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# REGULATION OF GENE TRANSCRIPTION BY BOTANICALS: Novel Regulatory Mechanisms

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■ **Abstract** Early investigations of gene regulation revealed that nutrients could modulate gene expression, an example being the discovery of metal-regulated gene transcription (11, 19, 44). Only more recently have we focused on the ability of non-nutritional botanicals or functional food components to affect gene expression at the transcriptional level. Significant findings include the discovery that hyperforin is an active ingredient of the herbal remedy St. John's wort, and activates gene transcription of cytochrome p450-3A4, causing significant botanical-drug interactions. Recently, the lipid-regulating peroxisome proliferator-activated receptors have been studied as receptors activated by soy isoflavones, perhaps explaining the lipid-lowering effect of soy intake. Epigallocatechin gallate has been shown to be an inhibitor of the proteolytic activity of the proteasome; this inhibition has a significant implication for cell proliferation and the stability of transcription factors in the nucleus. Very recently, the effects of botanicals have been studied as activators of sirtuins, important deacetylation enzymes that have been shown to enhance lifespan in a variety of organisms. Sirtuins have been implicated in the lifespan-enhancing effect of caloric restriction. Originally presumed to act mainly on compaction or accessibility of DNA, recent evidence shows important activity of sirtuins as controllers of transcriptional coactivator availability. This review focuses on novel mechanisms by which botanical products regulate cell function via gene transcription. Investigating these newly appreciated mechanisms will assist with the characterization and clarification of specific effects of botanicals on gene expression.

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## INTRODUCTION



The ability to control the expression of gene products is essential for single-celled and multicellular organisms dealing with a changing environment. Multicellular organisms must also be able to regulate gene expression to successfully complete the complex process of development. When considering mammalian physiology, homeostasis must be maintained despite constantly changing conditions related to diurnal, dietary, and other environmental challenges. At the level of the individual cell, maintenance of homeostasis is due in part to the ability of gene expression to be regulated. Simply put, at various times, proteins may need to be present or absent, their relative level increased or decreased, or receptor or enzyme activity altered by covalent modification such as phosphorylation or by the binding of regulatory proteins that may have positive or negative activity on target of interest. Genes may be regulated at many different levels, including transcription, mRNA processing, export from the nucleus, rate of translation, and half-life of both mRNA and polypeptide products (27, 57). Despite the wide array of regulatory mechanisms that control gene expression, it is gene transcription that is most often regarded as the most important aspect of genetic regulation. This role is due to at least two different factors. First, transcription is an initial event and therefore should be properly considered an initiating or committing step in the multistep process that is gene expression. Second, basal levels of gene transcription are often quite low in comparison with the elevated levels produced by the interaction of gene-specific activators and gene-specific DNA elements within the promoter regions of genes. This difference in magnitude between basal and elevated levels of transcription is often the most dramatic mechanism involved in the regulation of most genes. Although other factors such as mRNA turnover or translational efficiency also contribute to regulation, we most often see these mechanisms play a lesser role in regulation. Notable exceptions in nutrient regulation of gene expression do exist, e.g., the translational and post-translational control of transferrin and transferrin receptor by cellular iron concentrations is a hallmark example of post-transcriptional regulation (10, 60).

The activators of gene expression may serve to either inhibit or stimulate transcription by RNA polymerase II. Typically, activators have common structural features that contain activities such as DNA binding, transcription activation, and dimerization domains that may allow activators to bind as either homo- or heterodimers. The DNA-binding domains of transcriptional activators may fall into one of at least three different categories, including zinc-containing domains,

homeodomains, and leucine zippers (bZIP) or helix-loop-helix (bHLH) motifs. The transcriptional activating portions of activators are most often described as acidic, glutamine-rich, or proline-rich domains. Binding of activators to their regulatory sequences in DNA may be affected by availability of the activators themselves (i.e., levels of the activator are changed via either enhanced synthesis, increased import into the nucleus, or by dissociation from inhibitory factors that serve to sequester an activator). The activator may also be covalently modified, gaining enhanced activity as an activator (i.e., via cell signaling processes that may ultimately result in phosphorylation or ubiquitination of the activator). Ligand activation has the ability to modify dramatically the capability of activators to stimulate gene transcription; a notable example is the thyroid hormone receptor. Thyroid hormone (TH) has the ability to bind to regulatory sequences within the promoter of TH-regulated genes. The receptor may bind with its heterodimer partner, the retinoic acid X receptor (RXR) at regulatory sequences, serving as a repressor of transcription until the TH-receptor is activated by binding of TH to the receptor. This ligand-binding event may induce changes in other proteins surrounding the TH-receptor/RXR dimer that subsequently enhances RNA polymerase II binding and transcription (24).

The nuclear receptors are a family of zinc-containing proteins that interact with steroids, hydrophobic hormone molecules, or xenobiotics. The ability of nuclear receptors to bind to these ligands suggests that these proteins have an additional domain—a hormone-binding or ligand-binding domain. The structure of nuclear receptors includes an amino-terminus ligand-independent activation function; a DNA-binding domain containing two highly conserved zinc finger motifs that target the receptor to specific hormone response elements, and a large carboxy terminus region that includes a ligand-binding domain and ligand-dependent activation function (35). The steroid receptors were the first nuclear receptors to be cloned. Since then, a set of 48 related human genes have been identified, which is thought to represent the set of human nuclear receptors (46). The nuclear receptor family includes one set of receptors for which there is a high-affinity ligand-receptor interaction; examples include the estrogen, androgen, and vitamin D receptors. Another set of receptors within the nuclear receptor family include receptors that have a lower affinity for ligand. This class includes receptors that bind dietary or intracellular lipids, such as the liver X receptors (LXRs), the farnesoid X receptor (FXR), and the peroxisome proliferator-activated receptors (PPARs). Importantly, this set of nuclear receptors also includes the pregnane X receptor (PXR), sometimes also called the steroid xenobiotic receptor (SXR). The PXR receptor is among the more "promiscuous" nuclear receptors, allowing it to bind to steroids, toxic bile acids, environmental contaminants, and prescription drugs. Among the set of PXR ligands are many relatively hydrophobic botanical compounds. A third set of nuclear receptors are called the "orphan receptors." Orphan receptors by definition are the group of nuclear receptors for which a specific receptor ligand has not yet been identified. Some orphan receptors may in fact not have a ligand that will activate them.

Although the nuclear receptors offer an immediate model for how botanical products may interact with transcription machinery to affect gene expression, certainly several other mechanisms do exist that would permit botanical compounds to affect gene regulation. In addition to ligand activation, other processes have been implicated in the regulation of transcription factors: phosphorylation state, ubiquitin-dependent changes in protein half-life, and "SUMOlation" (small ubiquitin-related modifiers) of transcription factors (28). For example, SUMOlation may target transcription factors to inactive regions of the nucleus called "PML bodies," so named after localization of the promyelocytic leukemia protein to these poorly understood nuclear structures. The PML bodies have been proposed to serve a role as the storage site or reserve for transcription factors (52). Other covalent modifications of transcriptional machinery include methylation and acetylation. Some of these examples may have been caused by the activation of specific signal transduction pathways. Thus, there are many levels at which botanical compounds may be interacting with the process of transcription. The focus of this review is to provide a small set of examples of how botanical compounds may affect biological function by affecting the process of gene regulation. Our attempt here is to provide readers with a limited set of examples, and to focus on newer, less-reviewed topics rather than on more-documented or frequently summarized research areas.



## BOTANICALS AND NUCLEAR RECEPTOR REGULATION

At present, the most information available regarding the specific regulation of gene transcription by botanical compounds may be as ligands or regulators of nuclear receptors. Among the best described examples are those of PXR activation by hyperforin, an active ingredient of the herbal St. John's wort (SJW), and the regulation of cholesterol and bile acid metabolism by the Ayurvedic medication, guggulsterone. As the latter was the exclusive focus of two recent reviews (53, 54), including one in this journal, we follow below with a discussion of hyperforin as a botanical product with activity as a nuclear receptor ligand.

### The Pregnane X Receptor and Hyperforin

The herbal remedy SJW has long been touted as a natural antidepressant. As an herbal product, the extensive clinical testing associated with the drug development process was not required. Recently, clinical reports suggested that use of SJW was accelerating the metabolism of prescription drugs used concomitantly, such as the antiviral protease inhibitor indinavir (41), used in the treatment of AIDS; the immunosuppressant cyclosporine, administered to organ transplant recipients (48); and oral contraceptives (2). Because these drugs are metabolized by hepatic cytochrome P450-3A4 (CYP3A4), Kliewer and colleagues (22, 32) set out to determine if hyperforin, the active ingredient in SJW suggested to have the antidepressant activity, could in fact be an activator of CYP3A4, a

monooxygenase that hydroxylates many xenobiotics, a committing step in the oxidation and elimination of hydrophobic compounds. Estimates suggest that 60% or more of prescription drugs are metabolized and excreted by a CYP3A4-dependent pathway. A chloramphenicol reporter-based assay was performed in CV-1 cells to compare the ability of three different commercial SJW products with purified hyperforin and other compounds isolated from SJW. Testing also included the specific pregnane X receptor (PXR) ligand, SR12813, and a hallmark inducer of CYP3A4, the antibiotic rifampicin. The cell-based reporter assay demonstrated that extracts prepared from SJW products, SR12813, rifampicin, and hyperforin isolated from SJW were all potent inducers of the PXR-regulated murine CYP3A1-tk-CAT reporter. When used to compare activation of the 3A1 promoter in a dose-response study, hyperforin was found to be ~100-fold more potent than rifampicin. Rifampicin has been a well-studied inducer of CYP3A4 because of interactions known to be associated with antibiotic and other drug therapies. Kliewer and colleagues (22, 32) then demonstrated that hyperforin and SR12813 were nearly identical in their ability to act as a specific ligand for the PXR ligand-binding domain, as verified in a competition binding assay. A $K_i$ for hyperforin was measured at 27 nM. Further proof was provided by demonstrating increased levels of the CYP3A4 mRNA in human hepatocytes treated with extracts from commercially available SJW products along with both rifampicin and hyperforin. These data demonstrate the caution that must be exercised when using nonprescription botanical products. The combination of reporter-based assays, a direct assay of receptor binding, and the subsequent analysis of gene expression in human cells provided a clear picture of PXR activation along with a model for assessment of other botanicals suspected of PXR activation. Following these studies from Moore et al. (32), Watkins et al. (55) demonstrated that hyperforin could indeed bind to the ligand-binding domain of human PXR and was able to crystallize a PXR-hyperforin complex, the structure of which was determined at 2.1 Å resolution. Other groups (59) have confirmed results shown by Moore et al. (32) and Watkins et al. (55).

## Peroxisome Proliferator-Activated Receptors, Soy Isoflavones, and Resveratrol

Soy intake has long been recognized to favorably affect circulating cholesterol levels, especially in hypercholesterolemic individuals (1). Various components of soy have been investigated in an attempt to determine if one or more components might be hypocholesterolemic, including soy protein, soy lipids, the presence of bioactive peptides (or their production during the digestive process), and phytochemicals that include soy sterols, saponins, and isoflavones. Among the community of nutrition researchers, the mechanism responsible for improvements in lipid profiles due to soy intake is still debated. Our own working hypothesis is that there is likely more than one bioactive component in soy, and that the soy isoflavones have a bioactivity that affects lipid metabolism. A recent meta-analysis points to isoflavone intake

as a factor related to improvements in blood lipids (62), although other analyses have not confirmed this relationship (58). Isoflavones are particularly abundant in soy and in clover. In soy, the predominant isoflavones are genistein, daidzein, and glycitein. Metabolic conversion of daidzein into another isoflavone, equol, may be of particular importance because equol may have an unusually high bioactivity (50). The isoflavones were one of the first confirmed examples of an ingredient in a food source being an endocrine disruptor. Early studies identifying isoflavones as bioactive agents determined that clover isoflavones were agonists of estrogenic activity, interrupting the estrus cycle of sheep feeding exclusively on isoflavone-containing red clover (37). Starting with these early findings, a significant amount of research has been directed toward understanding soy isoflavones as phytoestrogens, especially in the context of bone metabolism, cancer, and reproductive function. Because there is a large body of research investigating the estrogenic action of isoflavones, we chose not to discuss them here, but we refer the reader to several reviews that provide a starting point for information on the important topic of isoflavones as phytoestrogens (3, 7, 49).

Although the evidence had become clear that soy isoflavones acted as phytoestrogens, the prospect of isoflavones acting as ligands or activators of other nuclear receptors had not been investigated until recently. Potential candidates would include receptors involved in lipid metabolism that display promiscuous ligand-binding capability. Such candidates would be the PPARs, the LXR, and the FXR. Mangelsdorf and colleagues (43) demonstrated how receptor ligands for the PPARs (fibrates or glitazones), LXR (oxysterols), or FXR (bile acids) could activate gene transcription and alter liver lipid levels, bile acid synthesis, net cholesterol absorption, and lipoprotein metabolism. Known effects of soy intake on blood lipids and lipid metabolism resembled some of the actions caused by activation of these lipid-regulating nuclear receptors. At a qualitative level, it appears that the space-filling chemical structure of isoflavones is similar to that of the fibrates, agonists of PPAR$\alpha$ used to treat hyperlipidemia and type 2 diabetes (8). Thus, in our own laboratories, we formulated the hypothesis that one effect of soy intake on lipid metabolism may be due to isoflavones acting as activators of PPARs. We began a variety of studies utilizing PPAR$\alpha$ and PPAR$\gamma$ expression vectors in a murine macrophage model and completed metabolic studies in the obese Zucker rat (29). Further, we used a panel of chimeric nuclear receptors in HepG2 cells to screen isoflavones and other soy components to determine which compounds might indeed be serving as ligands of nuclear receptors (45).

Two studies published in 2003 indicated that isoflavones are activating PPAR-directed gene transcription. Focusing on effects related to osteogenesis and the effects of the soy isoflavone genistein as an activator of the estrogen receptor and PPARs, Dang et al. (6) studied the effects of genistein on osteogenesis in KS483 and mouse bone marrow cells. Genistein was found to have a biphasic activity on markers of osteogenesis, peaking at a concentration of $\sim$1 $\mu$M in a cell culture trial. However, when following markers for adipogenesis, the dose-dependent effects of genistein were quite different, as the $\sim$1 $\mu$M concentration of genistein

that produced a maximal effect on osteogenic parameters was found to produce a negligible stimulation of adipogenesis. At higher concentrations of genistein, adipogenesis was promoted. When tested in combination with antiestrogen ICI 164,382, the osteogenic activity of genistein at $\sim$1 $\mu$M was reduced, whereas the adipogenic activity of genistein at the higher dose was not blocked by the antiestrogen. Dang and colleagues (6) hypothesized that the adipogenic activity of genistein was caused by activation of PPAR$\gamma$ in the adipocyte. They subsequently performed a PPAR-directed luciferase test and a direct-competition binding assay using genistein as a competitor of radiolabeled rosiglitazone. Genistein dose-dependently stimulated PPAR-directed gene expression in the micromolar range, and binding tests indicated that genistein bound to the PPAR$\gamma$ receptor and measured a $K_i$ for genistein of 5.7 $\mu$M. This important study was to our knowledge the first demonstration of genistein as a ligand for a PPAR receptor. Furthermore, the data from this report indicate that the bioactivity of genistein is likely mediated at least in part by activation of more than one nuclear receptor. At a given circulating concentration of genistein, the effect may be different from tissue to tissue depending on the relative expression of nuclear receptors within each tissue. The in vitro results of Dang et al. (6) predict an osteogenic effect of genistein at low concentrations resembling the effects of estrogen; while higher concentrations of genistein may more resemble the action of the antidiabetic medications, the glitazones. Glitazones are ligands for PPAR$\gamma$ and improve insulin sensitivity; however, they also stimulate adipogenesis (26).

Another study combining in vivo and in vitro trials demonstrated effects of an isoflavone-containing soy extract and the individual soy isoflavones genistein and daidzein as activators of both PPAR$\alpha$ and PPAR$\gamma$ (29). Male and female obese Zucker rats (OZRs) were fed diets with either casein or soy as the source of protein. Two different soy diets were used, with low or high content of isoflavones. Both male and female OZRs treated with high-isoflavone soy protein had reduced liver weight compared with the other diet groups. This reduced liver weight may have been due to reduced hepatic cholesterol and triglyceride content. Compared with casein-fed OZRs, male and female rats fed high-isoflavone soy had reduced levels of plasma cholesterol. Plasma triglycerides were not affected by diet treatment except for an unexplained rise in plasma triglyceride levels in female rats fed the high-isoflavone soy diet. The reduction in hepatic weight and lipid content is consistent with the effect of fibrates, agonists of the PPAR$\alpha$ receptor (25). In vivo studies also determined that the consumption of only the high-isoflavone diet improved glucose tolerance in female OZRs. In contrast to lipid weight and blood lipid changes resembling the action of PPAR$\alpha$, the latter findings are consistent with the effect of glitazones on PPAR$\gamma$. In vitro studies utilizing RAW264.7 cells determined that treatment with a mixture of unconjugated soy isoflavones (isoflavones without any covalently attached carbohydrate) and the isoflavones genistein and daidzein positively influenced both PPAR$\alpha$- and PPAR$\gamma$-directed gene expression. A third soy isoflavone, glycitein, did not share the same potent effect exhibited by the other two isoflavones. Taken together, the work of Dang

et al. (6) and Mezei et al. (29) provide strong evidence that soy isoflavones have action as PPAR activators.

Other studies provide supporting evidence for a relationship between isoflavones and PPARs. Iqbal et al. (17) performed a genetic screen examining gene expression patterns in the liver of female OZRs that were provided diets that contained casein, low-isoflavone soy, or high-isoflavone soy as the source of protein. Interestingly, one of the hepatic mRNAs confirmed to be induced in rats fed a soy-containing diet was carnitine palmitoyl transferase-I (CPT-I). CPT-I is a hepatic gene involved in the transfer of fatty acids into the mitochondria, promoting $\beta$-oxidation, and is known to be strongly induced by PPAR$\alpha$ (16). Another genetic screen identified a set of hepatic mRNAs regulated by the presence of high levels of dietary isoflavones provided to gerbils (30). Two mRNAs, encoding transferrin and phosphoenolpyruvate carboxykinase, were found in this screen to be down-regulated after consumption of high-isoflavone-containing soy. These two genes have been shown previously to be down-regulated by PPAR agonists (13, 56).

More recent confirmatory results were published by Kim et al. (21). A set of PPAR-regulated genes involved in the $\beta$-oxidation pathway were studied by real-time PCR after exposing HepG2 cells to 10 $\mu$M genistein for up to 48 hours. For the first 24 hours of treatment, relative mRNA levels for acyl CoA synthase, CPT-I, medium-chain acyl-CoA dehydrogenase, hydroxymethylglutaryl-CoA synthase, acyl CoA oxidase (isoforms 1 and 2), and enoyl-CoA hydratase were all found to be induced in HepG2 cells exposed to 10 $\mu$M genistein using a real-time PCR method. Protein levels for CPT-I were also measured by immunoblotting and found to be similarly elevated by the genistein treatment. Kim and colleagues then treated HepG2 cells simultaneously with genistein and the estrogen receptor inhibitor ICI182780. The concurrent treatment of HepG2 cells with genistein and antiestrogen on CPT-I induction resembled the effect of genistein alone, and they concluded that induction of CPT-I is mediated by PPARs and is independent of estrogen action. PPAR$\alpha$ receptor levels were also induced during exposure to genistein. It is not clear at present whether increases in PPAR-directed gene expression are occurring mainly due to genistein serving as a PPAR agonist or by enhancing PPAR responsiveness due to increased receptor number. In HepG2- and CV-1-based transfection assays, genistein was able to induce PPAR$\alpha$-directed luciferase expression when cells were exposed to concentrations of genistein as low as 0.1 $\mu$M.

Additional support indicating actions of isoflavones on PPARs comes from a very recent report (45) utilizing chimeric transcription factors containing nuclear receptor ligand-binding domains and Gal4 DNA-binding domains. These chimeras were transfected into HepG2 cells along with a Gal4-directed luciferase reporter. After exposure of HepG2 cells to various treatments for 24 hours, cell lysates were collected and luciferase expression measured as an indicator of transactivation. These studies confirmed many previous reports demonstrating activity of isoflavones as activators of estrogen receptors (ERs). A mixture of unconjugated isoflavones, genistein alone, and daidzein alone were all able to induce estrogen

receptor-dependent gene expression. However, when tested simultaneously in the presence of 1 $\mu$M estradiol, the presence of isoflavones did not stimulate estrogen-dependent gene expression to any greater degree. This was thought to be a result of the much higher affinity of estradiol for its receptor compared with the ability of isoflavones to bind to ERs. Aside from studies using the ER$\alpha$-Gal4 chimeric receptor, a panel of nine other chimeric receptors was tested to determine which receptors might be activated by isoflavones. Exposure to isoflavones produced significant induction when chimeras based on the PPAR$\alpha$, PPAR$\gamma$, PXR, and TR$\beta$ were used in conjunction with a Gal4-luciferase reporter. The individual isoflavones genistein and daidzein appeared to significantly induce these receptors as did a mixture of unconjugated isoflavones. A mixture of conjugated isoflavones was generally a less potent inducer, especially for PPAR$\gamma$. Other receptors that were unresponsive to isoflavones or marginally responsive were vitamin D, glucocorticoid, liver X, and farnesoid X. Soy saponin, a nonisoflavone soy compound, was generally not able to transactivate any nuclear receptor-Gal4 chimera. Paralleling the first study from Ricketts et al. (45), further tests were conducted to evaluate the ability of isoflavones to transactivate chimeric receptors in the presence of known agonists for each receptor. For the case of ER$\alpha$ mentioned above, no synergy of action was noted when cells were concurrently exposed to estradiol and an isoflavone. However, when cells transfected with PPAR$\alpha$-, PPAR$\gamma$-, or TR$\beta$-Gal4 chimeras were concurrently exposed to an isoflavone and the specific receptor agonist, there appeared to be enhanced luciferase expression compared with that observed in cells treated with agonist or isoflavone alone. This synergy was hypothesized to be caused by one of at least two different mechanisms. First, it may be that the specific receptor agonist and isoflavone form a duplex of sorts in the ligand-binding domain of the receptor. It has been determined previously that for at least one PPAR$\gamma$ ligand, two agonist molecules bind simultaneously in the ligand-binding pocket (47). An alternative to this explanation would be that isoflavones are acting through a second, nonligand-dependent mechanism, e.g., by altering the phosphorylation state of coactivators or corepressors, or by enhancing the effective half-life of nuclear receptors in the nucleus caused by a reduction in proteasome activity. Other evidence has demonstrated that the presence of isoflavones with clofibrate have a synergistic effect on PPAR$\alpha$-directed luciferase expression (31). Taken together, the data from several different research groups appear to point to an in vitro activity of isoflavones on PPAR-directed gene expression. Some of the trials demonstrating positive effects on PPAR-directed gene expression in cell culture systems used concentrations of isoflavones entirely consistent with blood levels reported in clinical studies. In vivo results described above demonstrate changes in lipid metabolism and glucose tolerance resembling the effects of fibrate and/or glitazone therapy. Taken together, it appears that soy isoflavones do have an in vivo activity on PPAR receptors.

A recent study completed to evaluate the effect of resveratrol as a PPAR activator suggests a rethinking of the bioactive role assigned to resveratrol. Resveratrol, the polyphenol abundant in grape skins and consumed in grape juice and wine, among

other products, has been thought to be a bioactive compound largely by virtue of its antioxidant and anti-inflammatory activities. Resveratrol has protective effects on the cardiovascular system including inhibition of platelet aggregation, it promotes vasodilation, and it reduces atherosclerosis. Many consider resveratrol the active ingredient associated with the cardioprotective effect of red wine consumption (9, 38). Although a wide-ranging number of studies of resveratrol have characterized the effects of this compound on cell proliferation, protection against oxidative damage, and several different cell signaling pathways, to our knowledge, the work of Inoue et al. (18) was the first to examine the potential of resveratrol to act as a PPAR agonist. Using a chimeric reporter system similar to that used by Ricketts et al. (45), resveratrol was found to selectively transactivate PPAR$\alpha$- and PPAR$\gamma$-Gal4 chimeras by 15- to 30-fold above control levels compared with 15 other nuclear receptor chimeras in which no appreciable degree of transactivation was noted. This increase was evaluated at 10, 50, and 100 $\mu$M and found to be dose-dependent at those concentrations. These effects were observed in the CV-1 cell line and in a number of blood vessel endothelial cells. In testing in vivo, treatment with resveratrol and PPAR$\alpha$ agonists reduced brain infarct volume in a mouse model for stroke. This attenuation was not observed when the same treatments were given to PPAR$\alpha$-knockout mice, which suggests that the protective effect of resveratrol may specifically involve PPAR$\alpha$. As we gain a greater appreciation of the interaction between cellular regulatory mechanisms and botanical compounds or food ingredients, a few principles are becoming clear. First, as with resveratrol, it is possible that one ingested dietary compound may interact with a number of systems such as PPAR regulation, antioxidative protection, and effects on cell signaling processes. Second, the promiscuous nature of nuclear receptors allows for the interaction of many different ingested compounds with the detoxification and lipid-regulating systems of the body. A diet high in phytochemical content may be providing a total input to a variety of promiscuous nuclear receptors that cannot be estimated by following only one food or botanical.



## UBIQUITINATION, THE PROTEASOME, AND EPIGALLOCATECHIN GALLATE

A somewhat recent interest in the study of gene regulation is the interaction of the proteolytic activity of proteasomes with the process of transcription. Proteasomal action may modify the half-life and environment of transcription factors and coactivators. Proteasomes are described as "chambered proteases" that consist of an array of polypeptide subunits with a variety of enzymatic activities arranged in a barrel-shaped or cylindrical configuration. A capped or gated pore allows access to the interior of the proteasome, in which proteolysis occurs. The eukaryotic 26S proteasome is among the best characterized. Within the interior of the proteasome, substrate degradation occurs in a processive manner and substrate unfolding must occur prior to proteolysis. Associated with proteasomes are chaperone

complexes that regulate access to the proteasome after an appropriate substrate (e.g., an unfolded protein) is recognized. Recognition and proteolysis are two separate events at the proteosome. The 26S proteasome typically recognizes substrates that have been tagged with a polymer assembled from ubiquitin subunits. It is recognized that there is a wide variety of enzymes that regulate the process of polyubiquitin tagging. Some of the events involved in ATP-requiring proteosome-mediated proteolysis include recognition of polypeptide substrates, unfolding of the polypeptide, and control of polypeptide entry into the protease-containing chamber. Another regulatory activity of the proteasome is removal of polypeptide-bound polyubiquitin chains. After deubiquitination, ubiquitin is released intact and may participate in further cycles of ubiquitination and targeting of polypeptides to proteasomes. Polyubiquitin removal has been shown to be tightly linked to subsequent events at the proteasome to ensure that proteolysis of the tagged substrates will occur. A recent review of the proteasome and its regulation provides additional detail (40).

What roles then does the proteasome serve in cells and how might proteasome activity be regulated by dietary components to affect gene expression? Proteasomal activity contributes to the amino acid pool to allow normal protein synthesis rates. Enzyme activity may be regulated by degradation. Importantly, proteasomal activity may be a most important mechanism for the removal of transcription factors that are no longer needed. Proteasomes are involved in the degradation of cyclins, and thus are critically involved in cell cycle regulation. The proteasome is a site for turnover of foreign proteins encoded by viruses or other invasive pathogens and is also the site of turnover of proteins that are folded incorrectly because of translation errors or mutations. A notable example of a direct disease link to proteasomal activity is in cystic fibrosis. Many cases of cystic fibrosis are thought to be caused by the accelerated degradation of a mutant version of the cystic fibrosis transmembrane conductance regulator chloride transporter. An example of proteasomal regulation of transcription factors has been studied for PPAR$\gamma$ (12). In 3T3-F442A cells, PPAR$\gamma$ levels were shown to be dependent on the specific PPAR ligand and also the concentration of the ligand. Unexpectedly, along with greater transcriptional activation by PPAR$\gamma$ at higher agonist concentrations, it was determined that the overall amount of the receptor was decreased. This reduction in the level of the receptor was determined to be post-translational in nature and mediated by the AF-2 region of PPAR$\gamma$. Inhibitors of proteasome activity blocked the ligand-dependent decrease in receptor levels. Finally, it was determined that ubiquitination of PPAR$\gamma$ was enhanced by the presence of ligand.

Several studies have begun to associate a biological activity of the polyphenols present in green tea, such as epigallocatechin-3-gallate (EGCG) to inhibition of proteasome activity. Nam et al. (36) found that EGCG, at concentrations typically observed in the serum of green tea drinkers, inhibited proteasome activity and caused growth arrest in the G1 phase of cell cycle in several tumor cell and transformed cell lines. In vitro analyses of proteolytic activity of the purified 20S portion of the proteasome were measured at various concentrations of EGCG and other

similar compounds. EGCG was found to inhibit the chymotrypsin-like activity of the 20S subunit at a concentration of ~100 nM. Other in vitro assays confirmed this inhibitory effect of EGCG on proteolytic action. Proteasomal activity was also reduced by EGCG in intact Jurkat cells. During treatment with EGCG, the accumulation of proteasomal target proteins p27, I$\kappa$B-$\alpha$, and other ubiquitinated proteins was observed. These decreases in proteasomal activity and increases in substrate were found to be independent of protein synthesis. Further, EGCG treatment caused Jurkat T and LNCaP cancer cells to enter growth arrest while in the G1 phase of the cell cycle. SV-40-transformed fibroblasts were more susceptible to EGCG compared with normal fibroblasts, as determined by relative entry into growth arrest during treatment. Subsequently, this same research group evaluated chemical modifications of ECGC and determined that enantiomeric analogs of EGCG were potent proteasome inhibitors, whereas modification of the hydroxyl groups of EGCG obliterated proteasomal inhibition (51). Most recently, it was found that EGCG acts to facilitate covalent modification of an amino-terminal threonine on the chymotrypsin-like $\beta$5 subunit of the proteasome.

In addition to playing a role in cell cycle regulation that may reduce the risk of certain cancers, consumption of EGCG-containing teas has also been shown to lessen the risk of atherosclerosis and to improve blood lipid levels (33). It may turn out that these clinically observed improvements are due to an effect of EGCG on the proteasome. However, rather than modifying targets of the cell cycle, in this case the target of proteasomal regulation may be sterol regulatory element–binding proteins (SREBPs) (14, 42). The SREBPs were discovered to be the pivotal regulatory proteins that allow cells to regulate intracellular cholesterol concentrations. Although not members of the nuclear receptor family, SREBPs are membrane-bound and normally localized to the endoplasmic reticulum. However, during times of low intracellular cholesterol concentrations, SREBPs are escorted to the golgi and are subsequently cleaved by a two-step process involving two separate golgi-specific proteases. These proteolytic events release a ~68 kDa N-terminal portion of the protein from the golgi. This processed protein has the bHLH structure characteristic of many transcription factors, and after cleavage, these "mature" SREBPs migrate to the nucleus, where they regulate a set of 30 or more genes involved in lipid and carbohydrate metabolism. Two SREBP genes encode three different isoforms; the isoform most directly related to cholesterol regulation is SREBP-2. Genes regulated by SREBP-2 include the LDL receptor and genes encoding enzymes of the cholesterol biosynthetic pathway, such as HMG-CoA reductase. Thus, activation or maturation of SREBP-2 leads to enhanced transcription and synthesis of receptors that will enhance uptake of cholesterol-containing lipoproteins; concurrently, intracellular cholesterol synthesis will be enhanced by increased levels of the biosynthetic enzymes.

Recently, Dou and colleagues (23) tested the hypothesis that EGCG affects lipid metabolism by controlling SREBP-regulated gene expression through inhibition of the proteasome. HepG2 cells were treated with 30 $\mu$M ECGC for 24 hours and cell protein was isolated at intervals during the treatment. Immunoblotting

demonstrated that ubiquitination of protein was transient, with a maximal amount of ubiquitination observed 2 hours after first exposure to EGCG. Levels of the known target proteins of the proteasome, p27 and I$\kappa$B-$\alpha$, were elevated after 2 hours of exposure to EGCG and stayed at these elevated levels for the remainder of the 24-hour treatment period. The levels of 68 kDa mature SREBP-2 (mSREBP-2) increased up to sevenfold 8 hours after the onset of exposure to EGCG and remained at this elevated level of expression throughout the rest of the 24-hour treatment period. Along with other proteasomal markers proteins, SREBP-2 levels were increased dose-dependently after 10 hours of exposure to 0, 1, and 10 $\mu$M EGCG. A target of mSREBP-2 regulation, the LDL receptor, was similarly up-regulated by EGCG. As described above, the ester bond in EGCG and related compounds was shown to be a requisite for ubiquitination and accumulation of proteasomal target proteins. Expression of mSREBP-2 and the LDL-receptor followed this pattern exactly. Regulation of proteasomal activity and protein levels were then evaluated in HeLa cells, and expression patterns paralleled those observed in HepG2 cells. In HeLa cells, the inhibitory effect of EGCG was determined to be on the chymotrypsin-like rather than trypsin-like activity of the proteasome. Immunostaining of both HepG2 and HeLa cells provided visual confirmation of immunoblotting experiments: LDL-receptor levels are increased in cells treated with EGCG or inhibitors of the proteasome such as LLnL.

Interestingly, Dou and colleagues have asked if the reported antiproliferative effects of isoflavones may be mediated through proteasomal inhibition (20). Computational modeling of the 20S proteasome predicts that genistein could bind to the chymotrypsin-like site of the 20S proteasome. Unlike prior studies indicating a possible covalent modification of the proteasome by EGCG (describe above), modeling of genistein to the proteasome predicts a steric block of Thr1. Energy calculations indicating a less stable genistein-proteasome interaction compared with EGCG-proteasome binding led to the prediction of weaker proteasome inhibition by genistein compared with EGCG. In vitro testing determined that genistein could inhibit activity of the 20S proteasome with an $IC_{50}$ of 26 $\mu$M. Inhibition of proteasomal chymotrypsin-like activity was also observed in cell extracts derived from LNCaP and MCF-7 solid tumor cells, with genistein being a more potent inhibitor than daidzein. Genistein was able to induce levels of ubiquitinated proteins and levels of proteasome target proteins at concentrations of genistein ranging from 50 to 200 $\mu$M. Protein markers of apoptosis and flow cytometry studies indicated induction of apoptosis by genistein at 50 to 100 $\mu$M. Following the previously discussed study (23), one might ask whether lipid-regulating proteins such as SREBP-2 would be affected if genistein was indeed inhibiting the activity of the proteasome. Recently, SREBP-2 levels were evaluated in HepG2 cells (34), and genistein treatment was observed to up-regulate mSREBP-2 and HMG-CoA reductase levels, although at lower doses than those tested by Kazi et al. (20). It remains to be seen if isoflavone up-regulation of mSREBP-2 levels is occurring via proteasomal inhibition, a second mechanism, or some combination of multiple mechanisms.

## SIRTUINS: DEACETYLASES AND CONTROLLERS OF TRANSCRIPTION COFACTORS

A short set of reports from just the past few years illustrates another pathway by which dietary compounds may interact with the process of gene transcription. As an introduction to this topic, we describe some of the findings of Sinclair and colleagues (5, 15), who investigated the connection between caloric restriction and increases in longevity. In vivo studies have shown that caloric restriction is the only dietary treatment that has produced the most consistently even lifespan-increasing results across species. Studies have documented the increase in lifespan due to caloric restriction in both yeast and *Caenorhabditis elegans*, mediated by extra copies of genes of specific deacetylases called sirtuins or by small-molecule agonists of the sirtuins. A set of plant polyphenols were identified that had a significant stimulatory effect on SIRT1 catalytic rate, mediated by increases in $K_m$ rather than $V_{max}$ for the enzyme. The sirtuin-activating compounds (STACs) resveratrol, butein, piceatannol, quercetin, and others were shown to have a stimulatory effect on SIRT1 ranging from ~5- to ~13-fold. When tested using *Saccharomyces cerevisiae*, resveratrol at 2–5 $\mu$M activated the yeast sirtuin Sir2 by approximately twofold, and butein, fisetin, and resveratrol increased lifespan by 31%, 55%, and 70%, respectively. The ability of Sir2 to extend lifespan in yeast is thought to be due to a role in the stabilization of repetitive DNA sequences. Assays quantifying extrachromosomal DNA indicated resveratrol was able to reduce levels of these potentially toxic molecules. A further assay evaluated the effect of STACs on human sirtuin SIRT1. The molecules tested included those from the stilbene, chalcone, flavone, isoflavone, flavanone, and anthocyanidin families. Among all compounds, the stilbenes resveratrol, butein, and piceatannol, and the flavones fisetin, luteolin, and quercetin were among the best activators of human SIRT1 when tested at 100 $\mu$M. In parallel with this in vitro testing was an in vivo test evaluating cell survival in response to ionizing radiation. Even in doses as low as 250–500 nM, the presence of resveratrol was found to be protective and dependent on active SIRT1. A subsequent study confirmed the effect of STACs in *C. elegans* and *Drosophila melanogaster* (61).

The ability of resveratrol to interact with sirtuins was followed up with a second report (15). Cohen et al. (5) demonstrated the increase in sirtuin SIRT1 levels in tissues from calorically restricted rats. When FaO cells were treated with serum obtained from ad libitum–fed or food-restricted rats, SIRT1 levels were increased in the cells exposed to serum derived from the food-restricted rats. This serum also was observed to decrease apoptosis in 293T cells. Addition of insulin and/or IGF-1 to the serum obtained from calorically restricted rats eliminated the ability of the serum to decrease apoptosis. These 293T cells, when treated with resveratrol or transfected with a SIRT1 expression vector, demonstrated suppression of Bax-mediated apoptosis within 24 hours; this decrease in apoptosis corresponds to a presumably longer lifespan for cells. Transfection of a dominant-negative form of SIRT1 increased susceptibility of cells to apoptosis. Speculation regarding

common responses in plant and animal stress situations include the suggestion that molecules indicating stress conditions within the plant may be utilized by fungal or animal cells as a signal of deteriorating environment and/or food supply. Further study of STACs, which are perhaps serving to enhance deacetylation of nuclear proteins and in turn help compact or condense regions of chromosomes, should prove to be a fascinating course of study and provide additional information on the ability of plant compounds to regulate mammalian processes. Other recent data provides support for a role for mammalian Sirt1 protein in helping mediate a response to caloric restriction in adipocytes by binding to PPAR$\gamma$ cofactors NCoR and SMRT. This association between Sirt1 and these cofactors serves to repress PPAR$\gamma$ activity. This response is attenuated in Sirt1 knockout mice (39). This



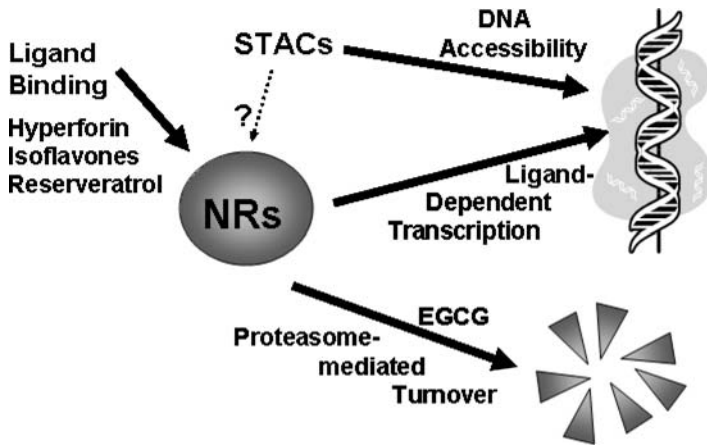


**Figure 1** Selected actions of botanical compounds on gene transcription. Botanical compounds may affect gene expression by more than one mechanism. In the case of hyperforin and probably other compounds, including isoflavones and resveratrol, these molecules are likely acting directly on nuclear receptors as ligands, thus promoting transcription of genes with specific promoter elements. In the case of hyperforin, activated pregnane X receptor/retinoic acid X receptor heterodimers bound to such target sequences enhance transcription rates. A second mechanism may be via the inhibition of proteasome-mediated turnover of nuclear receptors (NRs) or other transcription factors. In the presence of a hypothesized inhibitor such as epigallocatechin-3-gallate (EGCG), turnover of regulatory proteins may be reduced. This in turn will produce a greater effect on transcription by the given factors "spared" from the proteasome. Finally, sirtuin-activating compounds (STACs) may be regulated by plant polyphenols, including resveratrol. Regulation of sirtuins or other related enzymes may limit accessibility of transcriptional machinery to DNA. Some limited evidence suggests STACs may also be regulating cofactor interactions with nuclear receptors, indicated here as a question mark.

finding may help explain the recent discovery that longevity in mice is controlled within adipose tissue (4).

## SUMMARY

In this review, we have outlined three general mechanisms by which botanical products have been shown to regulate cell function by altering gene expression. These include activation of nuclear receptors, inhibition of the proteasome, and activation of sirtuins. These processes act directly on the process of gene transcription by controlling or influencing the processes of nuclear receptor activation, by controlling the half-life of transcription factors, and by regulating accessibility of condensed DNA or stabilization of coactivators. An overview of these mechanisms is presented in Figure 1. Just five to ten years ago, the prospect of recognizing the influence of botanicals on these processes would have been difficult to imagine. Although it is unclear if the immediate future will provide us with additional new mechanisms of control determined to be affected by botanicals, it is certain that these newly appreciated mechanisms will be investigated further to clarify the specific effects of botanicals on gene expression.

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