Review

Development of bioresources in Okinawa: understanding the multiple targeted actions of antioxidant phytochemicals

Yoko Aniya[1]*

[1] University of the Ryukyus, Senbaru-1, Nishihara, Okinawa 903–0213, Japan

Abstract: In research to develop healthy foods or preventive medicines from edible and medicinal herbs in Okinawa, we focused on the antioxidant activities of those bioresources. We first confirmed that the herbal antioxidant activities of such herbs increased upon ultraviolet irradiation treatment. This observation explains the high antioxidant activity of Okinawan vegetables, which grow under exposure to stronger ultraviolet light compared with those in other prefectures in Japan. Antidiabetic, hepatoprotective, cancer preventive, and cardioprotective actions were clarified using herbal extracts, and quercetin, chlorogenic acid, and gallic acid derivatives were isolated as antioxidant components from the herbs. Dimerumic acid was also isolated from the mold *Monascus anka*. All these antioxidants showed strong radical scavenging activities *in vitro* and beneficial effects in animal models. However, the concentrations of these compounds used *in vivo* seemed to be too low to have a physiologically important antioxidant effect based on their radical scavenging activities *in vitro*. Therefore, I performed a literature survey of antioxidant activities *in vivo*. Accumulating evidence has emerged that antioxidant phytochemicals show not only radical scavenging activities *in vitro* but also pleiotropic actions *in vivo*. The multitargeted, beneficial effects of antioxidant phytochemicals can be rationally explained using the xenohormesis concept, in which phytochemicals are the products of plant evolutionary adaptation to stress in plants, and their ability to induce a stress-adaptive response has been evolutionarily conserved in animals. (DOI: 10.1293/tox.2018-0041; J Toxicol Pathol 2018; 31: 241–253)

Key words: antioxidant, phytochemicals, multitargeted action, stress adaptive response, xenohormesis,

Introduction

Reactive oxygen species (ROS) such as superoxide anion ($O_2^-\bullet$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$), and lipid peroxyl radical ($LOO\bullet$) are produced in living organisms through normal cellular metabolism and environmental factors such as smoking, ultraviolet (UV) irradiation, or the ingestion of chemicals. ROS are highly reactive and can modify cellular components such as DNA, proteins, or membrane lipids resulting in cellular dysfunctions. Living organisms, including humans, have antioxidant enzymes by which ROS are neutralized: $O_2^-\bullet$ is converted to H_2O_2 by superoxide dismutase, H_2O_2 is converted to H_2O and O_2 by catalase and glutathione peroxidase, and lipid peroxide (LOOH) is converted to LOH and H_2O by glutathione peroxidase[1–3] (Fig. 1). An imbalance between oxidants and antioxidants in favor of the oxidants is termed oxidative stress[4]. Oxidative stress is involved in various pathological conditions, including cancer, neurological disorders, and others. Therefore, natural antioxidants that scavenge/neutralize ROS might be believed to ameliorate such pathological conditions, and numerous studies of antioxidants have been performed using traditional edible and medicinal herbs for the development of healthy foods/preventive medicines[5]. Accumulating evidence, however, has shown that phytochemicals with antioxidant activities not only can scavenge ROS but also can modulate various cellular functions by interacting with multiple proteins[6–8].

In this review article, I will introduce natural antioxidants that were obtained through a research project for the development of preventive medicines or healthy foods from traditional edible and medicinal herbs collected from the Okinawan Islands. Additionally, I will discuss how natural antioxidants can cause multitargeted, beneficial actions.

Antioxidant Activities of Okinawan Edible and Medicinal Herbs

The antioxidant activities of more than 30 edible and medicinal herbs collected from the Okinawan Islands were screened by measuring their radical scavenging activities *in vitro* and then confirming their effects *in vivo* using animal models. The effect of UV irradiation on antioxidant activity in herbs was studied using a greenhouse in which UV light
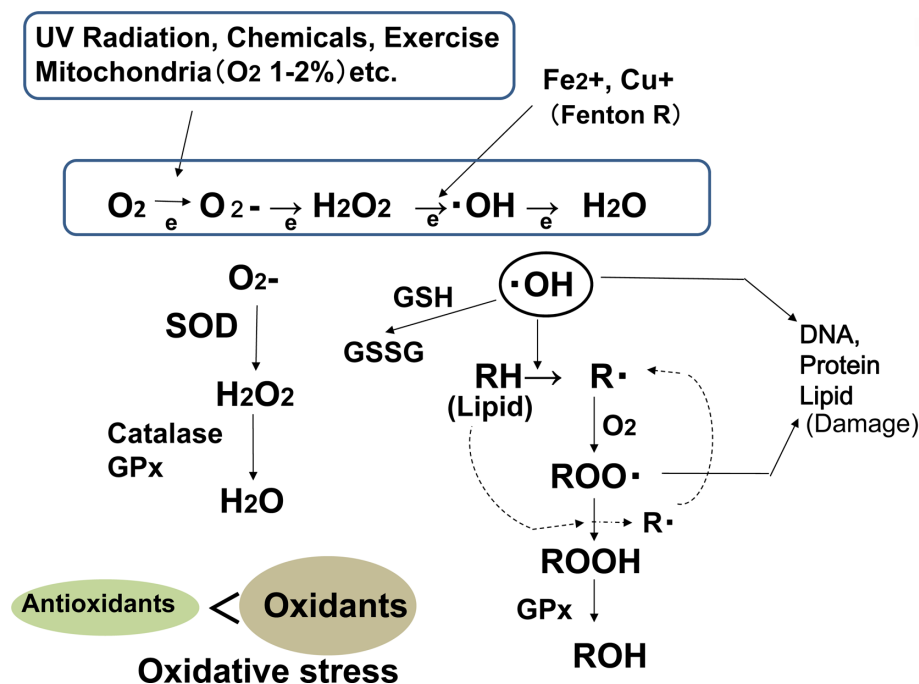
Fig. 1. Reactive oxygen species (ROS) and antioxidant enzymes. Oxygen (O_2) generates superoxide anion (O_2^-) through a one-electron reduction, hydrogen peroxide (H_2O_2) through a two-electron reduction, hydroxyl radical ($\cdot OH$) through a three-electron reduction, and water through a four-electron reduction. The hydroxyl radical is highly reactive and can react with lipid (LH) to generate a peroxyl radical ($ROO\cdot$), leading to a chain reaction that damages lipid membranes. The ROS thus produced are converted by antioxidant enzymes. O_2^- is converted to H_2O_2 by superoxide dismutase, H_2O_2 is converted to $H_2O + O_2$ by catalase, and peroxide (LOOH) is converted to LOH by glutathione peroxidase. The shift in the balance between oxidants (such as ROS) and antioxidants in favor of oxidants is termed oxidative stress. Gpx, glutathione peroxidase; SOD, superoxide dismutase; GSH, glutathione.

could be selectively blocked, and the results clarified that the antioxidant action of herbs increased in response to UV irradiation (Fig. 2). In some vegetables, the antioxidant activity was not detected without UV light irradiation. Several antioxidant components were isolated from the herbs, namely quercetin glucosides from *Psidium guajava* L. (guava), neochlorogenic acid from *Peucedanum japonicum* Thunb (botanboufu), isochlorogenic acids from *Crassocephalum crepidioides* (benibanaborogiku), chebulagic acid and corilagin from *Terminalia catappa* L. (momotamana), and gallic acid from *Limonium wrightii* O.K. (ukonisomatsu) (Fig. 3). Dimerumic acid was also isolated from *Monascus anka,* a mold that has been used for the fermentation of soybean curds (tofu)[9, 10].

The pharmacological actions of these herbal extracts observed *in vivo/in vitro* were as follows: 1) Antidiabetic action of guava leaves extract: The extract had a potent inhibitory action against aldose reductase activity, and treatment of streptozotocin-induced diabetic rats with the extract ameliorated the diabetic state, causing significant decreases in the concentrations of glucose and triglyceride in serum (Fig. 4). 2) Hepatoprotective action of herbs: When galactosamine/lipopolysaccharides or carbon tetrachloride were given to rats, severe oxidative stress-dependent hepatotoxicity was observed, and the pretreatments of the rats with various herbal extracts, including *C. crepidioides*[11], *T. cata-*
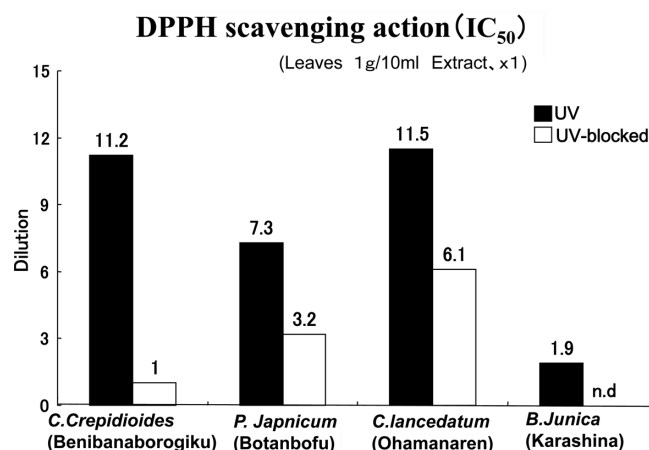

Fig. 2. Effect of ultraviolet irradiation on antioxidant activity in edible herbs. Herbs were cultivated inside or outside a greenhouse that was covered with special vinyl films that block UV rays. After drying the herbs, each was extracted with hot water (1 g/10 ml), and then measurements were performed to determine the concentration at which 50% of the radical 2,2-diphenyl-1-picrylhydrazyl was scavenged. The black columns show herbs cultivated under UV irradiation conditions, and the white columns show those cultivated under UV-blocked conditions. The antioxidant activities of the herbs were markedly increased by UV irradiation.
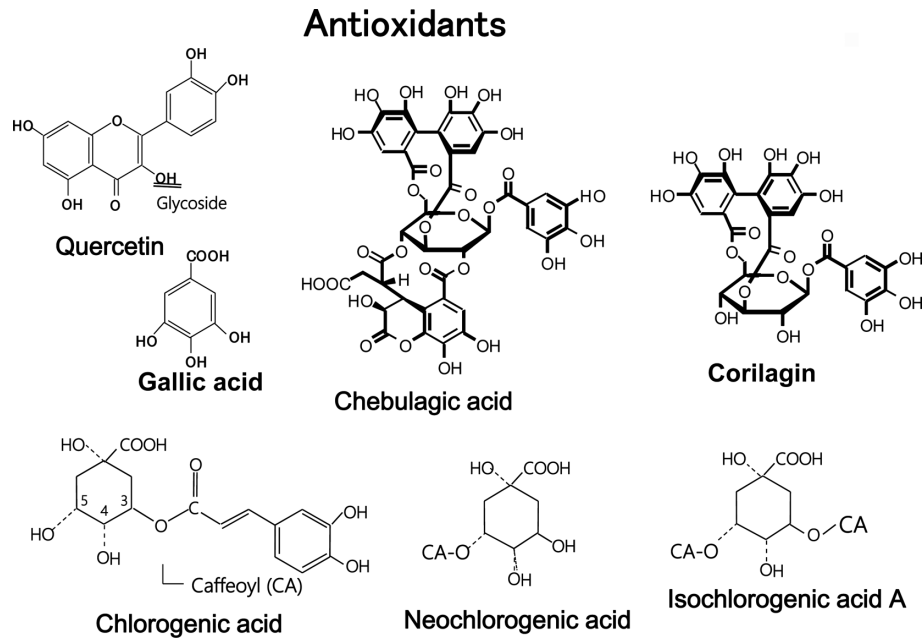
Antioxidants

Fig. 3. Structure of antioxidant phytochemicals isolated from herbs. Antioxidant components were isolated: quercetin glucosides from *Psidium guajava* (guava), gallic acid from *Limonium wrightii* (ukonisomatsu), neochlorogenic acid from *Peucedanum japonicum* (botanboufu), isochlorogenic acids from *Crassocephalum crepidioides* (benibanaborogiku), and chebulagic acid and corilagin from *Terminalia catappa* (momotamana).
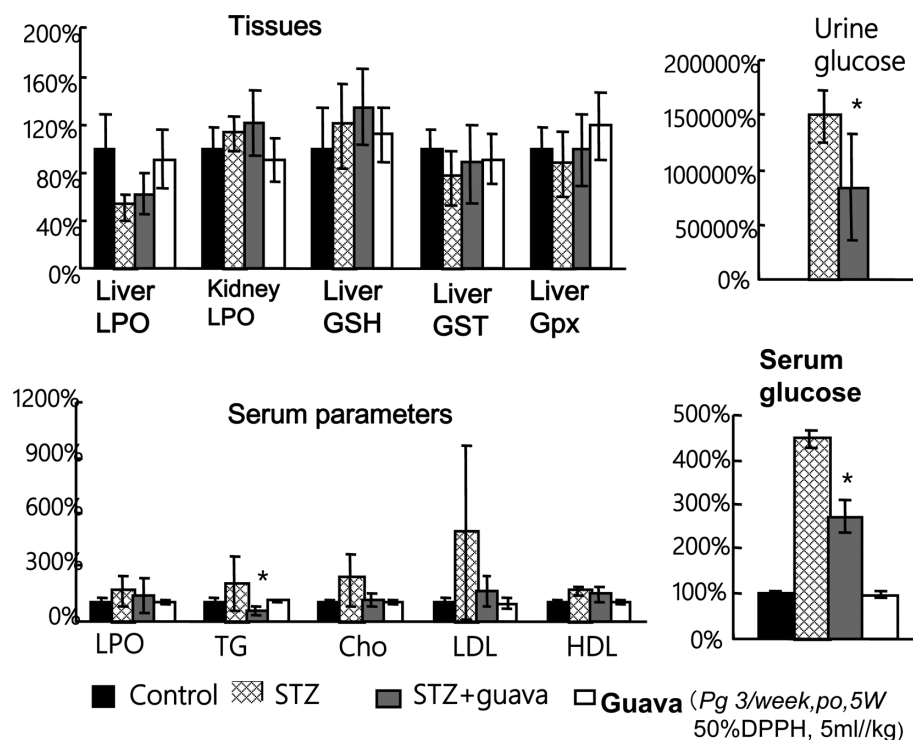

Fig. 4. Effect of the extract from guava leaves on streptozotocin (STZ)-induced diabetic rats. The extract at the dose showing 50% 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (5 ml/kg) was given orally to STZ-treated rats (5 ml/kg, 3times/week) or control rats for 6 weeks. Each parameter was measured in both tissue and serum. LPO, lipid peroxide; GSH, glutathione; GST, GSH *S*- transferase; Gpx, GSH peroxidase; TG, triglyceride; Cho, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein.*p<0.05 vs. STZ-treated.

ppa[12], and *Artemisia campestris* L (ryukyuyomogi)[13], ameliorated the hepatotoxicity significantly. 3) Preventive action against colonic carcinogenesis: Azoxymethane-induced rat colon carcinogenesis was inhibited by feeding a powder of dried leaves of *P. japonicum*[14] or *T. catappa*[15] as indicated by a significant decrease in preneoplastic lesions and proliferation indices. 4) Cardioprotective effect of herbal extracts: Aqueous extracts from leaves of *P. guajava* and *L. wrightii,* or their main antioxidants quercetin and gallic acid, respectively, improved the myocardial dysfunction caused by ischemia/reperfusion of rat hearts[16]. 5) Antimicrobial activity: Extracts from leaves of *T. catappa* showed strong antimicrobial and bactericidal activities[17]. 6) Antioxidant and hepatoprotective activities: The antioxidant and hepatoprotective activities of dimerumic acid from *M. anka* were clarified[9, 10].

While the antioxidants isolated from herbs have been presumed to contribute to their pharmacological actions, the concentration of each antioxidant in serum or tissues of experimental animals has been estimated to be far lower than that showing a radical scavenging activity *in vitro*. In our study on Okinawan herbs, the IC_{50} values (concentration at which 50% of the 2,2-diphenyl-1-picrylhydrazyl radical is scavenged) of antioxidants such as chlorogenic acid, isochlorogenic acid, and quercetin glycosides were 10–70 μM. However, the serum/tissue concentrations of each antioxidant given to the animals, as estimated from the antioxidant content of each extract, were assumed to be much lower than the concentration range that would be effective for scavenging radicals. It was therefore suggested that multiple antioxidants involved in herbal extracts might act synergistically *in vivo* or that the antioxidants modulate multiple cellular functions. Here, I will discuss the existing literature about antioxidant activities *in vivo*.

Action Mechanisms of Antioxidant Phytochemicals *in Vivo*: Effect on Transcription Factors

Nrf2/Keap1/ARE pathway

Nrf2 activation: Accumulating evidence has shown that the antioxidant activity of phytochemicals *in vivo* depends on increasing the cellular capacity for ROS neutralization via the upregulation/induction of antioxidant enzymes[18, 19]. Nrf2/Keap1/antioxidant response element (ARE) signaling is the most prominent pathway contributing to the upregulation of such antioxidant enzymes. Nrf2 is a redox-sensitive transcription factor that is the primary cellular defense against the cytotoxic effects of oxidative stress[20, 21]. Nrf2 is normally present in the cytoplasm, where it is bound to Keap1 and undergoes proteasomal degradation through a Keap1-associated Cul3-Rbx E3 ubiquitin ligase. However, in response to oxidative or electrophilic stress, Keap1 is modified and detaches from Nrf2, resulting in the nuclear translocation of Nrf2. Keap1 contains at least 25 reactive thiols and acts as a highly sensitive sensor of exogenous electrophiles. Oxidative thiol modifications of Keap1 such as oxido-reduction, alkylation, or thiol disulfide interchange cause it to undergo as conformational change, resulting

in the dissociation of Nrf2 from Keap1, which enables the nuclear translocation of Nrf2. In the nucleus, Nrf2 associates with small Maf proteins and then binds to an ARE on DNA, which results in the transcription of ARE-responsive genes[22] (Fig. 5). Phytochemicals with antioxidant activities could modify the reactive thiols of Keap1 and thereby potentiate the translocation of Nrf2 into the nucleus.

Nrf2-induced antioxidant and phase 2 enzymes: Many proteins that contribute to cellular antioxidant and detoxification functions are upregulated by the Nrf2/Keap1/ARE pathway. These Nrf2 target genes include superoxide dismutase, catalase, heme oxygenase-1, glutathione peroxidase, thioredoxins, thioredoxin reductase, peroxiredoxins, NAD(P)H-quinone oxidoreductase 1, and glutathione *S*-transferases[19, 21].

Phytochemical-mediated Nrf2 activation: Numerous antioxidant compounds/phytochemicals can act as Nrf2 activators by interacting with Keap1 sensor thiols[23]. Alkylating agents are the most potent Nrf2 activators, and many phytochemicals can alkylate Keap1 thiols as Michael acceptors, which are defined as acetylene compounds that are conjugated to an electron-withdrawing group and can form reversible alkylating reactions with Keap1 sensor thiols. Curcumin, sulforaphane, and organosulfides are potent Nrf2 activators that can act as Michael acceptors[23, 24]. By contrast, quercetin is oxidized and yields superoxide and a more reactive quinone, which can interact with Keap1 thiols to induce Nrf2 activation[25, 26]. In addition, quercetin binds to the Nrf2 protein and increases its half-life fourfold[26]. The phosphorylation of Nrf2 influences its abundance and activity, and direct or indirect inhibitors of protein kinase GSK3β can activate Nrf2 signaling[27, 28]. Chlorogenic acid[29], xanthohumol[30], and berberine[31] have been reported to be natural modulators of kinase activities that influence Nrf2 signaling[32]. It was therefore clarified that instead of directly scavenging ROS, ingested antioxidant phytochemicals induce endogenous antioxidant enzymes that neutralize ROS, resulting in the improvement of oxidative stress.

NFκB/ IκB pathway

NF-κB-mediated inflammation pathway: Cross-talk occurs between the Nrf2/Keap1 pathway and the transcription factors NF-κB and p53[33, 34]. NF-κB is a key transcription factor that regulates genes involved in inflammation, immune responses, apoptosis, development, and cell growth[35, 36]. Genes that encode inflammatory proteins, including TNF-α, IL-2 and IL-9, GM-CSF, iNOS, COX-2, and ICAM-1, are inducible via NF-κB[36]. Similar to Nrf2, NF-κB binds to the negative regulator IκBα. IκBα is phosphorylated by the cytosolic protein IKKβ, which it dissociates from NF-κB, and is subjected to proteasomal degradation, which leads to the translocation of NF-κB into the nucleus where it promotes the expression of its target genes[37] (Fig. 5). Interestingly, IKKβ can bind Keap1 and be targeted for ubiquitination like Nrf2. Thus, the binding of Keap1 to IKKβ reduces the concentration of free IKKβ proteins, which decreases IκBα degradation, resulting in the suppression of
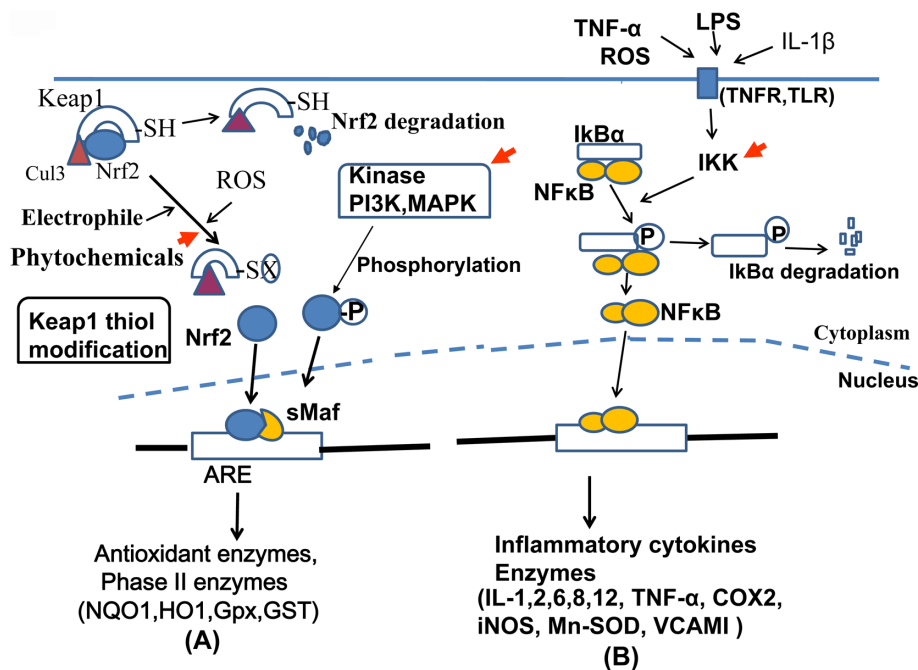
Fig. 5. Effects of antioxidant phytochemicals on Nrf2 and NF-κB signaling pathways. (A) Nrf2/Keap1 pathway. Nrf2 is normally present in the cytoplasm bound to Keap1 and sequestered by proteasomal degradation through Keap1-associated Cul3-Rbx E3 ubiquitin ligase. Nrf2 is activated through two mechanisms. The first mechanism is by modification of the thiols of Keap1, which leads to conformational changes in this protein and subsequently the release of Nrf2. The second mechanism involves the activation of kinases that phosphorylate Nrf2 and thereby free it from Keap1-mediated sequestration. After nuclear translocation, Nrf2 with sMaf binds to antioxidant responsive elements (AREs) on DNA and activates the transcription of antioxidant enzyme genes. Curcumin, sulforaphane, and quercetin activate Nrf2 by the first mechanism, whereas resveratrol and capsaicin function through the second mechanism. (B) NF-κB/IκB pathway. Oxidative stress and ligands of TNFRs and TLRs activate the upstream IκB kinases (IKKs) of NF-κB, resulting in the phosphorylation of IκB, which is usually bound to the inactive NF-κB dimer in the cytoplasm. IκB is then targeted for proteasomal degradation, and NF-κB moves into the nucleus, where it induces the expression of inflammatory cytokines and proteins involved in the adaptive stress response. Phytochemicals can modulate IKKs and thereby inhibit the inflammatory reaction. TLR, Toll-like receptor; TNFR, tumor necrosis factor receptor. Bold arrows indicate the targets of antioxidant phytochemicals.

NF-κB translocation into the nucleus[38]. This may be the elusive mechanism by which Nrf2 activation inhibits NF-κB activation. When Nrf2 is released by oxidative events, there is an increase in the intracellular pool of unbound Keap1 available to capture more intracellular IKKβ, consequently inhibiting the expression of the target genes of NF-κB. Thus, either the inhibition of NF-κB signaling or activation of Nrf2 signaling can exert an anti-inflammatory activity by inhibiting pro-inflammatory enzymes and/or inducing antioxidant enzymes[34].

Phytochemicals affecting the NF-κB pathway: Since NF-κB translocation into the nucleus is regulated by the phosphorylation of IκBα, inhibitors of IκBα phosphorylation are capable of exerting a physiological anti-inflammatory action. Various phytochemicals, including sulforaphane and curcumin, inhibit NF-κB by interfering with DNA binding of NF-κB and blocking the phosphorylation and degradation of IκB[39, 40]. Antioxidant polyphenols can inhibit enzymes associated with pro-inflammatory properties such as COX-2, LOX, and iNOS[41].

p53-mediated regulation of oxidative stress

p53 is a DNA sequence-specific transcriptional regulator that plays important roles in DNA damage response and repair, cell cycle regulation, and triggering apoptosis after cell injury. The role of p53 in the cell is determined by the type, intensity, and duration of imposed oxidative stress[42]. In response to low levels of oxidative stress, p53 exhibits antioxidant activities that contribute to the elimination of oxidative stress and ensure cell survival, whereas in response to high levels of oxidative stress, p53 exhibits pro-oxidative activities that further increase the levels of stress, leading to apoptotic cell death[43]. In the apoptotic response, p53 acts as a regulator of the apoptotic process that can modulate key control points in both the extrinsic and intrinsic pathways. Specifically, it can promote apoptosis by inducing the transcription of pro-apoptotic members of the Bcl-2 family such as Bax and by exerting direct effects on mitochondrial membranes (Fig. 6). The functional efficacy and stability of p53 are modulated by phosphorylation through the stress-responsive mitogen activated protein kinases (MAPKs). Therefore, phytochemicals that modulate MAPKs may prevent apoptosis and oxidative stress[44, 45].
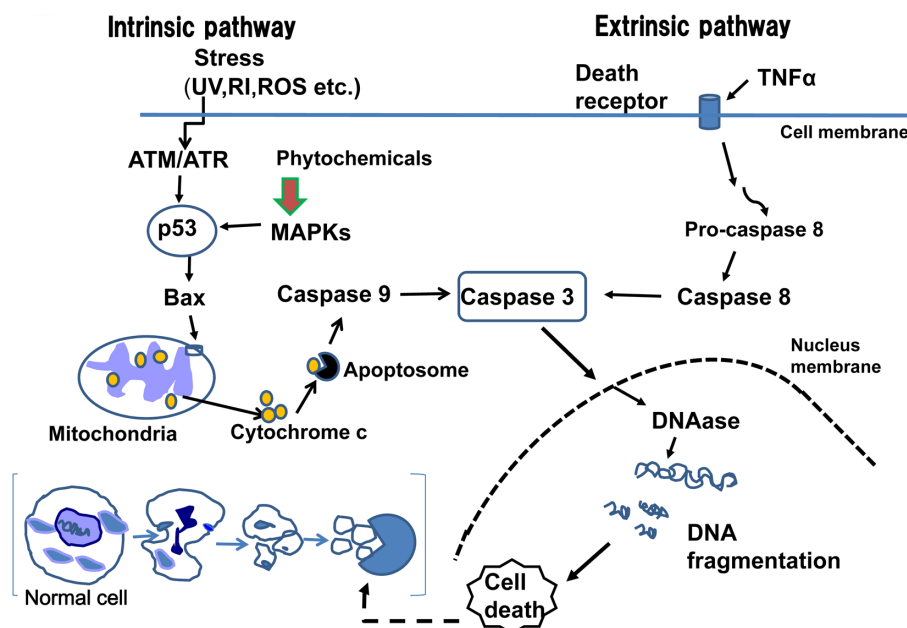
Fig. 6. Oxidative stress-induced apoptosis. In response to low levels of oxidative stress, p53 exhibits antioxidant activities that contribute to the elimination of oxidative stress and ensure cell survival, whereas in response to high levels of oxidative stress, p53 exhibits pro-oxidative activities that further increase the levels of stress, leading to cell death via apoptosis. p53 can promote apoptosis by inducing the transcription of pro-apoptotic members of the Bcl-2 family such as Bax and by its direct effects on mitochondrial membranes. TNFα causes apoptosis through death receptors (i.e., the extrinsic apoptotic pathway).

Effect of Antioxidants on Protein Kinase Signaling Pathways

As mentioned above, antioxidant phytochemicals directly or indirectly modulate transcription factors, including Nrf2, NF-κB, and p53, leading to antioxidant, anti-inflammatory, and apoptotic actions. Since the activities of these transcription factors are regulated by direct phosphorylation or the phosphorylation of their associated proteins, antioxidant phytochemicals capable of modulating protein kinase activity can influence them[46]. Protein kinases are a large family of approximately 530 highly conserved enzymes that catalyze the transfer of a γ-phosphate group from ATP to a variety of amino acid residues of proteins in a process known as cellular signal transduction.

MAPK pathways: Stressors or ligands bind to receptors on plasma membranes by which the stress signals are transmitted through a consecutive series of phosphorylation events which is termed the mitogen-activated protein kinase (MAPK) cascade and finally activate MAPKs. MAPKs include ERKs (extracellular signal regulated kinases), JNKs (c-Jun NH$_2$-terminal kinases), and p38 MAPKs. In response to a variety of cellular stimuli, including osmotic shock, pro-inflammatory cytokines, lipopolysaccharides, UV light, oxidative stress, and growth factors, MAPKs can phosphorylate various proteins, including transcription factors such as NF-κB, p53 and AP-1, which regulate cell proliferation and differentiation, cell cycle arrest, the activation of immunocytes, and apoptosis[46] (Fig. 7). Several flavonoids (antioxidant phytochemicals) have been shown to interact

with ERK, JNK, and p38 MAPKs[47, 48]. In a molecular docking study, quercetin could be docked to the MEK1 pocket, which is located separately from but adjacent to the ATP binding site. The binding of quercetin blocks the Raf/MEK/ERK/p90RSK pathway and leads to the suppression of AP-1 and NF-κB activities[49].

PI3K/Akt signaling pathway: Phosphatidylinositol 3-kinase (PI3K) typically becomes activated after a receptor with tyrosine kinase activity, such as an insulin receptor, is activated by its ligand binding to the receptor and then phosphorylates phosphatidylinositol-2, leading to the generation of phosphatidylinositol-3. Phosphatidylinositol-3 activates protein kinase B (Akt), which can phosphorylate various proteins, including GSK-3β, FOXO1, TSC1/2, p21, p27, etc.[50] (Fig. 7). The PI3K/Akt pathway is one of the strongest intracellular pro-survival signaling systems. Inhibition of the PI3K pathway abolishes cell survival and accelerates apoptosis, whereas the activated form of Akt blocks apoptosis[51]. It has been shown that various phytochemicals, including quercetin, resveratrol, epigallocatechin 3-gallate (EGCG), and curcumin, inhibit the PI3K/Akt pathway[51].

AMPK: AMP-activated kinase (AMPK) is a highly conserved sensor of increased levels of AMP and ADP originating from ATP depletion[52, 53]. AMPK is activated by an elevated AMP/ADP concentration via allosteric regulation and also by phosphorylation through several upstream kinases, including LKB1 and CaMKKβ. AMPK stimulates energy production from glucose and fatty acids during stress and inhibits energy consumption for protein, cholesterol, and glycogen synthesis. Many physiological
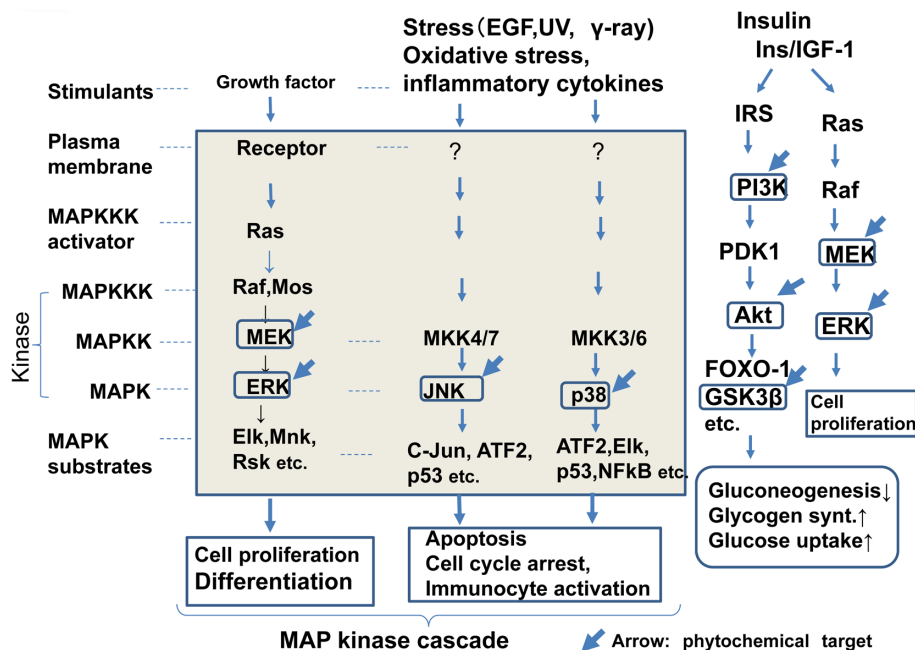
Fig. 7. Effects of antioxidant phytochemicals on protein kinase signaling pathways. Stressors or ligands bind to receptors on plasma membranes by which the stress signals are transmitted through a consecutive series of phosphorylation events, which is termed the mitogen-activated protein kinase (MAPK) cascade, and finally activate MAPKs, including ERK, JNK, and p38. MAPKs thus activated can phosphorylate multiple proteins, including transcription factors such as p53 and NF-κB, leading to the modulation of cell proliferation, differentiation, cell cycle arrest, apoptosis, and immunocyte activation. In insulin signaling, the signal is transmitted through the PI3K/Akt and MAPK pathways. Antioxidant phytochemicals target multiple kinases that are indicated with bold arrows. MEK, mitogen-activated kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IGF-1, insulin-like growth factor-1; IRS, insulin receptor substrate; GSK, glycogen synthase kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B.

conditions, including exercise and calorie restriction (CR), can stimulate AMPK activity, whereas nutritional overload seems to impair AMPK activity and concurrently induce insulin resistance in many tissues, thus promoting the development of metabolic syndrome[54]. AMPK can activate the SIRT1, ULK1, Nrf2, FOXO, and p53 pathways and inhibit the signaling of CRTC-1, mTOR, and NF-κB. Thus, phytochemicals that can activate or inhibit AMPK activity can modulate these signaling pathways. For example, quercetin causes AMPK activation, which in turn stimulates translocation of the glucose transporter GLUT4 from the cytosol into the plasma membranes, resulting in an enhancement of glucose uptake through GLUT4[55]. Phytochemicals such as resveratrol, epicatechin, EGCG, and curcumin have been found to activate AMPK[55].

mTOR: Mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is involved in the signaling pathways induced by growth factors, abundant nutrients, and a sufficient energy status and acts as a key controller of cellular aging[56]. mTORC1 is activated by insulin and other growth factors through the PI3K/Akt pathway and is inhibited by AMPK[57]. CR is a natural method that retards aging via mTORC1 inhibition and provocation of autophagy. Autophagy is a cellular housekeeping and protein quality control mechanism that can remove damaged or defective proteins and organelles such as mitochondria and recycles

amino acids during periods of starvation[58]. AMPK can be activated by CR, which in turn inhibits mTOR signaling, resulting in an enhancement of autophagy and consequently an extension of lifespan[59]. Inhibition of the mTOR pathway extends lifespan in model organisms and confers protection against age-related pathologies. Many phytochemicals, including quercetin, resveratrol, and EGCG, have been reported to modulate mTOR[60].

Modulation of Signaling Pathways by Sirtuins

While phytochemicals modulate intracellular signaling pathways by acting on transcription factors and protein kinases, the activity of those proteins is also modulated by acetylation/deacetylation reactions. Sirtuins (SIRT1–7) are NAD^+-dependent deacetylases that are distributed in almost all tissues and play central roles in cell survival, inflammation, energy metabolism, and aging[61]. CR has been considered as a potentially robust means of delaying the onset of aging-related diseases and slowing the aging process[62]. Sirtuins extend life span in a variety of species and mediate physiological adaptations to CR and many of the health benefits caused by CR[63]. SIRT1, which is predominately a nuclear protein, deacetylates the histones H3, H4, and H1 but also modifies more than 50 non-histone proteins, including transcription factors and DNA repair proteins[61]. It

is known that transcription factors, including p53, NF-κB, PGC1α, FOXO, and SREBP, are modulated by deacetylation via SIRT1. Additionally, SIRT1 can indirectly activate the energy sensor AMPK through deacetylation of the AMPK kinase LKB1; specifically, SIRT1 activates AMPK by enhancing the phosphorylation of AMPK via the deacetylation-induced activation of LKB1.

Various synthetic and natural compounds, including resveratrol, have been shown to directly activate SIRT1. These compounds, which are called sirtuin-activating compounds (STACs), can activate SIRT1 by binding to the allosteric, STAC-binding domain and primarily lowering the K_m for the peptide substrate, thereby increasing its catalytic activity. Deletion and mutation studies of SIRT1 have clarified that the N terminus of SIRT1 is a key mediator of allosteric activation, and the substitution of Glu230 with Lys at the N-terminus prevents its activation by resveratrol and synthetic STACs, indicating that both natural and synthetic STACs activate SIRT1 by a common mechanism[64]. Resveratrol, fisetin, and butein have been reported to activate SIRT1 as natural STACs and to extend life span in a wide variety of organisms, including yeast, flies, and obese mice[65].

Xenohormesis (Interspecies Hormetic Activity)

As mentioned above, antioxidant phytochemicals show multiple target actions, including the modulation of protein kinases, deacetylases, and transcription factors such as Nrf2, NF-κB, and p53 (Fig. 8). Recently, these biological actions caused by phytochemicals have been explained as a hormetic action (xenohormesis) or an adaptive response. Hormesis is a term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by a stimulatory or beneficial effect at low doses and an inhibitory or toxic effect at high doses[66]. In the fields of biology and medicine, hormesis is defined as an adaptive response of cells and organisms to moderate stress. For example, ischemic preconditioning, exercise, dietary energy restriction, and exposure to low doses of certain phytochemicals are known to cause a hormetic response. Graphically, hormetic stress response is defined by a nonlinear and biphasic dose-response curve, which could be a U-shaped or inverted U-shaped curve[67] (Fig. 9). Phytochemicals are structurally diverse secondary metabolites synthesized by plants and also by nonpathogenic endophytic microorganisms living within plants. Plants synthesize phytochemicals, in part, as a response to such hormetic environmental stresses as UV light, heat or cold stress, osmotic stress and high salinity, water deficit/dehydration, nutrient deprivation, and infection[68]. The phytochemicals thus synthesized are present within the plant at concentrations that are not toxic but create mild stress and protect the plant against higher doses of the environmental stress. Animals that ingest such phytochemicals may also mount a hormetic response against the phytochemicals.

Howitz and Sinclair proposed the concept of xenohormesis to explain why phytochemicals can cause hormetic responses in animals, including humans[69]. In xenohormetic responses, heterotrophs (i.e., animals and fungi) are able to sense chemical cues that are synthesized by plants and other

Cellular mechanisms of antioxidant phytochemicals

- Upregulation of antioxidant enzymes through Nrf2/Keap1
- Anti-inflammatory action via NF-kB
- Action on protein kinase signaling（MAPK,AMPK,mTOR etc.）
- Protein quality control via HSF-1
- Acetylation and deacetylation (SIRT1)
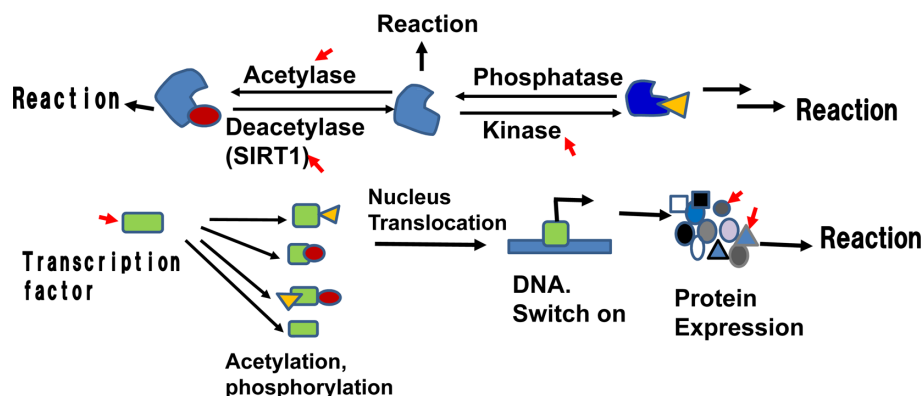- Epigenetic action

Fig. 8. Summary of the multiple targeted actions of antioxidant phytochemicals. Multiple proteins, including protein kinases, deacetylases, transcription factors and their associated proteins, and enzymes, are targeted with phytochemicals, leading to the modulation of intracellular signaling pathways. Bold arrows indicate the targets of these phytochemicals.

autotrophs in response to stress. In essence, xenohormesis refers to interspecies hormesis, such that an animal or fungal species uses chemical cues from other species about the status of its environment or food supply to mount a preemptive defense response that increases its chances of survival. It means that animals have evolved the ability to sense signaling and stress-induced molecules from other species and that they are under selective pressure to do so[68, 70, 71]. That is, xenohormesis is a biological principle that explains how environmentally stressed plants produce bioactive compounds that can confer stress resistance and survival benefits to animals that consume them (Fig. 10). The molecular mechanisms of the hormetic responses induced by phytochemicals have been shown, which include the activation of Nrf2, NF-κB, sirtuins, and protein kinases[34, 70], and the amplitude of the hormetic stimulation is modest, with the degree of activation of the proteins typically reaching a maximum of only 30–60% greater than the control group[67, 70].

It is intriguing that phytochemicals act as chemical signals between plants and animals and induce hormetic responses in animals, suggesting that the adaptive response mechanisms triggered by phytochemicals synthesized by

Hormetic response
(*Calorie restriction, Exercise, Phytochemicals*)

Maximum benefit
30~60% greater than the control group

Nrf2, Kinase, SIRT1 etc.

Beneficial

Harmful

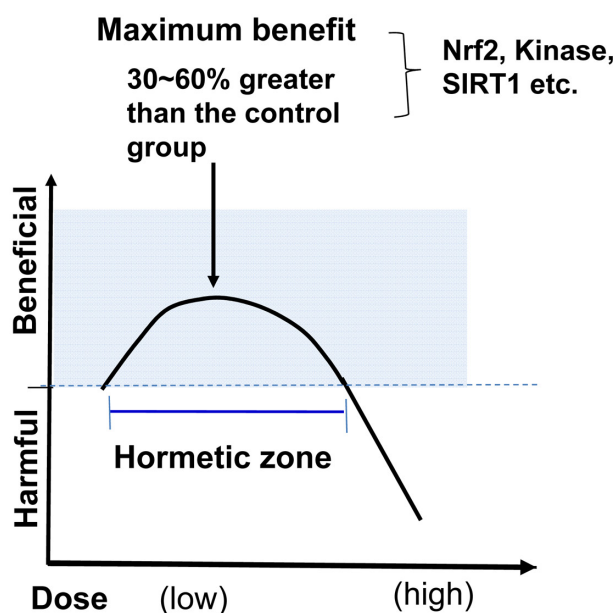**Hormetic zone**

Dose (low)   (high)

Fig. 9. Hormetic response curve. Antioxidant phytochemicals cause a hormetic response, with a stimulatory or beneficial effect at low doses and an inhibitory or toxic effect at high doses. Within the hormetic dose range, the maximum response is no more than 30–60% greater than the control group. The hormetic response is a stress-adaptive response that is mediated through the Nrf2/Keap1, NF-κB, sirtuin, and protein kinase signaling pathways.

plants may have been evolutionarily conserved between plants and animals. It is also apparent that such phytochemicals interact simultaneously with multiple proteins consisting of stress-responsive signaling pathways. Phytochemicals seem to show nonspecific binding with multiple proteins, leading to a systemic adaptive reaction.

Thus, various phytochemicals, including sulforaphane, resveratorol, curcumin, epigallocatechin gallate, and quercetin, have been shown to elicit a hormetic stress response in heterotrophic organisms[34, 68, 70]. These phytochemicals are likely to operate as hormetic stress agents within both the host plants synthesizing them and the heterotrophic organisms exposed to them. Molecular mechanisms of these hormetic antioxidant phytochemicals and their beneficial effects on neurodegenerative diseases and cancer have been comprehensively reviewed[34, 70].

Phytochemicals as Pan-assay Interference Compounds(PAINS)

Although numerous studies about the actions of dietary phytochemicals on multiple target proteins have been shown *in vitro* using cell lines, it should be taken in consideration that some phytochemicals have the features of PAINS *in vitro*[72, 73]. PAINS can display apparent bioactivity and/or interfere with assay readouts across multiple unrelated biological targets and testing methods. Promiscuous behaviors of PAINS that can contribute to assay interference include chemical aggregation, chelation, singlet oxygen production, compound fluorescence effects, redox activity, sample impurities, membrane disruption, cysteine oxidation, and nonselective compound reactivity with proteins[74]. Phytochemicals, including dietary polyphenolic molecules (flavonoids and diarylheptanoids), phytosterols, and monoterpenes, show several of these behaviors and are classified as PAINS. A previous review article described how curcumin can be classified as both a PAINS and an invalid metabolic panacea candidate[73]. That article explained why curcumin has not been developed as a therapeutic drug despite numerous research efforts, whereas artemisinin, which was discovered from a plant used in a traditional Chinese medicine (*Artemisia annua*) was developed as an effective therapeutic agent for malaria. In relation to PAINS, many of the effects on membrane proteins that are induced by amphiphilic phytochemicals such as polyphenols have been suggested to be due to cell membrane perturbations rather than specific protein binding[75].

The pan-assay interference property of phytochemicals seems to have been long overlooked in research on the development of healthy foods and preventive medicines or drug discovery from natural products. Thus, when researchers evaluate the functions of phytochemicals *in vitro*, the specific actions of the phytochemicals need to be carefully distinguished from the general actions of PAINS.
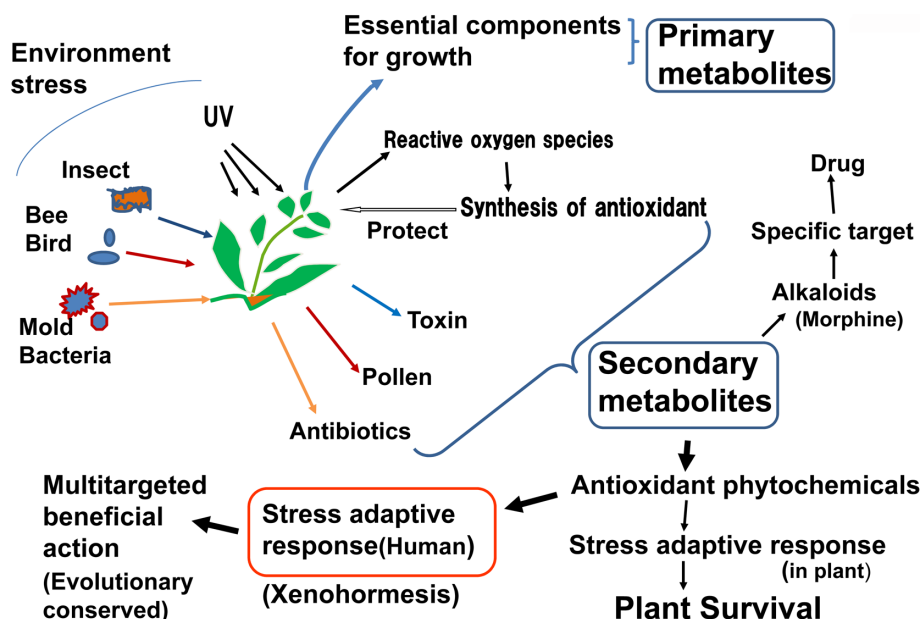
Fig. 10. Xenohormesis. Because plants cannot physically move away from environmental stresses, including temperature variation, water or nutrient availability, reactive oxygen species generation by UV light, and attack from predators, plants have evolved stress-adaptive responses involving the synthesis of phytochemicals as secondary metabolites. That is, phytochemicals are synthesized in plants as a response to environmental stimuli, by which plants protect themselves against stress through a stress-adaptive response, known as a hormetic action. Animals cannot synthesize the phytochemicals, but their cells can sense them and subsequently undergo a stress-adaptive response that appears to have been evolutionarily conserved between plants and animals. This hormetic action, phytochemical-induced hormetic action, which is found in animals including humans, is recognized as xenohormesis. Xenohormesis is a biological principle that explains how environmentally stressed plants produce bioactive compounds that can confer stress resistance and survival benefits for animals that consume them.

Summary and Personal Views

In our research to develop healthy foods and preventive medicines from edible and medicinal herbs in Okinawa, we focused on the antioxidant activities of those resources. As expected, an increase in the antioxidant activities of edible herbs caused by UV irradiation was confirmed, and typical antioxidant phytochemicals such as quercetin, chlorogenic acid, and gallic acid derivatives were isolated from the herbs. The extracts from these herbs showed antidiabetic, hepatoprotective, cancer preventive, and cardioprotective actions *in vivo*; however, the concentrations of the herb-derived antioxidant compounds used *in vivo* seem to be lower than the concentrations that show radical scavenging activities *in vitro*.

The gap between the phytochemical concentrations in animals and *in vitro* has been pointed out by numerous researchers based on studies in which antioxidant phytochemicals have caused multiple biological effects at low concentrations that seem to be unrelated to their direct radical scavenging activity. For example, certain antioxidants promote the genetic expression of antioxidant enzymes via the activation of the Nrf2 transcription factor. Thus, accumulating evidence has emerged that antioxidant phytochemicals can act not only on transcription factors (e.g., Nrf2, NF-κB, and p53) but also on various enzymes, including deacety-

lases (e.g., SIRT1) and protein kinases such as AMPK, MAPKs, PI3K/Akt, or mTOR, which can modulate cellular signaling pathways. This means that instead of acting solely through radical scavenging, antioxidant phytochemicals interact with multiple proteins that are constituents of cellular signaling pathways and thereby modulate the signaling activities of those pathways.

Why and how can antioxidant phytochemicals exert such multitargeted and beneficial effects in animals? The xenohormesis concept has been proposed as an answer to this question[69]. Phytochemicals are synthesized in plants as secondary metabolites in response to environmental stimuli and protect plants against stresses related to such stimuli, which is defined as a hormetic or stress-adaptive response. Animals, including human, cannot synthesize phytochemicals, but our cells can sense them and subsequently undergo a stress-adaptive response that appears to have been evolutionarily conserved between plants and animals. Xenohormesis explains from the viewpoint of stress response evolution why plants produce bioactive phytochemicals and how the human body can undergo beneficial adaptations in response to such phytochemicals. This intriguing evolutionary perspective on mammalian responses to phytochemicals encourages us to reconsider the biological activities of plant-derived antioxidants as well as xenobiotics, including drugs and toxins.

Taking into consideration of xenohormetic effects of

antioxidant phytochemicals, the beneficial effects of a high consumption of vegetables or fruits, which lowers the risk for lifestyle-related diseases, can be explained by the stress-adaptive response to dietary phytochemicals. Indeed, recent studies on plant polyphenols as preventive medicines for age-related diseases seem to have recognized that the multitargeted, beneficial, and nontoxic effects of polyphenols on animals come from the xenohormetic action of polyphenols[76].

In conclusion, it is important to realize that antioxidant phytochemicals are the products of evolutionary adaptation to stress by plants, that humans have evolved stress-adaptive responses to these compounds, and that multitargeted, beneficial effects of antioxidant phytochemicals on humans result from a cellular stress-adaptive response of our cells to the phytochemicals. For the development of antioxidant phytochemicals as healthy foods and preventive medicines, the following points should be considered: 1) antioxidant phytochemicals often show a biphasic dose-response curve with beneficial effects at low doses, 2) the biological actions of phytochemicals should be carefully distinguished from those of PAINS, 3) the bioavailability of phytochemicals depends not only on their metabolism in the small intestine but also on their catabolism by colonic microbiota[77], and 4) polypharmacology and network pharmacology approaches focused on understanding the pleiotropic effects of antioxidant phytochemicals are needed.

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