ABSTRACT
Polyphenols in food plants are a versatile group of phytochemicals with many potentially beneficial activities in terms of disease prevention. In vitro cell culture experiments have shown that polyphenols possess antioxidant properties, and it is thought that these activities account for disease-preventing effects of diets high in polyphenols. However, polyphenols may be regarded as xenobiotics by animal cells and are to some extent treated as such, ie, they interact with phase I and phase II enzyme systems. We recently showed that dietary plant polyphenols, namely, the flavonoids, modulate expression of an important enzyme in both cellular antioxidant defenses and detoxification of xenobiotics, ie, γ-glutamylcysteine synthetase (GCSh). This enzyme is rate limiting in the synthesis of the most important endogenous antioxidant in cells, glutathione. We showed in vitro that flavonoids increase expression of γ-glutamylcysteine synthetase and, by using a unique transgenic reporter mouse strain, we showed increased expression in vivo, with a concomitant increase in the intracellular glutathione concentrations in muscles. Because glutathione is important in redox regulation of transcription factors and enzymes for signal transduction, our results suggest that polyphenol-mediated regulation of glutathione alters cellular processes. Evidently, glutathione is important in many diseases, and regulation of intracellular glutathione concentrations may be one mechanism by which diet influences disease development. The aim of this review is to discuss some of the mechanisms involved in the glutathione-mediated, endogenous, cellular antioxidant defense system, how its possible modulation by dietary polyphenols such as flavonoids may influence disease development, and how it can be studied with in vivo imaging.

KEY WORDS Polyphenols, blueberries, glutathione, γ-glutamylcysteine synthetase, gene regulation

OXIDATIVE STRESS AND ANTIOXIDANTS
The hallmark of oxidative stress is increased production of reactive oxygen species (ROS), in amounts that exceed cellular antioxidant defenses. The consequence of oxidative stress may be oxidative damage of lipids, proteins, and DNA, with subsequent disease development and aging (1). ROS production may result from exogenous factors such as radiation and drug exposure or endogenous factors such as increased mitochondrial respiration and oxidative enzymes in infections and inflammation. A possible mechanism for the protective effects of fruits and vegetables with respect to disease is that bioactive compounds in these food items reduce oxidative stress. Fruits and vegetables contain several thousand structurally diverse phytochemicals, of which a large fraction are polyphenols (2). Many polyphenols have antioxidant properties (ie, reductants) and may react directly with reactive chemical species, forming products with much lower reactivity. Alternatively, compounds in a plant-based diet may increase the capacity of endogenous antioxidant defenses and modulate the cellular redox state. Changes in the cellular redox state, conveying physiologic stimuli through regulation of signaling pathways, may have wide-ranging consequences for cellular growth and differentiation (3). In addition, it has been well documented that polyphenols modulate protein kinase activities (4), serve as ligands for transcription factors (5), and modulate protease activities (6).

Many dietary polyphenols are antioxidants, and the possibility exists that they protect against oxidative damage by directly neutralizing reactive oxidants. We recently measured antioxidant capacity (ie, electron- or hydrogen–donating capacity) systematically in a variety of dietary plants, including various fruits, berries, vegetables, cereals, nuts, spices, and pulses used throughout the world. Our results (7) demonstrated a >1000-fold difference in total antioxidant capacity among dietary plants. Interestingly, berries (particularly blackberries, blueberries, and elderberries) high in antioxidant capacity are among the plants with the highest concentrations of polyphenols. This is intriguing, because many berries have been shown to protect against oxidative stress-related pathologic conditions in vivo. For example, Joseph et al (8, 9) demonstrated that long-term feeding of blueberries to rats retarded and even reversed the onset of age-related neurologic dysfunctions, such as a decline in neuronal signal transduction, and cognitive, behavioral, and motor deficits. Stoner and coworkers (10, 11) showed that supplementation with black raspberries in the diet reduced the multiplicity and incidence of esophageal tumors in N-nitrosomethylbenzylamine-treated rats.

POLYPHENOLS AND γ-GCSH GENE REGULATION
The most important endogenous antioxidant defense systems are composed of the thiol-containing tripeptide glutathione and...
small thiol-containing proteins such as thioredoxin, glutaredoxin, and peroxiredoxin. Of these, glutathione is found at millimolar concentrations in most cells and is the major contributor to the redox state of the cell. Glutathione exists in cells in both a reduced form (GSH) and an oxidized form (GSSG); it may also be covalently bound to proteins through a process called glutathionylation (12, 13). The ratio of GSH to GSSG is determined by the overall redox state of the cell. Glutathione is synthesized enzymatically by γ-glutamylcysteine synthetase (γGCS) and glutathione synthetase, with the former being the rate-limiting enzyme (14).

One important task for cellular glutathione is to scavenge free radicals and peroxides produced during normal cellular respiration, which would otherwise oxidize proteins, lipids, and nucleic acids. One mechanism operating to counteract oxidative damage involves transactivation of genes encoding enzymes that participate in glutathione metabolism and synthesis. Typically, these enzymes belong to the phase I and II families of detoxification genes. Analyses of promoter regions of these enzymes suggest that several gene response elements may be involved in such transcriptional regulation, including xenobiotic response elements and antioxidant/electrophil response elements (AREs/EpREs) (15, 16). The latter is defined by a specific consensus sequence of nucleotides (17) and responds to substances with antioxidant properties (18).

Polyphenols are among the most abundant phytochemicals in human food items and, of these, flavonoids are probably the most well studied. The mechanism of action of flavonoids in cellular processes is still not clearly understood. In a series of experiments, we showed that relatively low concentrations of flavonoids stimulated transcription of a critical gene for GSH synthesis in cells (19). Transcriptional control of the catalytic subunit of γGCS, γGCSH, is mediated by a 5' flanking region containing several response elements, including AP-1 sites, one NF-κB site, and several AREs/EpREs (20). Both onion extracts and pure flavonoids transactivated γGCSH through antioxidant response elements in the promoter in both COS-1 cells (19) and HepG2 cells (Figure 1), with quercetin being the most potent flavonoid. Structurally similar flavonoids were not as potent; myricetin, with only one hydroxyl group more than quercetin, was inactive, which emphasizes the apparent specificity of γGCSH induction.

Flavonoids in food items are conjugated to various sugar molecules, which likely influence their intestinal absorption, transport, and entry into cells. Conjugates of quercetin were found to be totally inactive in tissue cell cultures (19), which emphasizes the importance of in vivo experiments, in which the activity of tested substances reflects bioavailability, metabolism, cellular activity, and excretion of micronutrients. To this end, we developed a transgenic mouse strain with ~3.8 kilobases of the γGCS promoter upstream of luciferase incorporated into its genome. We used this mouse strain to test the ability of polyphenol-rich berries to modulate γGCSH gene expression, with a novel in vivo whole-body or ex vivo whole-organ imaging technique (20) and analysis of gene expression in tissue homogenates and sections (21) (Figure 2). For monitoring of basal γGCSH promoter activity in the transgenic mice, β-luciferin was injected intravenously and the mice were placed on their dorsal sides in a light-sealed chamber and underwent imaging with an ultrasensitive video camera. Strong luminescence was detected from the whole animal, reflecting ubiquitous expression of γGCSH and glutathione synthesis. Several organs were then excised and subjected to ex vivo imaging (Figure 2). Interestingly, strong luminescence was emitted from brain, muscle, and epididymis/testis, whereas kidney and lung demonstrated medium luminescence and liver, spleen, and heart showed much lower activity. When transgenic animals were fed juice or homogenates made from antioxidant-rich berries for 3–4 wk, we observed that γGCSH promoter activity was modulated in organs such as skeletal muscle, brain, and liver (21). We also measured glutathione concentrations in the organs in which modulation of the γGCSH promoter was observed. The only organ in which we could detect statistically significant increases in total glutathione concentrations was gastrocnemius muscle (Figure 3).

In vivo feeding experiments with polyphenol-rich diets revealed large differences in γGCSH promoter activity responses among individual animals. Some animals responded and some did not. One possible explanation for this phenomenon may be related to differences in bacterial populations in the gut microbial flora influencing the extent of enzymatic hydrolysis of polyphenol conjugates. Indeed, Aura et al (22) showed that conjugates of flavonoids are hydrolyzed by the gut flora among humans, and several studies demonstrated that sugar moieties influence enterocyctic uptake and thus tissue distribution (reviewed in ref. 23).

MECHANISMS OF ACTION OF FLAVONOIDS

Gene regulation through binding of the transcription factor Nrf2 to AREs/EpREs has been described in some detail (for review, see ref. 23). Although our studies indicate that the effects...
of polyphenols are mediated through one or several Nrf2 binding sites (ie, AREs/EpREs), several activities of flavonoids could be responsible for the effect on the γGCSH promoter. Nrf2 is inactivated through cytosol sequestering through binding to the cytoskeleton-associated protein Keap1. Binding of Nrf2 to Keap1 is thought to be dependent on critical thiols in Keap1, thiols that are possibly sensitive to the cellular redox state. Therefore, release and subsequent translocation of Nrf2 to the nucleus are presumably sensitive to cellular oxidative stress, thiol-reactive compounds, and antioxidants (24) (Figure 4). Because ARE/EpRE-containing gene promoters are thought to be regulated by substances with antioxidant properties, a possible explanation for the different γGCSH promoter–inducing activities could be that some flavonoids are better antioxidants than others. However, when we measured the ability of flavonoids to reduce Fe3+ in the ferric ion-reducing activity assay, which is commonly used to measure total antioxidant capacity in biologic samples (25), we found that the antioxidant capacity did not correlate with γGCSH-inducing activity in COS-1 cells. Myricetin had a higher antioxidant capacity than did kaempferol but it did not transactivate the γGCSH promoter (19) (Figure 5), which suggests that transcriptional γGCSH regulation depends on other properties of the flavonoids.

Several activities of flavonoids should be considered in this respect. First, flavonoid antioxidant scavenging of free radicals often involves formation of a radical of the flavonoid itself. Quercetin is oxidized to a quinone when serving as an antioxidant, and Boots et al (26) recently showed that such quinones react with thiols. Therefore, it could be speculated that free radical-oxidized quercetin reacts with thiols in Keap1, the key regulatory protein in transcriptional regulation of antioxidant-responsive genes through Nrf2.

Second, the chemical properties of some antioxidants may also give them prooxidant properties (27), and this should be considered with respect to mechanisms for induction of cellular antioxidant defenses. Flavonoids can auto-oxidize (27), and products of auto-oxidation can possibly react with or otherwise reduce cellular concentrations of glutathione. Quercetin and myricetin are known to auto-oxidize at physiologic pH (28), and subsequent reduction of glutathione concentrations can possibly explain transcriptional up-regulation of both γGCS subunits (29).

Third, it can be speculated that the effect is mediated by increased mitochondrial production of hydrogen peroxide and superoxide anion. Hodnick et al (30) showed that quercetin and myricetin cause mitochondrial respiratory bursts. However, myricetin was found to be the most efficient enzyme inhibitor and producer of mitochondrial respiratory bursts, whereas kaempferol was the least efficient and quercetin was intermediate. Therefore, there seems to be no correlation between the ability to
modulate mitochondrial respiration and the γGCS promoter induction observed in our studies (19).

The aforementioned explanations depend on potentially deleterious effects of flavonoids. How deleterious effects may be translated into disease-preventing effects may not be obvious, but the principle of hormesis has been suggested to explain how repeated challenges, such as repeated low oxidative stress, may adapt cells and organs and in the long term make them more resistant to severe oxidative stress (31). The hormetic principle has been proposed to explain why physical activity and exercise, which increase oxidative stress in various tissues, can significantly decrease the risk of disease development (32). It is possible that repeated mild cellular oxidative stress induced by flavonoids through the diet boosts cellular antioxidant defense systems and in the long term shifts these defense systems to a higher steady state, which prevents disease development or reduces the impact of oxidative stress when disease occurs. Nrf2-mediated transactivation is also influenced by phosphorylation (33), making it possible that the protein kinase inhibitory activities of flavonoids may be involved.

CELLULAR EFFECTS OF ALTERED GLUTATHIONE METABOLISM

Detoxification of xenobiotics requires sufficient glutathione synthesis for conjugation and excretion of GSH-conjugated metabolites. Therefore, polyphenol-stimulated glutathione synthesis could well be beneficial in cellular handling of carcinogenic substances, for example. The role of glutathione in detoxification was recently reviewed (34).

The intracellular glutathione concentration and the redox status of the cell are likely to influence regulation of protein function through glutathionylation, ie, covalent binding of glutathione to the protein through disulfide bonds. Until recently, glutathionylation of proteins had been described for only a few cellular proteins. A proteomic approach taken by Fratelli et al (35) identified 38 proteins that are glutathionylated in oxidatively stressed T lymphocytes.

Two mammalian transcription factors known to be glutathionylated are AP-1 and NF-κB (36), but the role of such covalent modification in the cell nucleus is still under debate. Determination of the roles of transcription factors in the regulation of many disease-associated genes in cancer and chronic inflammatory diseases requires detailed studies to establish the influence of cellular glutathione on their activity. Many transcription factors contain thiol groups that have been shown to influence DNA binding. Redox switching and regulation of transcription factors were recently described in great detail, particularly for single-cell organisms (37). Because glutathione is the fundamental redox regulator in eukaryotic cells, it is conceivable that principles of GSH-mediated redox switching of transcription factors can be extrapolated from single cells to multicellular organisms.
Neurodegenerative diseases such as Parkinson’s disease, involving dopaminergic neurons, are associated with neuronal oxidative stress. It was recently shown that the dopamine-synthesizing enzyme tyrosine hydroxylase is inhibited by reversible glutathionylation during oxidative stress (38).

Cellular turnover of proteins is mediated to a large extent by proteasomes. Recent evidence from Demasi and Davis (39) showed that protein turnover attributable to proteasomes is regulated by intracellular glutathione. Those authors found that glutathionylation of 20 S proteasomes and hydrogen peroxide–induced oxidative stress inhibited the proteolytic activity of proteasomes.

Enzymes such as protein kinases and phosphatases have been shown to be modulated by critical thiol groups (for a recent review, see ref 40), and protein kinase A can be regulated directly through glutathionylation (41). Intracellular concentrations of glutathione clearly play a role in the regulation of protein tyrosine phosphatase SHP-1, which is oxidized and inactivated by hydrogen peroxide and can be re-reduced and activated by thiols such as glutathione (42).

Intracellular glutathione also influences immune responses. Production of major immune regulatory cytokines such as interleukin-12 has been shown to be regulated by intracellular glutathione (43). Even cell cycle transitions involving cyclin D1 appear to be redox regulated, because thiol-containing antioxidants delay the transition from G₀ to G₁ and S phases in embryonic mouse cells (44).

GLUTATHIONE AND DISEASE

Oxidative stress is a general term intimately linked to production of reactive oxygen, nitrogen, or iron species and the overall redox state of the cell. Oxidative stress can be assessed by measuring GSH and GSSG, and it is frequently expressed as the ratio between the two. Indeed, the ratio has been suggested as a clinical marker in diseases in which oxidative stress is particularly important. Interestingly, GSH is oxidized to GSSG in an age-dependent manner, possibly reflecting accumulating oxidative stress (45). A decreased ratio between GSH and GSSG is also associated with progression of tumors (46), and decreased total glutathione concentrations were found among patients with chronic diseases, including genitourinary, gastrointestinal, cardiovascular, and musculoskeletal diseases (47). Oxidative stress in general and GSH concentrations in particular are also associated with neurodegenerative disorders and HIV. In Parkinson’s disease, ROS concentrations are increased in parts of the substantia nigra and glutathione concentrations are decreased (48).

Some improvement among patients with early Parkinson’s disease has been observed with administration of glutathione (49). Decreased concentrations of glutathione among HIV-infected individuals may have dual effects. First, decreased concentrations are known to promote apoptosis in the CD4⁺ subtype of T cells (50). Second, it has been shown that intracellular thiols regulate HIV transcription (51) and that GSH increases transcriptional activation of HIV through NF-κB binding sites in the HIV long terminal repeat (52). These combined effects suggest that decreased concentrations contribute significantly to HIV/AIDS development and that supplementation with glutathione precursors (such as N-acetylcysteine) may be of some benefit for HIV/AIDS patients. Corroborating this, Herzenberg et al (53) found that GSH concentrations were associated with survival rates in HIV. Other viruses also seem to be dependent on the intracellular redox state for replication. Expression of late influenza viral proteins was found to be increased when cells were treated to reduce intracellular glutathione concentrations (54).

Alteration of glutathione concentrations may also be associated with tumor development through dependence on glutathione S-transferases and glutathione peroxidases. These enzymes are essential in detoxification of carcinogens and scavenging of ROS. Polymorphisms in various classes of glutathione S-transferases have been associated with increased development of tumors of the lung, bladder, and colon (55) and head and neck (56). Decreased activity of key enzymes involved in glutathione synthesis, accompanied by decreased availability of cysteine synthesis, contributes to mucosal glutathione deficiency in inflammatory bowel disease (57). Glutathione is also critical in the lung, and altered concentrations have been associated with pulmonary diseases such as acute respiratory distress syndrome (58) and chronic obstructive pulmonary disease (59).

CONCLUDING REMARKS

Dietary antioxidants have long been suspected to scavenge reactive ROS and thereby avert deleterious effects on proteins, lipids, and nucleic acids in cells. This has been put forward as one of the major mechanisms for the disease-preventing effects of fruits and vegetables. Our results (19, 21) add modulation of intracellular GSH concentrations to the list of possible disease-preventing effects of polyphenols, with the implication that they modulate GSH-dependent cellular processes, such as detoxification of xenobiotics, glutathionylation of proteins, and regulation of redox switching of protein functions in major cellular processes.

The potential beneficial effects of flavonoids have prompted commercial interest in manufacturing supplements containing high concentrations of flavonoids. However, their concentration-dependent cellular effects, some of which may be harmful, should be of concern with respect to high-dose supplementation. Flavonoids at high concentrations produce reactive radicals through auto-oxidation and increasing mitochondrial respiratory bursts (27, 30). Although the redox potentials of most flavonoid radicals are lower than those of the superoxide and peroxyl radicals (60), the effectiveness of the radicals in generating lipid peroxidation, DNA adducts, and mutations may still be significant in disease development (61). Also of concern is the observation that some flavonoids inhibit enzymes (such as topoisomerases) involved in DNA structure and replication (62), and it has been suggested that high intake of flavonoids predisposes subjects to the development of certain childhood leukemias (63, 64). Flavonoid supplementation as a general recommendation to increase cellular glutathione concentrations may also be troublesome, because glutathione has a major role in overall redox regulation of cell functions and is not suitable as a therapeutic target for substances that alter cellular concentrations by orders of magnitudes. Therefore, the current recommendation is to increase fruit and vegetable consumption. Because the active compounds and the mechanisms involved in disease-preventing effects are still poorly understood, the recommendation is that eating a variety of fruits and vegetables provides the best protection. It remains to be determined whether dietary polyphenols modulate cellular glutathione concentrations among humans and whether they contribute to regulation of major cellular signaling.
pathways, which would explain the indisputable fact that fruits and vegetables protect against disease.

REFERENCES