide (100 μM) for 6 h. After culturing with hydrogen peroxide, 10 μl of an MTT solution was added to each well, and the absorbance was measured with an microplate reader (Benchmarkplus, Bio-Rad laboratories, Hercules, CA, USA) at 570 nm according to the manufacturer's instructions after incubation at 37 °C for 2–3 h in a humidified CO_2 incubator. The obtained values were compared with each of the controls incubated with vehicle only.

2.2.10 Statistical analysis

All values were expressed as means ± SD. Statistical significance was analyzed by Student's paired two-tailed *t*-test or a one-way ANOVA followed by Tukey's HSD using R software. A *p*-value of 0.05 was regarded to be statistically significant

2.3 Results

2.3.1 DOPAC has radical scavenging and prooxidative activities.

I compared radical scavenging activities of quercetin, DOPAC, OPAC, PCA and HPA using a DPPH method. As shown in Fig. 2.3A, catechol-type phenolic acids including DOPAC and PCA showed a potent radical scavenging activity, whereas OPAC and HPA did not show any scavenging activity. The activity of DOPAC is equivalent to that of quercetin and significantly higher than that of PCA. Since the superoxide anion radical is one of the precursors of ROS and their metabolites, the SOD-like activity of the phenolic acid metabolites in an XA/XOD system was next examined. As shown in Fig. 2.3B DOPAC and PCA showed a significant inhibitory effect on the superoxide-dependent NBT reduction. The superoxide-scavenging activity of DOPAC was significantly higher than that of PCA, but lower than that of quercetin. Although a representative catechin from green tea, (−)-epigallocatechin-3-gallate, is an effective antioxidant if free radicals exist nearby, it can undergo autoxidation and behave as a pro-oxidant (Tang *et al*., 2014; Wu *et al*., 2009). The ROS generating ability of phenolic compounds has also been demonstrated to play important roles in some biological or chemical systems, oxidative DNA damage and activation of transcriptional factors (Galati *et al*., 2003). Hence, I examined whether hydrogen peroxide is produced during the autoxidation of the quercetin metabolites using a FOX assay. As shown in Fig. 2.3C, DOPAC as well as quercetin produced a significant amount of hydrogen peroxide, whereas the other metabolites did not. These results suggested that DOPAC possesses a redox-active character in a manner similar to quercetin.

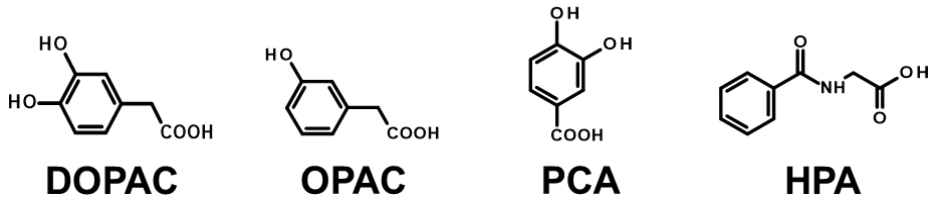

Fig. 2.2. Chemical structures of phenolic catabolites of quercetin glycosides.

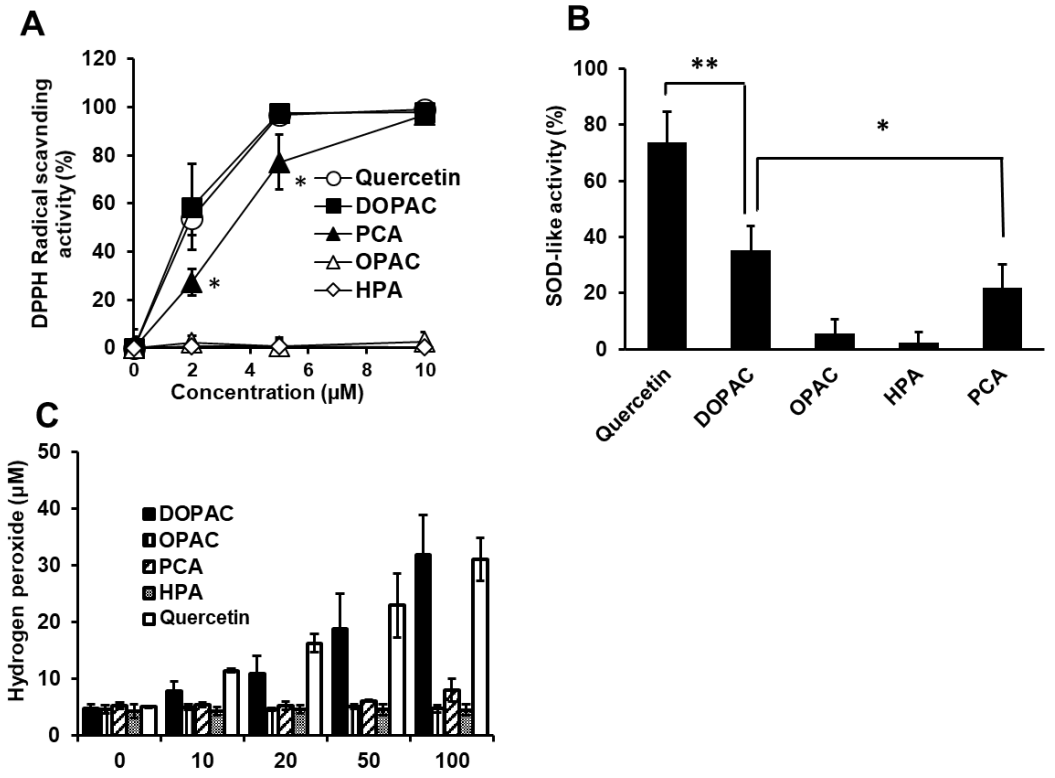

Fig. 2.3. Radical scavenging and prooxidative potentials of the phenolic acid catabolites. (A) Free radical scavenging activity against DPPH radical of quercetin (open circle), DOPAC (closed square), PCA (closed triangle), OPAC (open triangle) and HPA (open diamond). A test compound was incubated with 0.5 mM DPPH for 20 min at room temperature in the dark. The DPPH radical scavenging activity was evaluated by the absorbance at 517 nm. All values were expressed as means ± SD of three separate experiments (*p < 0.05 compared with quercetin or DOPAC). (B) SOD-like activity of the phenolic acid catabolites. The SOD-like superoxide scavenging activity of the text compounds (50 μM) was estimated by measuring the NBT reduction in an XA/XOD system. All values were expressed as means ± SD of three separate experiments (*p < 0.05, **p < 0.01 compared between the indicated groups). (C) Prooxidant activity of the phenolic acid catabolites. The hydrogen peroxide concentration was determined using a FOX assay. All values were expressed as means ± SD of three separate experiments.

2.3.2 DOPAC induces phase 2 drug-metabolizing enzymes in Hepa1c1c7 cells.

I next investigated whether the phenolic acid metabolites of quercetin glycosides have the ability to induce the gene expression of the drug-metabolizing enzymes, including NQO1, glutamate-cysteine ligase, catalytic subunit (GCLC), xCT, HO-1 and cytochrome P450 1A1 (CYP1A1). The incubation of Hepa1c1c7 cells with 50 μM DOPAC for 24 h led to a significant increase in the mRNA levels of GCLC, NQO1 and HO-1 (Fig. 2.4), whereas the other metabolites at the same concentration showed no significant effect. Although quercetin at 50 μM significantly induced the expression of CYP1A1 as well as all phase 2 enzymes, DOPAC at this concentration showed no effect on this phase 1 enzyme expression. We next checked the dose-dependent effect of DOPAC on the expression of the drug-metabolizing enzymes. As shown in Fig. 2.5, the expressions of NQO1, GCLC and HO-1 were dose-dependently increased by the treatment of DOPAC at concentrations lower than 50 μM. However, the mRNA levels of xCT and CYP1A1 were increased only at concentrations higher than 100 μM. These results suggested that DOPAC induces the expression of the drug-metabolizing enzymes through different pathways between its lower and higher concentrations.

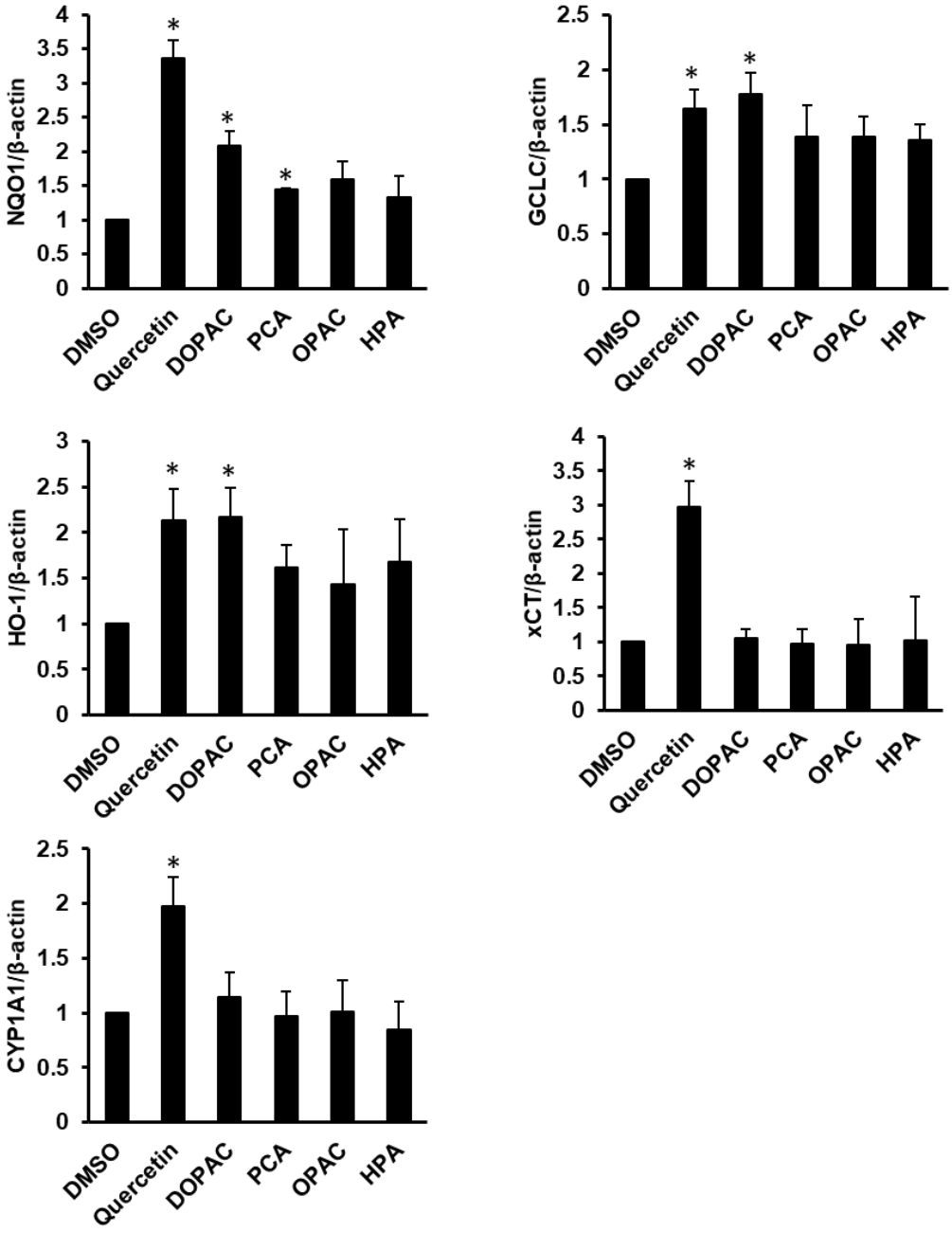

Fig. 2.4. Modulating effects of the phenolic acid catabolites on the gene expression of the drug metabolizing enzymes. The total RNA was extracted from Hepa1c1c7 cells treated with quercetin or the phenolic acids at 50 μM for 24 h, then a RT-PCR analysis for NQO1, GCLC, HO-1, xCT and CYP1A1 was carried out. All values were expressed as means ± SD of three separate experiments (*p < 0.05 compared with control).

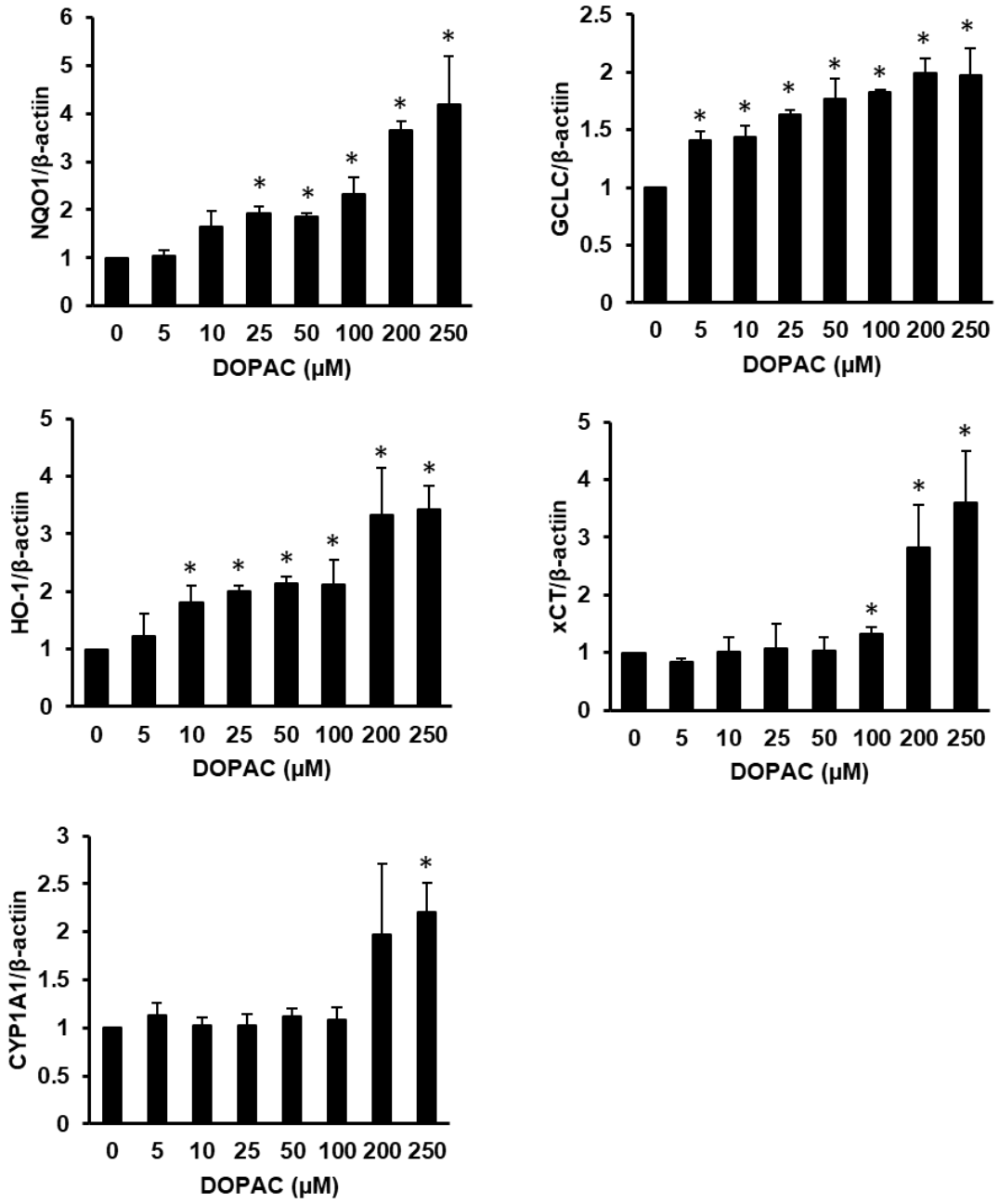

Fig. 2.5. Concentration-dependent effects of the DOPAC on the gene expression of the drug metabolizing enzymes. The total RNA was extracted from Hepa1c1c7 cells treated with DOPAC at the indicated concentrations for 24 h, then a RT-PCR analysis for NQO1, GCLC, HO-1, xCT and CYP1A1 was carried out. All values were expressed as means ± SD of three separate experiments (*p < 0.05 compared with DOPAC 0 μM).

2.3.3 DOPAC increases glutathione *S*-transferase activity in RL34 cells and cellular glutathione content in Hepa1c1c7 cells.

To gain further evidence for the induction of the drug-metabolizing enzymes by DOPAC, the effects of DOPAC on the activity of GST, one of the representative phase 2 drug-metabolizing enzymes, and the intracellular total GSH level were determined. As shown in Fig. 2.6A, the treatment of normal hepatocyte RL34 cells with 100 μM DOPAC for 24 h resulted in an enhancement of the total GST activity by approximately 1.4-fold. Quercetin showed equivalent potency only at concentration of 10 μM, but this concentration induced the strongest GST activity than higher concentrations (Fig. 2.6 C). The treatment of Hepa1c1c7 cells with higher than 200 μM of DOPAC for 24 h exhibited a significant alteration in the intracellular GSH level (Fig. 2.6B) These results indicated that the key step for the DOPAC-stimulated GSH up-regulation might be the substrate transport by xCT, but not the GSH biosynthesis by GCLC.

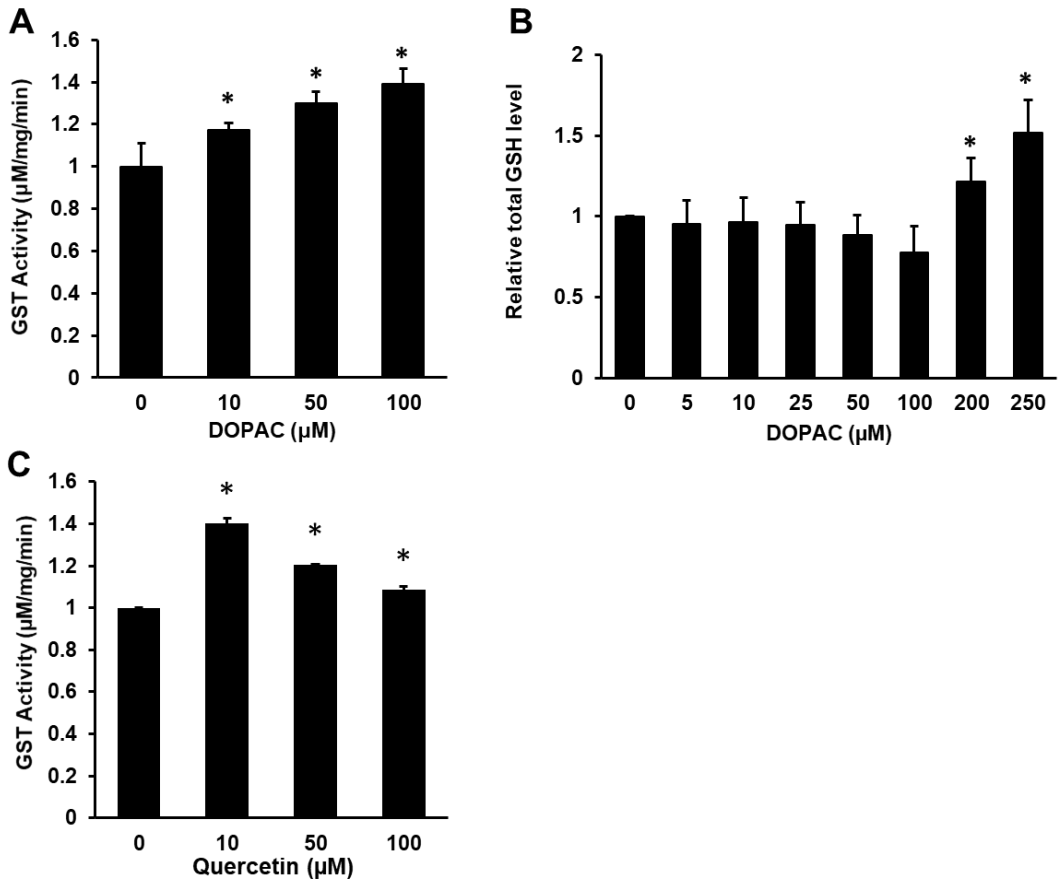

Fig. 2.6. Modulating effects of the DOPAC and quercetin on the total GST activity and the intracellular GSH level. RL34 cells were treated with DOPAC (A) or quercetin (C) for 24 h and the total GST activity was measured by a CDNB assay. (B) Hepa1c1c7 cells were treated with DOPAC for 24 h and the total GSH level was measured by a DTNB assay. All values were expressed as means ± SD of three separate experiments (*p < 0.05 compared with DOPAC 0 μM).

2.3.4 DOPAC protects Hepa1c1c7 cells against hydrogen peroxide induced oxidative stress.

A number of cellular defenses composed of non-enzymatic and enzymatic components are involved in the protection against excessive ROS. As already mentioned, DOPAC showed a significant scavenging effect against the stable free radical DPPH (Fig. 2.3A) as well as an inducing effect on some antioxidant enzymes such as HO-1 and GST (Figs. 2.5 and 2.6A). These results prompted me to examine whether DOPAC inhibits the ROS-induced oxidative stress through its direct antioxidant action or indirect antioxidant action regulated by the transcriptional level. The protective effect of DOPAC against the hydrogen peroxide-induced cytotoxicity was evaluated by an MTT assay. Although the incubation of DOPAC even at 100 μM 30 min before the hydrogen peroxide treatment showed no effect on the cytotoxicity caused by oxidative stress (Fig. 2.7A), pretreatment with DOPAC for 24 h inhibited it in a concentration-dependent manner (Fig. 2.7B). Thus, the treatment with DOPAC at the dose required for the induction of the phase 2 drug-metabolizing enzymes, but not for the GSH up-regulation, actually exhibited an antioxidant effect in Hepa1c1c7 cells. For the purpose of comparison, I evaluated the effect of quercetin on hydrogen peroxide induced toxicity. Since 10 μM of quercetin had almost the same GST activity inducing ability as 50 μM of quercetin (Fig 2.3C), 10 and 50 μM of quercetin was pretreated. As with DOPAC, every concentration of 30 min pretreatment had no effect on the cell viability (Fig. 2.7C), but 24 h pretreatment of 10 μM of quercetin recovered the cell viability completely (Fig. 2.7D). 50 μM of quercetin itself possessed toxicity to cells, thus it did not abolished the cytotoxicity of hydrogen peroxide (Fig. 2.7D). 10 μM of As shown in Fig 2.2C, DOPAC produces ROS by autooxidation reaction. Since transcription of phase 2 enzymes are induced by ROS accumulation, I checked whether ROS production is involved in antioxidative cytoprotection of DOPAC. The pretreatment of hydrogen peroxide (20 and 40 μM) showed no effect on the hydrogen peroxide induced cytotoxicity (Figs. 2.7C and 2.7D), suggesting that hydrogen peroxide from the autooxidation of DOPAC is not account for cytoprotection.

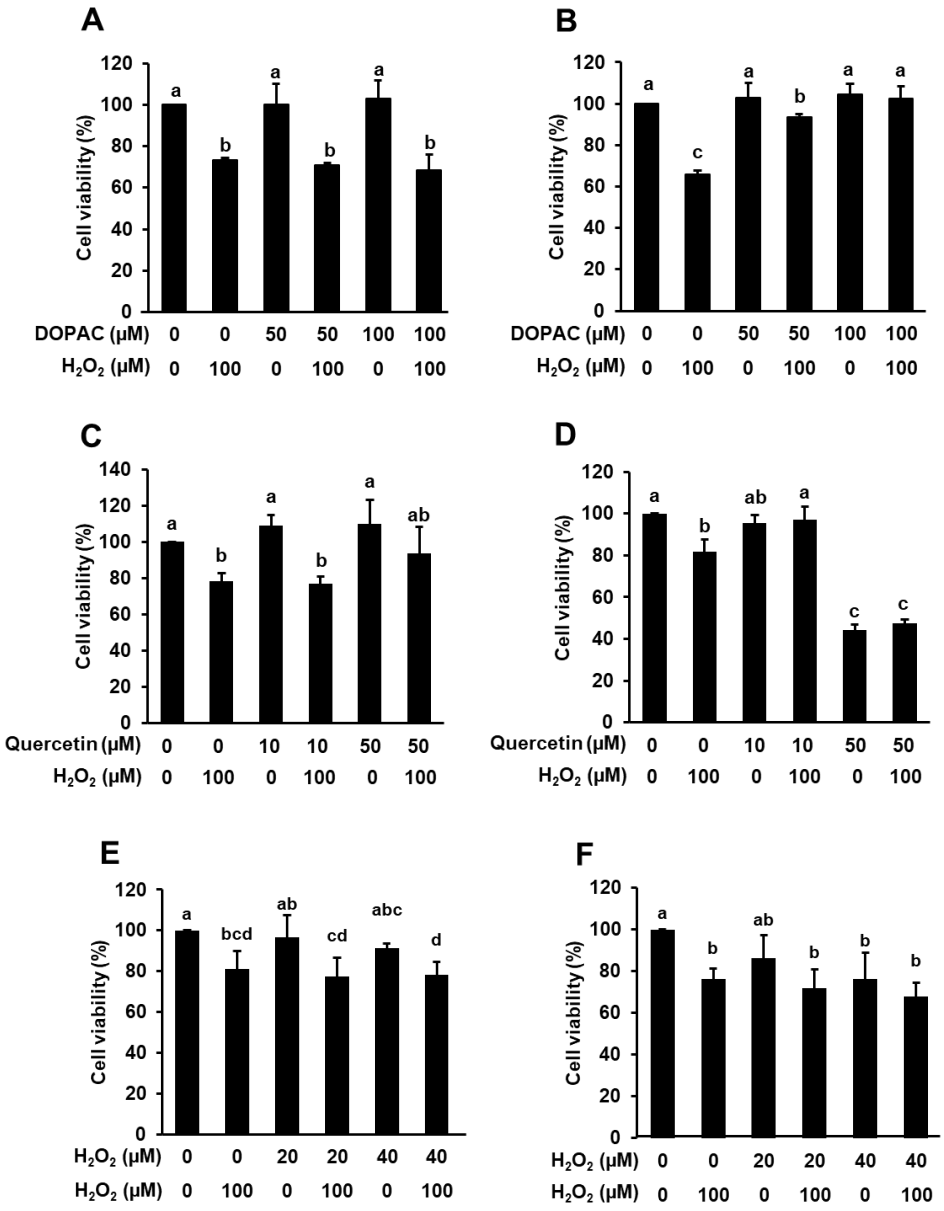

Fig. 2.7. Effect of DOPAC on the hydrogen peroxide-induced cytotoxicity in Hepa1c1c7 cells. Hepa1c1c7 cells were pretreated with DOPAC (50 or 100 μM), quercetin (10 or 50 μM), or hydrogen peroxide (20 or 40 μM) for 30 min (A) , (C) and (E) or 24 h (B), (D) and (F), then treated with hydrogen peroxide (100 μM) for 6 h. Cell viability was measured using an MTT assay. All values were expressed as means ± SD of three separate experiments. Different letters above the bars indicate significant differences among the treatments for each condition *(p* < 0.05).

2.4 Discussion

In the present study, I identified DOPAC as the most biologically active phenolic acid derived from the catabolism of quercetin glycosides in terms of antioxidative potentials. DOPAC has been regarded as one of the major catabolites from quercetin glycosides, such as Q4′G (Mullen *et al*., 2008), rutin (quercetin 3-*O*-rutinoside) (Aura *et al*., 2002), and hyperoside (quercetin 3-*O*-galactoside) (Yang *et al*., 2013). An *in vitro* catabolic study of quercetin using human fecal bacteria also demonstrated the formation of DOPAC (Peng *et al*., 2014), supporting the idea that DOPAC is a predominant catabolite from quercetin glycosides not only in rodents, but also in humans. An increased urinary excretion of DOPAC as well as other phenolic acid catabolites has been observed in human subjects after the consumption of polyphenols from chocolate (Rios *et al*., 2003). In addition to its radical scavenging activity, DOPAC inhibits the secretion of the pro-inflammatory cytokines in lipopolysaccharide-stimulated peripheral blood mononuclear cells (Monagas *et al*., 2009) and the expression of P-selectin in resting platelets (Rechner *et al*., 2005). Although DOPAC shows relatively weaker biological activities than those of the quercetin aglycone, DOPAC has some advantages for application as a food chemical because of its lower cytotoxicity. This idea is supported by the fact that DOPAC is also known as a metabolite of a neurotransmitter dopamine, suggesting the existence of a metabolic pathway of DOPAC in humans (Gesi *et al*., 2001). DOPAC and PCA showed a significant radical scavenging activity against not only the DPPH radical, but also the superoxide anion radical, whereas OPAC and HPA did not show any scavenging activity (Figs. 2. 3A and B). The o-diphenol moiety, but not the monophenol, might be essential for the radical scavenging activity, which is consistent with previous reports (Nakamura *et al*., 1998; Tsuda *et al*., 1996). For example, the number of hydroxyl groups on the phenolic B-ring of anthocyanins plays an important role in the superoxide-scavenging activity (Tsuda *et al*., 1996). On the other hand, the radical scavenging activities of DOPAC were significantly higher than those of PCA (Figs. 2.3A

and B). Previous studies regarding the antioxidative mechanism for BHT (2,6-di-tert-butyl-4-methylphenol) have indicated that an aryl proton at the 4-methyl group of BHT is easily removed by free radicals (Guyton *et al*., 1991). It was also reported that dihydroferulic acid and dihydrosinapic acid with a saturated sidechain showed a more potent radical scavenging activity than ferulic acid and sinapic acid with an unsaturated side chain, respectively (Shimoji *et al*., 2002). These studies would support the idea that the methylene group bound to the benzene ring of DOPAC plays an enhancing role not only in the radical scavenging effects, but also in the autoxidation-dependent hydrogen peroxide production (Fig. 2.3C). There is substantial evidence that the phase 2 drug-metabolizing enzymes including GST, NQO1, HO-1, and glutamate-cysteine ligase, play important roles in the detoxification of prooxidative or electrophilic toxicants, and their induction contributes protection against chronic diseases (Nakamura *et al*., 2010). Among the tested phenolic acids, only DOAPC at the concentration of 50 μM significantly enhanced the mRNA levels of GCLC, NQO1 and HO-1 (Figs. 2.4 and 2.5). These 3 genes were transcriptionally up-regulated by the lower concentrations of DOPAC, whereas the inducible expression of a phase 1 drug-metabolizing enzyme gene, CYP1A1, as well as xCT required higher concentrations (Fig. 2.5). Lower concentrations of DOPAC also enhanced the total GST activity in normal rat hepatocytic RL34 cells (Fig. 2.6A),whereas only higher concentrations of DOPAC increased the intracellular level of GSH (Fig. 2.6B). The former phenomenon is consistent with a previous study showing that DOPAC significantly up-regulated the gene expression of a GST isozyme, GSTP1, in RL34 cells (Ishii *et al*., 2009). However, the concentration dependency of DOPAC for the intracellular GSH level was correlated with that for the gene expression of xCT, but not GCLC, suggesting that the up-regulation of the GSH level might be regulated by the substrate uptake and not by the enhanced biosynthesis. Quercetin has been reported to activate both the Nrf2-dependent pathway (Surh *et al*., 2008) and the aryl hydrocarbon receptor (AhR)-dependent pathway (Ciolino *et al*., 1999). Nrf2 binds

to antioxidant response element (ARE) and induces a number of Nrf2-dependent phase 2 drug-metabolizing genes, including NQO1, HO-1, GCLC, etc. (Nakamura *et al*., 2010). AhR, the transcription factor binding to XRE, plays a pivotal role in the inducible expression of CYPs including CYP1A1 (Ciolino *et al*., 1999). A previous study indicated that cross talk between AhR and Nrf2 is mediated by the mechanism that the gene promoter of Nrf2 includes at least one functional XRE (Miao *et al*., 2005). Both the functional XRE and ARE elements exist in proximity to the promoters of NQO1 (Favreau *et al*., 1991). The expression of CYP1A1 was significantly up-regulated only at concentrations higher than 100 μM DOPAC (Fig. 2.5). Moreover, the inducible gene expression of NQO1 or HO-1 by DOPAC at concentrations higher than 100 μM was more remarkable than by its lower concentrations (Fig. 2.5). These results strongly suggested that the higher concentrations of DOPAC might activate the AhR/XRE pathway and thus synergistically enhance the Nrf2/ARE pathway. It has recently become clear that the phase 2 gene inducers also act as indirect antioxidants in a manner different from the radical-scavenging direct antioxidants (Nakamura *et al*., 2010). For example, several GSTs have a GSH peroxidase activity towards hydroperoxides.HO-1 also generates carbon monoxide and biliverdin/bilirubin. A previous study demonstrated that the overexpression of the HO-1 protein inhibited the hydrogen peroxide-induced cytotoxicity and carbon monoxide is responsible for this protective effect (Lin *et al*., 2007). The results in the present study demonstrate that DOPAC significantly inhibits the hydrogen peroxide-induced cytotoxicity independent of its radical scavenging potential (Figs. 2.7A and B). The antioxidant effect in Hepa1c1c7 cells was actually exhibited by the 24-h treatment of DOPAC at 50 μM, which is required for the induction of the phase 2 drug-metabolizing enzymes, but neither for the GSH up-regulation nor for the CYP1A1 induction, suggesting the significant role of GST and/or HO-1 in the cellular antioxidant action. The pretreatment of 10 μM quercetin for 24 h, but not that for 30min, showed the effect similar to that of DOPAC (Figs. 2.7C and D). This concentration of

quercetin is also able to enhance the GST activity (Fig. 2.6C). These results suggested that the cytoprotective activity of quercetin is 5 times more than potent compared to that of DOPAC. However, the treatment of 50 μM quercetin itself showed a significant toxicity, even though it could reduce the hydrogen peroxide-induced cytotoxicity (Fig. 2.7D). In addition, the pretreatment of hydrogen peroxide (20 and 40 μM) showed no effect on the hydrogen peroxide induced cytotoxicity (Figs. 2.7E and F), suggesting that hydrogen peroxide produced by the autoxidation of DOPAC could be ruled out in its cytoprotective mechanisms.

In conclusion, I identified DOPAC as a predominant biologically-active catabolite quercetin glycosides formed by the colonic microbiota in the large intestine. DOPAC may contribute to preventive effects of dietary quercetin against oxidative stress related diseases via not only direct radical scavenging activity, but also phase 2 gene expression inducing property.

CHAPTER 3

3,4-Dihydroxyphenylacetic acid has cytoprotective effects against oxidative stress through direct modification of Keap1 and aryl hydrocarbon receptor

3.1 Introduction

Oxidative stress is defined as imbalance between the production of ROS and antioxidant defense in the cells and tissues. Although ROS plays important role in regulation of cellular signaling pathways (Finkel, 2011), because of its high reactivity, it can cause damage to cellular molecules such as DNA, proteins and lipid, leading various diseases including chronic inflammation, autoimmune disease, neurological disorders, cardiovascular diseases, and cancer (Birben *et al*, 2012; Brieger *et al*, 2012). As ROS is produced not only by external factors but also by biological processes like cellular respiration and metabolic processes, cells are equipped with antioxidative systems. Cellular defenses against oxidative stress are divided into two types: direct and indirect. Direct antioxidative system is the way to scavenge ROS using low-molecular-weight compounds such as vitamin C and E, glutathione and β-carotene. Indirect antioxidative system is mediated Keap1/Nrf2/ARE pathway, which regulates transcription of a series of cytoprotective enzymes (phase 2 enzymes) including NQO1, HO-1, and GST (Dinkova-Kostova *et al*., 2008).

Expression of phase 2 enzymes is induced by activation of Keap1/Nrf2/ARE pathway. Under basal condition, Keap1 binds to Nrf2 in the cytoplasm and Nrf2 is polyubiquinated and consequently degraded by the proteasome. Under oxidative stress, Keap1 is inactivated by modification of cysteine residue, leading stabilization and nuclear translocation of Nrf2. After entering the nucleus, Nrf2 binds to ARE together with small Maf proteins and drive transcription of phase 2 enzyme genes (Taguchi *et al*, 2011; Kansanen *et al*, 2013). Increasing evidence suggests interaction between Keap1-Nrf2 pathway and AhR pathway. AhR is a transcription factor retained in the cytoplasm.

Upon ligand binding on AhR, AhR translocates into the nucleus and forms heterodimer complex with ARNT, resulting in binding to XRE located in promoter region of AhR responsive genes. Miao *et al*. showed *nrf2* transcription was regulated by AhR via XRE elements suggesting the presence of XRE in *nrf2* gene promoter (Miao *et al*., 2005). Additional studies reported that *nrf2* and *glutathione-S-transferase* contain XRE region (Nioi *et al*., 2004; Yeager *et al*., 2009).

In chapter 2, I found that DOPAC had antioxidative properties via inducing phase 2 enzymes, but an underlying mechanism of this phenomenon have still unclear. DOPAC has catechol structure, which can be subjected to oxidation by phenol oxidases including tyrosinase and converted into ortho-quinone in cells (Ishii *et al*., 2009; Nakamura *et al*., 2014). *O*-quinone react with nucleophiles such as GSH or protein cysteine residue in 1,4-Micheal fashion to form a catechol-nucleophile conjugate. This characteristic motivated us to seek the target proteins of DOPAC to reveal the detailed mechanism for its antioxidative effects.

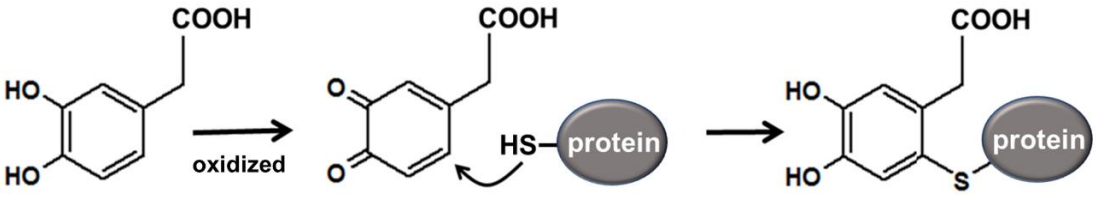

Fig. 3.1. DOPAC modifies a protein sulfhydryl group in a phenol oxidase-dependent manner.

In the present study, I designed a novel DOPAC probe using CuAAC, a representative click reaction. I introduced an alkyne moiety into DOPAC by esterification with 2-propyn-1-ol to afford the DOPAC propargyl ester (DPE). I made sure that DPE had the same phase 2 enzyme inducing efficacy as DOPAC by RT-PCR suggesting DPE behaves in the same fashion as DOPAC in Hepa1c1c7 cells. Thus I carried out CuAAC reaction with an azide-labeled biotin in lysate of

Hepa1c1c7 cells treated with DPE, visualized western blotting using the horseradish peroxidase (HRP) conjugated streptavidin, which showed a variety of molecular weight proteins. Finally I performed pull-down assay using anti-Keap1and anti-AhR antibody and observed direct interaction between DPE and Keap1 and AhR, indicating that DOPAC induced phase 2 enzyme gene expression through direct modification of Keap1 and AhR.

3.2 Materials and methods

3.2.1 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-biotinconjugate 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-AhR antibody, anti-Keap1antibody, 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 healthcare (Little Chalfont, UK). All other chemicals such as benzyl azide were purchased from Wako Pure Chemical Industries (Osaka, Japan).

3.2.2 Instrumental methods of chemical analysis

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.

3.2.3 Cell culture and treatment

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, 4mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. For experiments, cells were seeded in complete medium and treated with each reagent or DMSO vehicle.

3.2.4 Synthesis of DOPAC propargyl ester

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% NaHCO3 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–CH_2–O–), 6.63–6.72 (3H, *m*, **H**–Ar–).

3.2.5 CuAAC reaction with DPE and benzyl azide

DPE (23 mg, 112 mmol) and benzyl azide (14 μl, 112 mmol) 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 $C_{18}H_{16}N_3O_4$).

3.2.6 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 mg) 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 genespecific primers: HO-1, (F) 5′-ACATCGACAGCCCCACCAAGTTCAA-3′ and (R) 5′-CTGACGAAGTGACGCCATCTGTGAG-3′; 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).

3.2.7 Competitive inhibition of DPE-modification in cells and in cell lysate

As for treatment of cells, confluent Hepa1c1c7 cells were preincubated 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 mg 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.

3.2.8 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 mM 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.

3.2.9 Statistical analysis

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.

3.3 Results

3.3.1 Design and synthesis of click chemistry probe.

I designed an alkyne-functionalized clickable DOPAC probe to capture its target proteins. The strategy for detecting DOPAC-interacting proteins is as follows: 1) DPE is synthesized via fisher esterification. 2) DPE is conjugated with the target protein. 3) A DPE-protein conjugate is subjected to click reaction with biotin azide to form a biotin-DPE-protein complex. 4) The complex is detected with HRP-streptavidin.

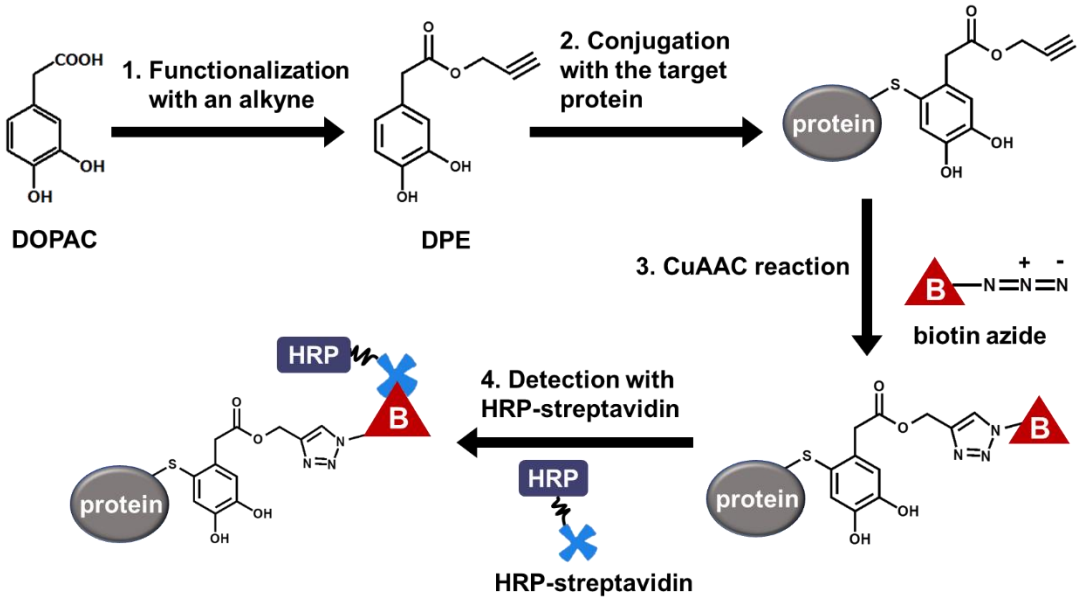

Fig. 3.2. The strategy for detecting DOPAC-targeting proteins using DPE as a click chemistry probe.

To introduce an alkyne functionality into DOPAC, I esterified DOPAC with 2-propyn-1-ol via the Fischer esterification method, providing DPE in 40% yield (Fig. 3.3). 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.

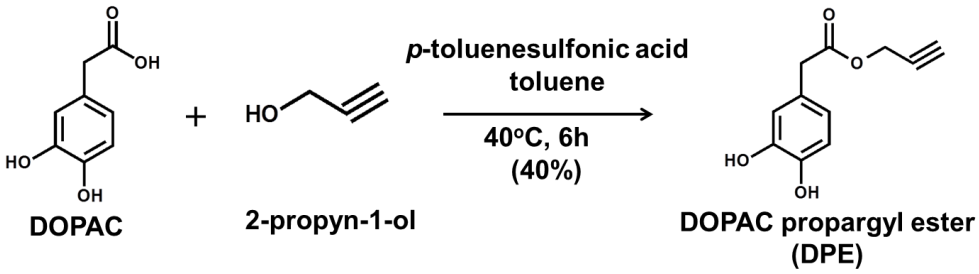

Fig. 3.3. Esterification of DOPAC with 2-propyn-1-ol to give DPE.

3.3.2 Evaluation of chemical and biological properties of DPE.

To examine whether the esterification of DOPAC influences the ability to bind to protein sulfhydryls, I compared the thiol modification ability of DPE with that of DOPAC. I 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 (Nakajima *et al*., 2007). Laccase was used as a polyphenol oxidase to oxidize DOPAC and DPE into the *o*-quinone structures via a two-electron oxidation as previously reported (Nakamura *et al*., 2014) . As shown in Fig. 3.4A, 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, I examined the biological features of DPE such as inducing property of the phase 2 drug-metabolizing enzyme gene expressions. As shown in Fig. 3.4B, DOPAC dose-dependently increased the gene expression of HO-1 and NQO1, which is consistent with results in chapter 2. 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.

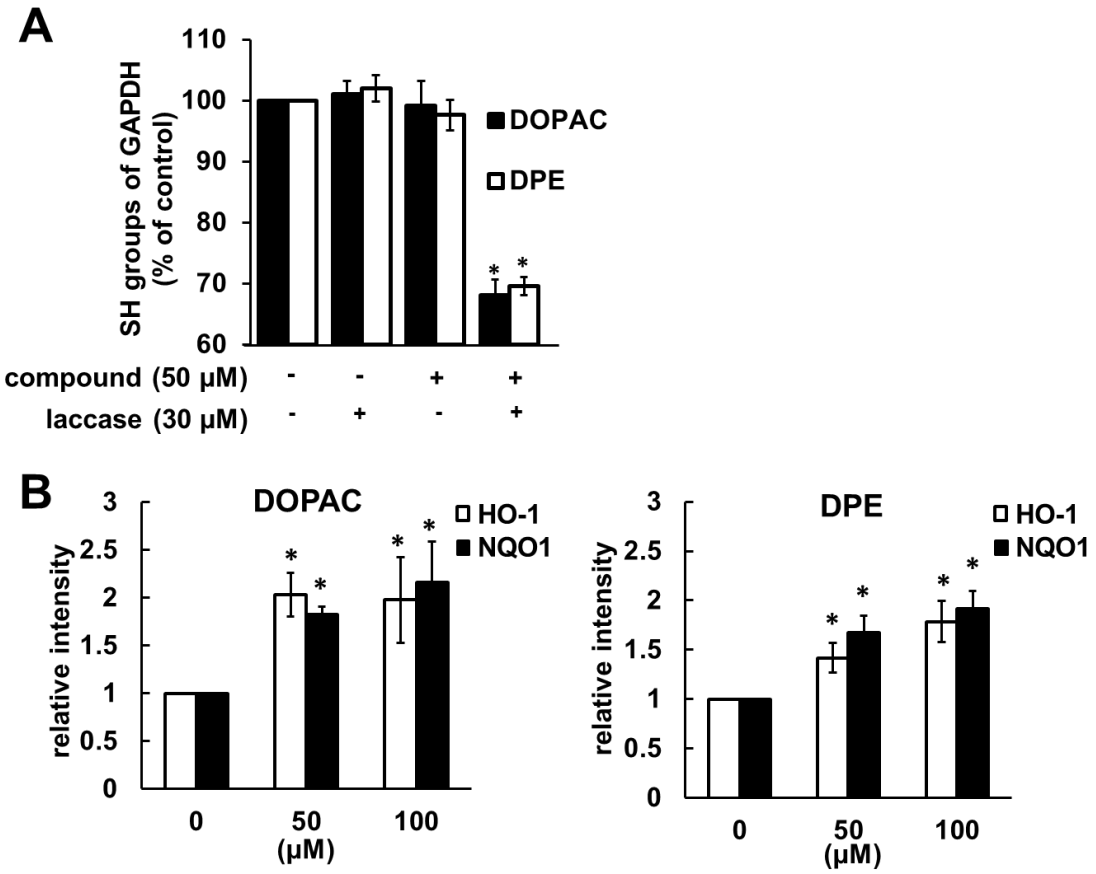

Fig. 3.4. Chemical and biological properties of DPE and DOPAC. (A) Modification of sulfhydryl groups in GAPDH by DOPAC and DPE. GAPDH (500 μg/ml) was incubated with DOPAC or DPE in 50 mM sodium phosphate buffer (pH 7.2) for 1 h at 37 °C in the presence or absence of laccase (30 units). The level of residual sulfhydryl groups in GAPDH was measured by the spectrophotometric method using DTNB. (B) 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.).

3.3.3 Detection of DPE-modified model proteins *in vitro*.

I 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 (Rubia *et al*., 1977). Based on the spectral data, the product was identified as a 1,2,3-triazol compound with the DOPAC ester and benzyl moiety (Fig. 3.5A).

To confirm the efficacy of DPE to tag the target protein, I 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. 3.5B). 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.

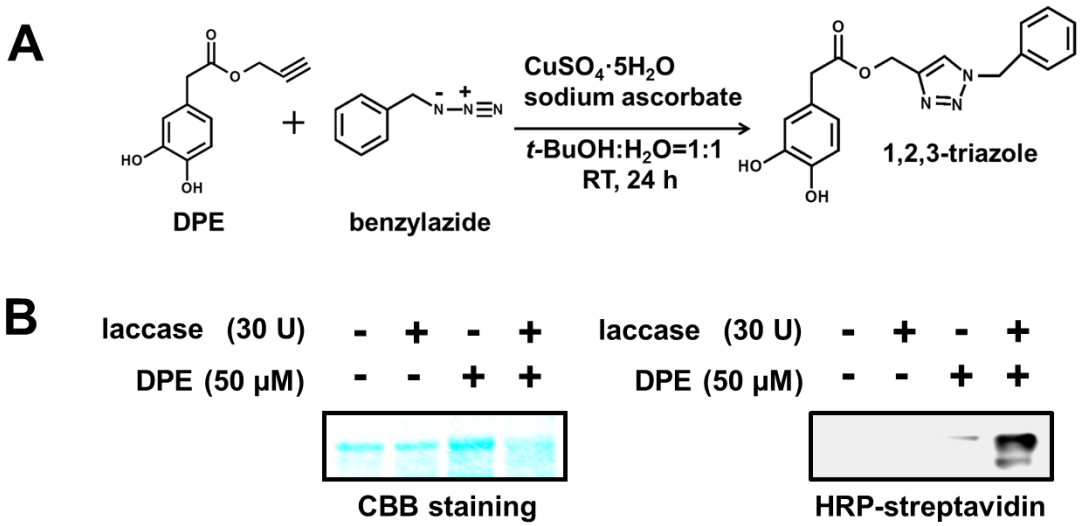

Fig. 3.5. CuAAC reaction of DPE with an azide-linked tag molecule. (A) Formation of 1,2,3-triazole by CuAAC reaction with DPE and benzyl azide.(B) Detection of GAPDH tagged by CuAAC reaction with DPE and the azide-labeled biotin. GAPDH (1mg/ml) was incubated with or without 50 μM DPE in the presence 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). 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).

3.3.4 Detection of the intracellular DPE-modified proteins.

I applied this probe to cultured cells to label the cellular proteins. DPE contains an ester bond, which would be hydrolyzed by carboxyl esterase present in the cells. Therefore, we used BNPP as an esterase inhibitor to prevent cleavage of DPE in the cells (*Hatfield* et al., 2011). 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. 3.6A, the DPE-modified proteins were observed in the groups not only with but also without the BNPP treatment. These results 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, I 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. 3.6C). To investigate the effect of the esterification, I next synthesized another DOPAC ester using *n*-butanol instead of 2-propyn-1-ol to afford DBE (Fig. 3.6B). DBE inhibited DPE-modified proteins even at a concentration lower than that of DOPAC (Fig. 3.6C). 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, I compared the inhibitory ability of DOPAC and DPE using cell lysate. As shown in Fig. 3.6D, 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.

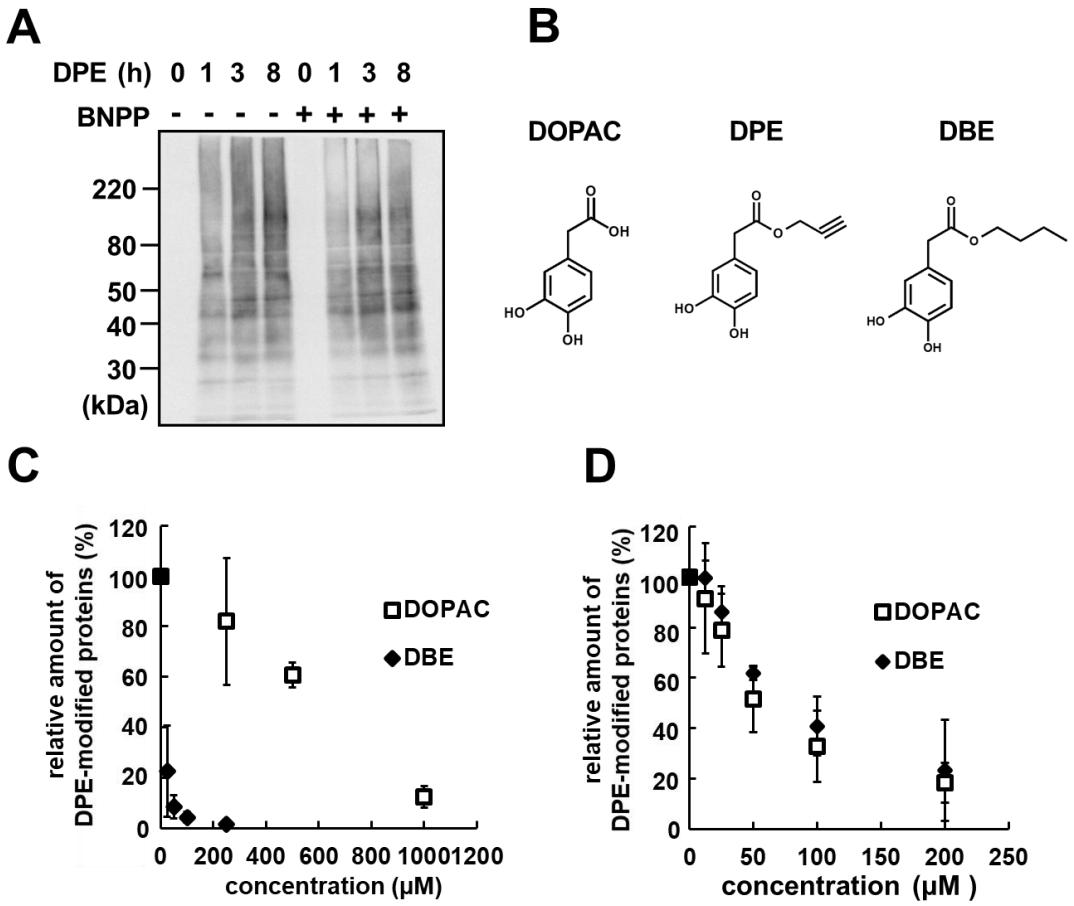

Fig. 3.6. Detection of the intracellular DPE-modified proteins. (A) Detection of the DPE-modified proteins in Hepa1c1c7. Confluent Hepa1c1c7 cells were pre-incubated with 0.1% DMSO or 100 μM BNPP and incubated with 100 μM DPE for indicated time periods in serum-free MEM-α. The DPE-tagged cellular proteins were detected by HRP-streptavidin. (B) Structural comparison of DBE with DOPAC and DPE. (C) Effect of the pretreatment of DOPAC or DBE with DPE on the DPE-modified protein formation in Hepa1c1c7 cells. Hepa1c1c7 cells were preincubated with DOPAC (open circle) or DBE (closed circle) for 30 min and incubated with 25μM 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.

3.3.5 Identification of the target proteins of DOPAC.

I finally attempted to detect the modification of Keap1and AhR in the cells exposed to DPE, because the expression of a phase 2 enzyme, NQO1, is regulated by the Keap1/Nrf2/ARE pathway and AhR/ 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 (Taguchi *et al*., 2011; Jaramillo *et al*., 2013). 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 (Hayes *et al*., 2009). Electrophilic quinones have reported to have capability to covalently bind to and activate AhR (Abiko *et al*., 2015). These led us to assume that direct modification of Keap1and/or AhR of DOPAC may regulate the gene expression. To confirm this, Hepa1c1c7cells 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. 3.7). These results indicate that Keap1 and AhR are potential targets of DOPAC for the up-regulation of phase 2 enzymes in Hepa1c1c7 cells.

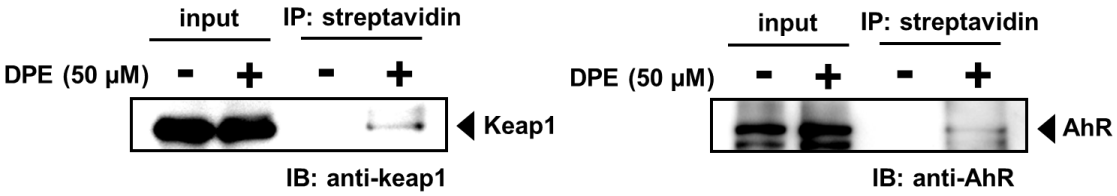

Fig. 3.7 Detection of the DPE-modified Keap1 and AhR by pulldown assay. Confluent Hepa1c1c7 cells were incubated with 50 μM DPE for 5 h in serum-free MEM-α. The cell lysate was incubated with Streptavidin Mag Sepharose beads for 30 min. The DPE-modified Keap1 (left) or AhR (right) were detected by immunoblot analysis.

3.4 Discussion

In recent years, the idea has been widely accepted that phytochemicals modulate signal transduction via direct modification of biomolecules, leading to beneficial effects on human health. Therefore, researchers have made efforts to identify the target molecules interact with food chemicals. Tachibana *et al*. found Epigallocatechin-3-Gallate binds with the 67 kDa laminin receptor and exerts anti-cancer effects (Tachibana *et al*., 2004) and Hong *et al*. demonstrated sulforaphane activates Keap1/Nrf2 pathway by direct modification of Keap1 (Hong *et al*., 2005). Catechol is easily oxidized by catechol oxidase to ortho quinone, which covalently modify sulfhydryl groups of protein cysteine residues (Ishii *et al*., 2009). Cysteine modification is known to serve as an activity switch in various proteins such as Keap1 (Dinkova-Kostova *et al*., 2001) and IkBα kinase (Gupta *et al*., 2010). These facts as well as the phase 2 drug-metabolizing enzyme inducing property of DOPAC motivated me to investigate direct targets of DOPAC.

In this study, I developed a novel tag-free probe for detecting DOPAC-targeting proteins using click reaction. Click chemistry is a general term for a reactions that are fast, simple to use, easy to purify, regiospecific, and high yielding. CuAAC is nearly perfect and the most broadly applicable click reaction which forms 1,4-disubstituted 1,2,3-distributed in the presence of Cu(I) catalysis. Since being discovered by the groups of Meldal in Denmark (Tornøe *et al*., 2002) and Sharpless and Folkin in the U.S. (Rostovtse *et al*., 2002) in 2002, CuAAC has been used in a wide range of research area including bioconjugation (Wang *et al*., 2003), drug discovery (Manetschery *et al*., 2004), polymers (Helms *et al*., 2004) and radiochemistry (Schirrmacher *et al*., 2007). DOPAC was alkyne-functionalized via condensation with propargyl alcohol to be labeled with biotin azide via CuAAC for detection. I found that introduction of terminal alkyne do not affect thiol-modification (Fig 3.4A) and phase 2 drug-metabolizing enzyme inducing activities (Fig 3.4B), suggesting DPE captures the same proteins as DOPAC. DPE contains an intramolecular ester bonds, and thus I was

concerned about the possibility that DPE is subjected to hydrolysis by intracellular carboxylesterases. However, DPE without BNPP detected as various proteins as with BNPP, suggesting that DPE is resistant to cellular esterase, thereby is useful for using in cells (Fig 3.6A). Also advantage of this probe is compactness, which enable to interact with a variety of proteins. Previous developed biotin-tagged DOPAC probe detected much less variety of proteins, probably due to sterically bulky biotin tag (Ishii *et al*., 2009). It should be noted that coincubation of DPE with quercetin decreased amount of DPE-modified proteins compared to single incubation of DPE (data is not shown), indicating that this probe detect not only targets of DOPAC but also polyphenols with catechol moiety .

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 pull down 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.

CHAPTER 4

Aureusidin, an aurone-type flavonoid with an unique structure, as a potential cytoprotective agent against oxidative stress

4.1 Introduction

Flavonoids, one group of plant secondary metabolites, are widely present in plant origin foods and beverages, such as vegetables, fruits, tea, wine, and cocoa. Flavonoids commonly share the C_6-C_3-C_6 basic skeleton, where two benzene rings (ring A and ring B) are connected through three carbons that form ring C. Flavonoids are classified into several different subclasses dependently on variation of these three rings, in particular the degree of oxidation of ring C, including flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins, isoflavones, and aurones (Ross *et al*., 2002).

Aurones [2-benzylidenebenzofuran-3(2H)-ones] are classified as one subclass of flavonoids, even though their C ring consists of five membered ring instead of six membered ring as other major flavonoid subclasses have. Aurones serve as bright yellow pigments in several edible and ornament flowers, such as snapdragon (*Antirrhinum majus*) and dahlia (*Dahlia variabilis*) (Ono *et al*., 2006). Since aurones are biosynthesized only in limited number of plants, their biological activities have not well been studied compared to other flavonoids. So far, natural and synthetic aurones have been reported to show some chemical and biological properties, including P-glycoprotein modulation (Boumendjel *et al*., 2002; Hadjeri *et al*., 2003), free radical scavenging (Venkateswarlu *et al*., 2004; Detsi *et al*., 2009), antimalarial (Carrasco *et al*., 2014) and anticancer activities (Lawrence et al., 2003; Cheng *et al*., 2010)

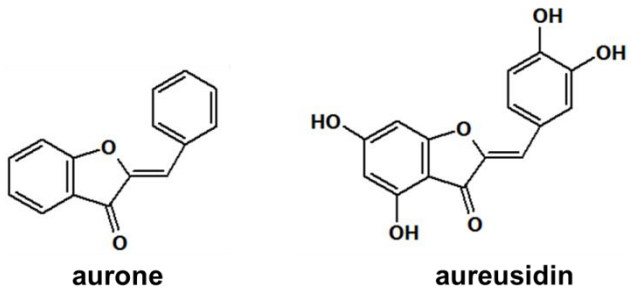

Fig. 4.1 Chemical structure of aurone and aureusidin.

In this study, I tried to evaluate cytoprotective activities of aureusidin (AU). As shown in Fig. 4.1, AU contains an *o*-dihydroxyl structure (catechol structure) in the B-ring. Possession of catechol structure in the B ring of flavonoids is expected to exert a radical scavenging activity, because it can donate a proton and an electron to hydroxyl, hydroperoxyl, and peroxynitrite radicals, stabilizing them and resulting in occurring a relatively stable flavonoid radical (Cao *et al*., 1997). Also, the catechol type polyphenol can be oxidized into *o*-quinone, which reacts with cysteine residue on Keap1 via a Michael addition reaction, resulting in the phase 2 cytoprotective gene expression (Ishii *et al*., 2009; Kumar *et al*., 2014). Based on these facts, I hypothesized that AU has a potential to act as a potent antioxidant. To test this hypothesis, I first synthesized AU and then examined its cytoprotective effect against hydrogen peroxide-induced oxidative stress. Furthermore, I investigated the effect on the gene expression of phase 2 enzymes and CYP1A1, an AhR-regulated gene to reveal the mechanism underlying AU-induced cellular antioxidative effects.

4.2 Materials and methods

4.2.1 Materials

Diethyl ether anhydrous, phloroglucinol anhydrous, and phosphorus (V) oxide were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Hydrogen chloride solution 1.0 M in diethyl ether and chloroacetonitrile and 3,4-dihydroxybenzaldehyde were obtained from Sigma-Aldrich (St. Louis, MO, USA). TLC silica gel 60 F_{254} was purchased from Merck (Darmstadt, Germany). 28% Sodium methoxide methanol solution was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other chemicals were purchased from Nacalai tesque (Kyoto, Japan).

4.2.2 Cell culture and treatment

The mouse hepatoma cell line Hepa1c1c7, obtained from the American Type Culture Collection, was grown and maintained at 37°C in α-MEM 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 (final 0.1%, v/v).

4.2.3 Synthesis of aureusidin (AU)

AU was synthesized from phloroglucinol in 4 steps as previously reported by David *et al.* (2005) and Nguyen *et al.* (2014) with some modification. Procedures for each steps are as described below.

*2-(2-Chloro-1-iminoethyl)-1,3,5-benzenetriol hydrochloride (**1**).*

Phloroglucinol anhydrous (6.0 g, 50 mmol) was dissolved in 25 ml of diethyl ether anhydrous at 0°C. Chloroacetonitrile (3.8 g, 50 mmol), zinc chloride (0.2 g, 1.5 mmol) and 75 ml of hydrogen chloride solution, 1.0 M in diethyl ether were added sequentially. The solution was stirred at 0°C for

3 h and another 21 h at room temperature. The precipitate was collected by filteration, washed with anhydrous diethyl ether and then completely dried to afford 5.73 g (48%) of **1**. ^{1}H NMR (DMSO-*d6*): δ5.45 (s, 2H, CH_2), 6.07, 6.28 (each d, *J*=2.0, 1.5 Hz respectively, 2H, H-3 and H-5), 10.91 (s, 2H, CH_2), 9.90, 11.54, 12.57 (each s, total 3H, OH).

*4,6-Dihydroxy-3(2H)benzofuranone (**2**).*

The compound **1** (2.38 g, 10 mmol) was dissolved in 1 M hydrochloric acid (500 ml). After the solution was refluxed for one hour, it kept at 0^{o}C overnight. The precipitate in the solution was collected by filtration, washed with ice-cold water and dried using phosphorous pentoxide in a vacuum desiccator giving 1.06 g of a mixture compounds of (**2**) and 2-Chloro-1-(2,4,6-trihydroxyphenyl)ethanone (**3**). The mixture (1.0 g) and 10 ml of 28% sodium methoxide methanol solution was refluxed for 2 h. The solvent was evaporated under reduced pressure and 1 M hydrochloric acid was added until occurring yellow solid. The residue was collected by filtration and dried to obtain 0.53 g (25%) of **2**. ^{1}H NMR (DMSO-*d6*): δ4.55 (s, 2H, CH_2), 5.91, 5.93 (each d, *J*=1.5, 1.8 Hz respectively, 2H, H-5/H-7), 10.62 (br. s, total 2H, OH).

*Aureusidin (AU) (**3**).*

The compound **2** (0.17 g, 1.0 mmol) and 3,4-dihydroxybenzaldehyde (0.14 g, 1.0 mmol) was mixed in 200 µl of 36% HCl solution in 14 ml methanol. The reaction mixture was stirred at room temperature for 24 h and the progress of the reaction is monitored by TLC. After the reaction was stopped by neutralized with sodium hydroxide, the solution was evaporated under reduced pressure. The crude products were separated by preparative TLC on silica gel (chloroform: methanol=15:85) and yellow band was scraped off, diluted with ethyl acetate and methanol and dried to afford yellow solid. The obtained product was subjected to further purification using solid phase extraction. The

yellow solid was dissolved in water/methanol (80:20) and loaded on to a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) equilibrated with methanol. After the cartridge was washed with water containing 0.1% acetic acid, the absorbed material was eluted with water/methanol (20:80) and dried completely *in vacuo*, giving 24 mg (8.4%) of AU. ^{1}H NMR (MeOH-*d6*): δ6.00 (d, *J*=1.5 Hz, H, H-5), 6.18 (d, *J*=1.5 Hz, H, H-7), 6.56 (s, H, H-10), 6.82.(d, *J*=8.40 Hz, H, H-5′), 7.19 (dd, *J*=8.10 and 2.10 Hz, H, H-6′), 7.47 (d, *J*=2.10 Hz, H, H-2′)

4.2.4 RNA extraction and RT-PCR

Confluent Hepa1c1c7 cells were treated with AU at the indicated concentrations for 6 h or 24 h. RNA extraction and RT-PCR ware performed as described in chapter 2.

4.2.5 Cell viability determination with MTT assay

Hepa1c1c7 cells were seeded at 5.6×10^4 cells per well in a 96-well plate. After overnight preculture, the cells were incubated with AU (0, 5, 10, 25, 50 μM) for 16 h, followed by additional incubation with hydrogen peroxide (100 μM) for 6 h at 37 ºC in CO_2 incubator. 10 μl of MTT solution (5 mg/ml) was added to each well and incubated for 2 h at 37 ºC in CO_2 incubator. After aspiration of the medium completely, 200 μM of DMSO was added and the plate was shook for 10 min in room temperature. The absorbance was measured with an microplate reader (Benchmarkplus, Bio-Rad laboratories, Hercules, CA, USA) at 530 nm. The obtained values were compared with each of the controls incubated with vehicle only.

4.2.6 Statistical analysis

All values were expressed as means ± SD. Statistical significance was analyzed by Student's paired two-tailed *t*-test or a one-way ANOVA followed by Tukey's HSD using R software. A *p*-value of 0.05 was regarded to be statistically significant.

4.3 Results

4.3.1 Synthesis of AU by four-step synthesis.

In advance of the cellular experiments, I first prepared AU by four-step synthesis as previously reported by Bolek *et al*. in 2005 and Nguyen *et al*., in 2014 with some modification. Phloroglucinol was condensed with chroloacetnitrile in the presence of zinc chloride as a catalyst to give 2-(2-chloro-1-iminoethyl)-1,3,5-benzenetriol hydrochloride (**1**) in 48.3% yield. Next, the iminium salt (**1**) was hydrolyzed in 1 M HCl under reflux to afford the mixture of 4,6-dihydroxy-3(2H)benzofuranon (**2**) and the acetophenone derivatives of 2-chloro-1-(2,4,6-trihydroxyphenyl)ethanone (**3**) . The mixture was treated with sodium methoxide in methanol to cyclize (**3**) to (**2**). The product (**2)** was obtained in 25.5% yield. The purified compound (**2**) and 3,4-dihydroxybenzaldehyde were dissolved in methanol containing 2.4 equivalents of hydrochloric acid and refluxed to proceed condensation. By monitoring TLC, the yellow spot was observed, which might be AU. AU was purified using preparative TLC and solid phase extraction methods to afford AU in 8.4 %. The overall yield from phloroglucinol to AU was calculated to be 1.3%. The structure of (**1**), (**2**) and AU were confirmed by NMR analysis.

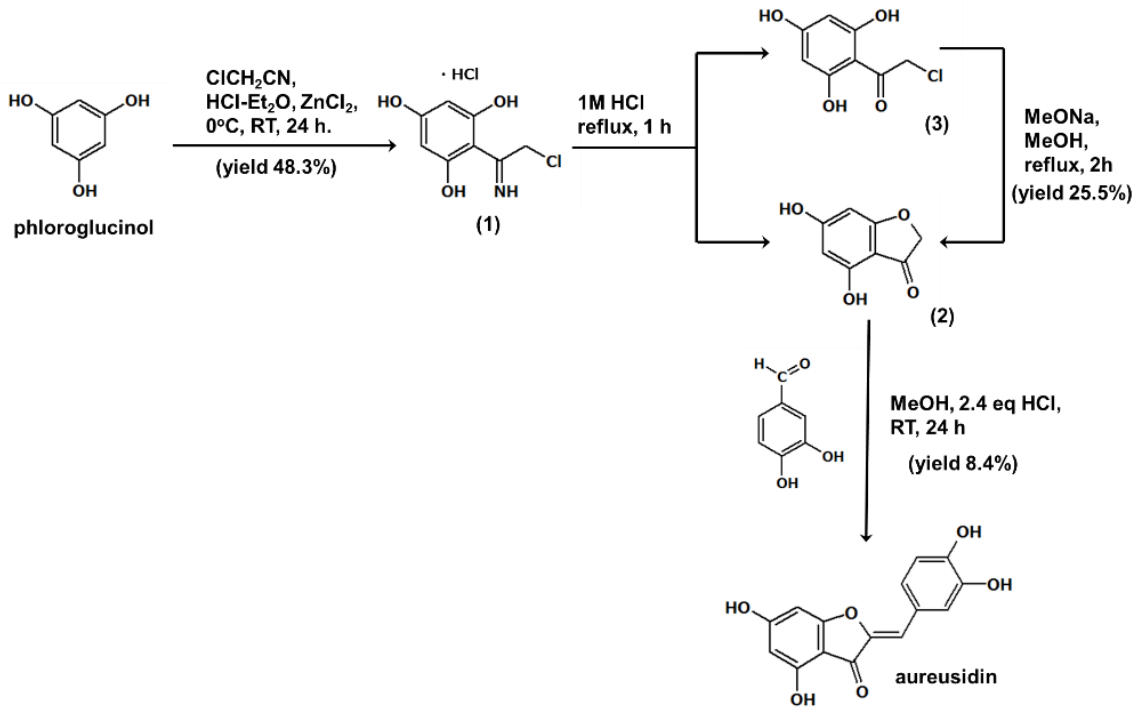

Fig. 4.2. Synthesis of AU in four steps from phloroglucinol.

4.3.2 Cytoprotective effects of AU against hydrogen peroxide-induced oxidative stress.

The protective effects of AU on the hydrogen peroxide-induced cytotoxicity were determined by an MTT assay. As shown in Fig. 4.2, the 6-h treatment of 100 μM hydrogen peroxide significantly decrease the cell viability, whereas the treatment with AU alone had no effect on viability. The treatment of AU prior to the hydrogen peroxide exposure for 16 h significantly mitigated cell cytotoxicity, suggesting that AU indirectly protects cells from hydrogen peroxide-induced cell damage.

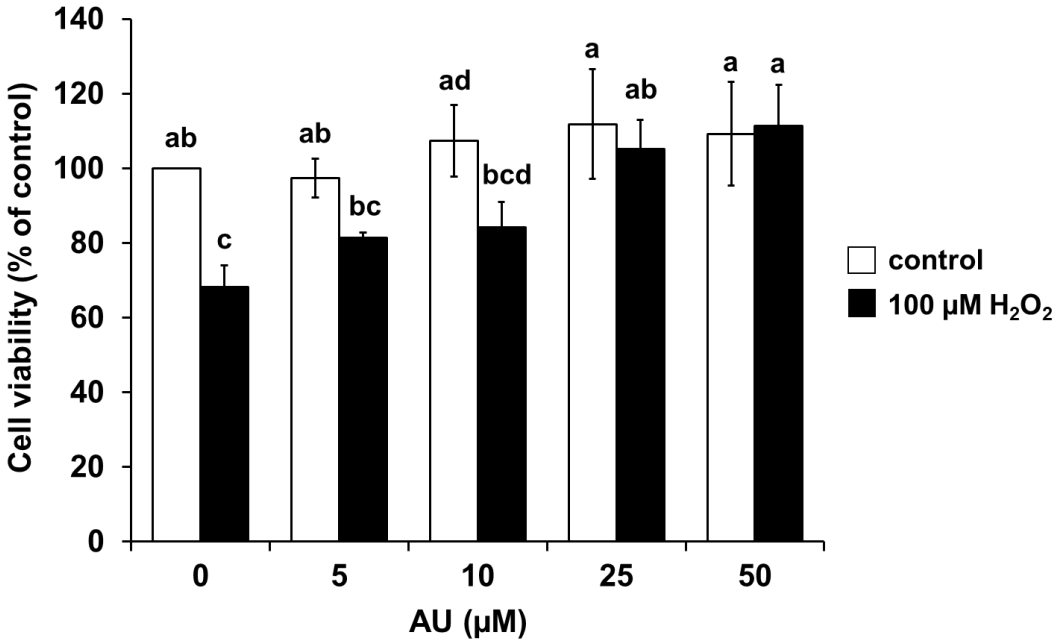

Fig. 4.3. Cytoprotective effects of AU on hydrogen peroxide-induced toxicity. Hepa1c1c7 cells were treated with various concentration of AU for 16 h, and then exposed to 100 μM hydrogen peroxide for 6 h. Cell viability was measured using an MTT assay. All values were expressed as means ± SD of four separate experiments. Different letters above the bars indicate significant differences among the treatments for each condition ($p < 0.05$).

4.3.3 Modulating effects of AU on phase 1 and 2 gene expression.

Flavonoids containing catechol moiety has a potential to activate Keap1-Nrf2 pathway leading to the induction of phase 2 enzyme expression. Therefore, I evaluated effects of AU on HO-1 and NQO1 gene expressions using RT-PCR. The 6-h treatment of AU enhanced the gene expression of HO-1 and NQO1 in a dose-dependent manner (Fig. 4.4A). The 24-h treatment of AU similarly increased the NQO1 gene expression, but not so significantly on HO-1 (Fig. 4.4B).

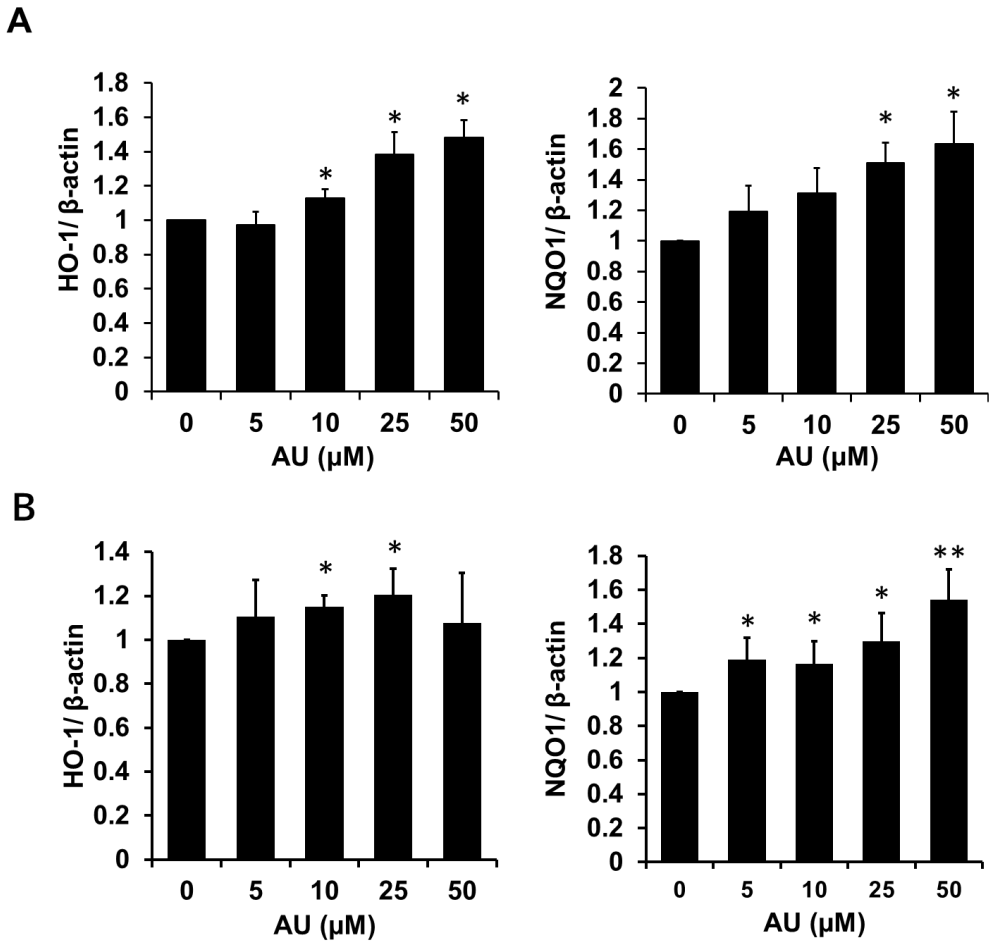

Fig. 4.4. Modulating effects of AU on HO-1 and NQO1 gene expression. Hepa1c1c7 cells were incubated with various concentrations of AU for 6 h (A) or 24 h (B) and then RT-PCR analysis for HO-1 and NQO1 was performed. All values were expressed as means ± SD of four three experiments. (*p < 0.05, **p< 0.01 compared with AU 0 μM).

Since some types of flavonoids can act as an agonist of AhR (Zhang *et al*., 2003), I investigated whether AU induces the CYP1A1 gene expression, which is known as a sensitive indicator of AhR activation. As shown in Fig. 4.5, CYP1A1 gene transcription is dose-dependently enhanced by the AU treatment, suggesting that AU has an agonist activity towards AhR.

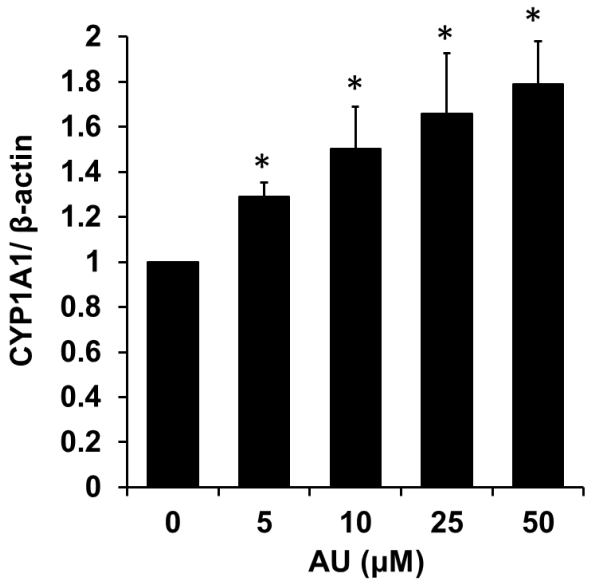

Fig. 4.5. Modulating effects of AU on CYP1A1 gene expression. Hepa1c1c7 cells were incubated with various concentrations of AU for 6 h and then RT-PCR analysis for CYP1A1 was performed. All values were expressed as means ± SD of four three experiments. (*p < 0.05 compared with AU 0 μM).

4.4 Discussion

Flavonoids are classified into several subclasses according to variations of the substitution pattern. The typical properties shared by almost all flavonoid subclasses are radical scavenging activities and some biological activities, including anti-inflammatory (Panche *et al*., 2016) and anticancer (Chahar *et al*., 2011), but the degree of effectiveness and the underlying mechanisms are varied due to their structural differences (Wang *et al*., 2018). Therefore, systematic understanding of relationship between chemical structures and biological activities is beneficial for the progress of flavonoid research and development.

Although AU was synthesized in advance of evaluating its biological activities, the low overall yield (1.3%) is partly due to difficulties in the AU purification. Although preparative TLC with silica gel plates was carried out for purification of AU, attached AU was not completely recovered by eluting owing to its high affinity with silica. Moreover, ethyl acetate and methanol eluted not only AU but also some kind of substances contained in silica gel plates. Therefore, to remove contaminants, the eluted products are further subjected to solid phase extraction. Future efforts will be concerned with the improvement of the AU purification methods to increase the total yield.

This study demonstrates that AU exerts cytoprotective effects against oxidative stress via induction of phase 2 enzymes. Catechol type polyphenols are known to act as Nrf2 activators since catechol moiety is oxidized into *o*-quinone and then reacts with cysteine residues of Keap1, leading to the activation of Nrf2 (Lee-Hilz *et al*., 2006). As shown in Fig.4.4, AU significantly enhanced the expression of phase 2 detoxifying enzyme genes. Lee *et al* previously reported that several 4,6-dimethoxyurones with different substituted groups showed the potent NQO1 induction and Nrf2/ARE activating properties (2010). The data suggested that these inducing activities are partly due to electrophilicity of the aurone compounds.

Phase 2 gene expression is not only regulated by Keap1/Nrf2 signaling pathway, but also

AhR/XRE signaling pathway. As shown in Fig. 4.5, AU enhanced CYP1A1 gene expression, a representative AhR target gene. Jin *et al*. investigated the relationship between chemical structure and AhR responsiveness among 14 different flavones and flavonols using Caco2 colon cancer cells and consequently indicated responsiveness to AhR influenced by the number (penta- > hexa-> tetra-/tri-hydroxyflavones) and position of the hydroxyl groups (Jin *et al*., 2018). Lee *et al*. reported several functionalizes aurones activate AhR signaling in Hepa1c1c7 cells and pointed out the possibility that the differences in potency are related to their planarity and size (2010). Although I found that AU enhances CYP1A1 gene expression, further studies are needed to determine the structural factors in AU as well as signal transduction mechanisms for AhR/XRE signaling activation.

CONCLUSION

In the present study, I evaluated cytoprotective effects of dietary flavonoids and their colonic catabolites against oxidative stress, and also investigated underlying molecular mechanisms through assessment of effects on phase2 drug-metabolizing enzyme expressions and identification of the target proteins using hepatoma cells.

In the chapter 2, I assessed direct and indirect antioxidative properties of phenolic acids formed by microbiota from quercetin glycosides in Hepa1c1c7 cells and RL34 cells. The results are summarized as follows:

1. DOPAC had the strongest radical scavenging activities in all the tested catabolites *in vitro*.
2. DOPAC exhibited the most potent phase 2 enzyme inducing efficacy in all the tested catabolites in Hepa1c1c7 cells.
3. DOPAC enhanced GST activity and increased cellular GSH content in RL34 cells and Hepa1c1c7 cells, respectively.
4. Pretreatment of DOPAC for 24 h, not 30 min significantly attenuated hydrogen peroxide-induced cytotoxicity.

These results suggested that DOPAC possesses cytoprotective properties against oxidative stress through induction of phase 2 drug-metabolizing enzymes.

In chapter 3, I synthesized a DPE as a click chemistry probe for detection DOPAC-targeting proteins and evaluated the efficacy of the probe. Moreover, I used the probe for detection and identification of DOPAC-interacting proteins in Hepa1c1c7 cells. The results are summarized as follows:

1. DPE exhibit almost the same thiol-modifying and phase 2 drug-metabolizing inducing ability as DOPAC.
2. Incubation of DPE with GAPDH in the presence of laccase *in vitro* forms the detectable DPE-modified GAPDH by HRP-streptavidin.
3. Treatment of Hepa1c1c7 cells with DPE with or without carboxylesterase inhibitor resulted in detection of various DPE-modified proteins.
4. A pulldown assay detected interaction of DPE with Keap1 and AhR.

These results suggested that DPE is a useful probe for detecting DOPAC-targeting proteins and DOPAC induce enhanced phase 2 enzyme expression via directly modification with Keap1 and AhR.

In chapter 4, I synthesized AU and examined its antioxidative effects in Hepa1c1c7 cells. The results are summarized as follows:

1. AU was synthesized in four steps from phloroglucinol in 1.3% overall yield.
2. AU up-regulated gene expression of HO-1, NQO1 and CYP1A1.
3. AU significantly inhibited hydrogen peroxide induced cytotoxicity.

These results suggested that AU increases phase 2 enzyme expression via activation of not only Keap1/Nrf2 pathway but also AhR pathway and consequently protects cells from oxidative stress.

Newly revealed antioxidative actions and their mechanisms of dietary flavonoids and their catabolites in this study provide additional scientific evidence to support health promoting effects of flavonoids.

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SAYAKA NAKASHIMA

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