Activation of MAP Kinases, Apoptosis and Nutrigenomics of Gene Expression Elicited by Dietary Cancer-Prevention Compounds

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INTRODUCTION

Many dietary components have been shown to be potent cancer-preventive agents against chemically induced carcinogenesis models in animals. Many of these chemopreventive agents are promising because they are generally non-toxic substances that interfere with the process of cancer development or carcinogenesis. Although substantial progress has been made in the basic understanding of carcinogenesis, the identification of molecular and cellular targets for effective chemoprevention is lacking. Further, the cellular signal transduction events related to these molecular targets elicited by many of these agents are not well characterized. By using two classes of potential chemopreventive compounds, the phenolic compounds and the isothiocyanates (ITCs), we review the potential utility of two signaling events, the mitogen-activated protein kinases (MAPKs) and the caspase-apoptosis signaling pathways, in addition to the gene expression profile modulated by these agents in human cell lines and in animal models. The biological consequences of the modulation of the MAPK signaling pathway, caspase pathway, and some other unidentified pathways might be important to predict the pharmacologic responses related to cell survival or apoptotic cell death and potential cytotoxic effect of these dietary cancer-chemopreventive compounds.

MODULATION OF MAPK BY CHEMOPREVENTIVE AGENTS

The MAPK Pathways

The MAPK family, characterized as proline-directed serine/threonine (ProXSer/ThrPro)[1,2] kinases,[3] is activated in response to a wide variety of extracellular stimuli and mediates signal transduction cascades that play an important regulatory role in cell growth, differentiation, and apoptosis.[4] In mammalian systems, the biochemical properties of three MAPKs has been characterized in detail: the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), also referred to as stress-activated protein kinase, and the P38 MAPK.[5] Each kinase cascade consists of a module of three kinases: a MAPK kinase kinase (MAPKKK), which phosphorylates and activates a MAPK kinase (MAPKK), which in turn phosphorylates and activates a MAPK. The ERK subgroup of MAPKs is activated primarily by mitogenic stimuli such as growth factors.[6] In contrast, the JNK and P38 pathways are activated primarily by a diverse array of cellular stress including ultraviolet irradiation, hydrogen peroxide, DNA damage, heat, and osmotic shock.[5] Many chemotherapeutic drugs have been known to activate JNK and p38 MAPK, and their activation has been implicated in apoptosis.[7,8] Once activated, these three MAPKs (ERK, JNK, and p38) can phosphorylate many transcription factors, such as c-Myc, p62TCF/Elk-1, c-Jun, activating transcription factor 2 (ATF2), CCAT/enhancer-binding protein [C/EBP]-homologous protein/growth arrest DNA damage-inducible 153 (CHOP/GADD153), myocyte enhancing factor-2c (MEF2C), and stress-activated protein-1, and ultimately lead to different biological consequences.[9,10] Because MAPKs are activated by such a wide range of factors, these signaling cascades may serve as a common mechanism and integrate with other signaling pathways to control cellular responses to various extracellular stimuli, including natural products.

Activation of the MAPK Pathways Leading to ARE-Mediated Induction of Phase II Detoxifying Enzymes

Two groups of dietary components, 1) phenolic compounds including green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG), 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), a synthetic food antioxidant that has been primarily used as a food preservative, and its demthylated metabolite *tert*-butyl-hydroquinone (tBHQ) and 2) ITCs, found mostly in cruciferous vegetables, including phenethyl ITC (PEITC) and sulforaphane, have been shown to be potent chemopreventive agents against tumor formation from a variety of carcinogens in rodents.[11–21] They are also potent inducers of phase II detoxifying enzymes such as glutathione-*S*-transferase (GST) and quinone reductase (QR).[14,22–25] Treatment of animals with BHA, tBHQ, and other potential chemopreventive chemicals that preferentially induce phase II xenobiotic detoxifying enzymes reduces the conversion of chemical carcinogens (e.g., aflatoxin B1, benzo[a]pyrene, azoxymethane and *N*-nitrosomethylbenzylamine) to mutagenic metabolites and enhances their detoxification and excretion with the formation of conjugated metabolites. Consequently, this treatment reduces toxicity, mutagenicity, and carcinogenicity of various chemical carcinogens.[19,20,26–29]

Studies from our laboratory showed that EGCG potently activate MAPKs including JNK1, p38, and ERK2 activities in many mammalian cell lines such as human hepatoma HepG2 cells, human colon adenocarcinoma HT-29 cells, and human cervical squamous carcinoma HeLa cells[30,31] in a time- and concentration-dependent manner. Similarly, PEITC and sulforaphane activated JNK1, ERKs, and p38 in HepG2 cells and HT-29 cells.[32,33] Exposure of HepG2 cells to green tea polyphenols potently activated JNK1 and ERK2 activities, although with different kinetics and dose responses.[34] One of the consequences of the activation of

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MAPK by extracellular stimuli including green tea polyphenols implicates the induction of gene expression. Indeed, green tea polyphenol transcriptionally activated the antioxidant response element (ARE) or electrophile response element chloramphenicol acetyltransferase reporter gene, which is present in many stress-response genes including many phase II drug metabolizing enzymes, GST, QR,[35–37] and genes encoding for defense against oxidative stress including heme-oxygenase-1.[38] To elucidate the physiologic consequences of MAPK activation by chemopreventive compounds, we found that the ERK MAPK cascade positively transcriptionally activates ARE-mediated reporter gene induced by tBHQ and sulforaphane[39] and that JNK1 positively transcriptionally activates ARE-mediated reporter gene induced by PEITC,[40] whereas activation of the p38 MAPK cascade by tBHQ may lead to downregulation of ARE-mediated gene expression.[41] These results suggest that the coordinate modulation of MAPK cascades may be critical in the regulation of phase II genes through the ARE/electrophile response element DNA enhancer element induced by various xenobiotics and reactive oxidative stress.

Recent studies from several laboratories have implicated the basic leucine zipper transcription factors, including nuclear factor-E2–related factor-1 (Nrf1),[42] Nrf2,[42,43] and small Maf,[43] in the binding and transcription activation of ARE sequences. In knockout studies, the induction of QR and GST by BHA was largely eliminated in the liver and intestine of $Nrf2^{-/-}$ mice,[43] and the gene expression of several detoxification enzymes including QR were markedly reduced in the lung of $Nrf2^{-/-}$ mice.[44] This lack of phase II enzyme induction in $Nrf2^{-/-}$ mice suggests that Nrf2 is the most likely transcriptional factor involved in the transcriptional activation of ARE-mediated phase II gene induction. Recent data from our laboratory demonstrated that cotransfection of different amounts of Nrf2 expression plasmid with ARE-luciferase (LUC) reporter construct increases the transcriptional activation of ARE-LUC reporter gene in a dose-dependent manner.[45] The dominant negative mutant (deletion of N-terminal 1 to 430 amino acid residues) of Nrf2 blocked transcriptional activation of the ARE-LUC reporter gene[45] and transcriptional induction of ARE-LUC by inducers of phase II genes including BHA, tBHQ (unpublished data), and PEITC.[40] Further, we found that overexpression of the MAPKKK (MAPK/extracellular signal-regulated kinase kinase kinase-1, transforming growth factor-beta-activated kinase 1 (TAK1), and apoptosis signal-regulating kinase 1 (ASK1) induces expression of heme-oxygenase-1 and that cotransfection of the dominant negative mutant of Nrf2 abolishes the induction.[45] Taken together, these results indicate that MAPK pathways activated by MAPK/extracellular signal-regulated kinase kinase kinase-1, TAK1, and ASK1 may link chemically induced stress response to Nrf2, leading to transcriptional activation of the ARE promoter present in many stress-response genes including phase II detoxifying enzymes. Future studies will elucidate the role of phosphorylation in the transcription activity of Nrf2 on ARE-mediated gene expression.

Inhibition of Signaling Kinases and MAPK Pathways Leading to Attenuation of AP-1–Mediated Gene Expression

Recent studies have shown that EGCG and theaflavins in a dose-dependent manner inhibit cell transformation induced by epidermal growth factor or 12-*O*-tetradecanoylphorbol-13-acetate. At the dose range (5–20 μM) that inhibited JB6 mouse epidermal cell transformation, EGCG and theaflavins also inhibited activated protein-1 (AP-1)–dependent transcriptional activity and DNA binding activity, particularly through the inhibition of a JNK-dependent mechanism.[46] A polyphenolic fraction isolated from grape seeds caused irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting MAPK activation and inducing G1 arrest and differentiation.[47] In addition, this grape seed extract inhibited human prostate cancer DU 145 cell growth, and these anticancer effects were mediated by impairment of the pathway connecting the epidermal growth factor receptor, ERK1/2, Elk1 and AP-1.[48] We recently found that resveratrol, a polyphenolic compound derived from grapes, inhibits AP-1 activity induced by phorbol 12-myristate 13-acetate and ultraviolet C and the MAPK pathway by interfering with c-Src protein tyrosine kinase and protein kinase C, which may provide a mechanism for the anticarcinogenic activity of resveratrol.[49] However, more recently, our studies with other human cell lines such as prostate PC-3 and colon HT-29 cell showed that the ITCs and EGCG could activate MAPK in addition to AP-1 and ARE-LUC activities but inhibited nuclear factor-κB luciferase activity under the same conditions (unpublished results). Hence, the inhibition or activation of MAPK and AP-1 elicited by these cancer chemopreventive compounds probably depends on the types of cell lines and the stimulatory agents and on the concentrations or doses of the agents being used.

APOPTOSIS AND CANCER CHEMOPREVENTION

Apoptosis, also known as programmed cell death, plays important roles in many biological processes including carcinogenesis, tumorigenesis, and cancer. For instance, apoptosis may play a central role by which genetically damaged cells can be removed from the body. Apoptosis of preinitiated and/or neoplastic cells may represent a protective mechanism against neoplastic transformation and development of tumor by eliminating genetically damaged cells or cells that may have been inappropriately induced to divide by mitogenic and/or proliferative stimuli. Death receptors such as tumor necrosis factor-α receptor, Fas (CD95 or Apo1), death receptor-3 (also called Apo3, WSL-1, TRAMP, or LARD), death receptor-4, or death receptor-5 (also called Apo2, TRAIL-R2, TRICK2, or KILLER)[50] induce apoptosis. This is accomplished by activating downstream intracellular apoptotic machinery such as the ICE/Ced-3 family proteases (caspases). In addition to these physiologic regulators of apoptosis via the death ligands and death receptors complex, many environmental stresses cause apoptosis. Recent studies have suggested that oxidative stress may play a critical role in apoptosis through these signals.[51,52]

The mitochondrion plays a central role in cell death signaling through its ability to differentially regulate the trafficking of pro- and antiapoptotic proteins, such as Bcl-2,[53,54] within its intermembrane space, depending on the type of stimulus. The release of cytochrome-c is mediated by at least two mechanisms, dependent and independent of mitochondrial permeability transition. In the mitochondrial permeability transition–dependent mechanism, the mitochondrial pores open, followed by the movement of cytochrome-c from the intermembrane space, which then forms the apoptosome together with procaspase-9, adenosine triphosphate, and Apaf1, where caspase-9 is activated.[55] The subsequent activation of downstream caspase-3 then occurs and leads to the apoptotic processes.[56]

Many chemopreventive agents have been found to induce apoptosis. These agents include retinoic acid, sulindac, perillyl alcohol, curcumin, PEITC, sulforaphane, EGCG, apigenin, quercetin, chrysin, silibinin, silymarin, and resveratrol.[57–68] Induction of apoptosis by some of these agents is reported to be at least partly responsible for their chemopreventive activities.[62–66] To understand the signaling events leading to apoptosis elicited by chemopreventive agents, we studied the role of the pathway connecting mitochondria, cytochrome-c release, and caspase activation induced by PEITC. We found that treatment of HeLa and HepG2 cells with various ITCs such as PEITC activated caspase-3 activity and induced apoptosis.[69] Further, PEITC was found to induce cytochrome-c release followed by activation of caspases such as caspase-3 and -9, but not caspase-8, leading to apoptosis in human colon carcinoma HT-29 cell line.[33] Similar mitochondrial signaling events were observed in HeLa and HepG2 cells when treated

with BHA and in HT-29 cells when treated with EGCG.[31] Interestingly, the kinetics of activation of caspase activities induced by BHA or PEITC were rather rapid (within 2 to 4 h), and apoptosis occurred shortly thereafter. In contrast, the stimulation of caspase-3 activity by EGCG in HeLa cells and HT-29 cells did not appear until 12 h after treatment with EGCG,[30,31] and similar results were observed for quercetin- and chrysin-induced caspase-3 activity in HeLa cells and in human A549 lung adenocarcinoma cells (unpublished observations). Interestingly, we observed the delayed activation of caspase activities elicited by tamoxifen- and benzo(a)pyrene-induced cell deaths in cell lines and in vivo rat mammary tumors.[70–72] We are currently investigating the mechanisms of the differences in the induction of caspase activities and apoptosis between BHA and PEITC (relatively fast) versus EGCG, quercetin, and chrysin (relatively delayed) types of chemopreventive compounds.

Several upstream signaling events have been suggested as activators for the mitochondrial caspase cascade, one of which is JNK activation.[73,74] The mechanism that accounts for the proapoptotic actions of JNK has not been completely elucidated. One potential mechanism is through the proapoptotic members of the Bax subfamily of the Bcl-2–related proteins.[75] Recently, the critical role of JNK in ultraviolet-induced apoptosis was shown by using *Jnk1*$^{-/-}$ *Jnk2*$^{-/-}$ double-null mouse embryonic fibroblasts.[73] Our recent findings showed that SP600125, a specific JNK inhibitor, can block cytochrome-c release and apoptotic cell death induced by PEITC[33] and EGCG,[31] therefore suggesting that JNK activation is necessary for stress-related release of cytochrome-c, mitochondrial dysfunction, and apoptosis, although the exact role of JNK in these processes is yet to be elucidated. Further, JNK inhibitor increased EGCG-induced ERK activation, and ERK inhibitor increased JNK activation, indicating cross-talk between these two pathways.[31] Indeed, there are extensive interactions among the upstream kinases of three MAPKs,[76] and most recently, it has been reported that sustained JNK activation uncouples ERK activation from upstream MAPK/extracellular signal regulated kinase kinase, demonstrating a negative cross-talk between the JNK pathway and the ERK pathway.[77]

NUTRIGENOMICS OF GENE EXPRESSION ELICITED BY CHEMOPREVENTIVE COMPOUNDS

Nutrigenomics is a new field of study that integrates high-throughput functional genomic technologies into nutrition research.[78] Relations between nutrition and cancer have been indicated by mechanistic, epidemiologic, and clinical studies. However, the mechanisms by which dietary components modulate cancer are not clear, partly because of the lack of appropriate research tools to identify the complex mechanisms involved. The mechanisms were studied mostly by using functional assays or looking at the expression of a limited number of genes, proteins, or physiologic end points. With the emergence of nutrigenomics, it is now possible to exploit the genome-wide changes in gene expression profiles related to nutrition and cancer. The science of nutrigenomics is in its infancy, but it has the potential to transform the science of nutrition.

Some papers related to nutrigenomics and cancer have been published, and most of them have focused on gene expression analysis. A study on EGCG in prostate carcinoma LNCaP cells found 25 EGCG-responsive gene candidates out of 250 kinase and phosphatase genes.[79] Of these, most of the EGCG-repressed genes were related to the G-protein signaling network, thereby implicating a role for G-proteins in the early stage of prostate cancer prevention. Aside from the effect of EGCG on growth inhibition, the induction and inhibition of some other genes imply a potential role for EGCG in the regulation of cell migration and cell volume control. We used DNA microarray filters (Clontech Human Apoptosis Array, 205 genes) to study the effect of EGCG on apoptosis-related genes in HeLa cells (unpublished data). Some pro-apoptosis genes were identified to be upregulated, such as BAD and death receptor-5. Several cell cycle–related proteins, such as CDK-6 and CDK-7, were downregulated. In addition, transcription factors that include E2F-3 and E2F-5 were upregulated.

A transcriptional profile of the small intestine of wild-type (nrf2$^{+/+}$) and knockout (nrf2$^{-/-}$) mice treated with vehicle or sulforaphane was generated by using the Murine Genome U74Av2 oligonucleotide array.[80] Comparative analysis of gene expression changes between different treatment groups of wild-type and nrf2-deficient mice identified Nrf2-regulated genes, including NAD(P)H: quinone oxidoreductase (NQO1), GST, gamma-glutamylcysteine synthetase (GCS), and UDP-glucuronosyltransferase (UGT), and a number of new genes. Also identified were genes encoding for cellular nicotinamide adenine dinucleotide phospate–regenerating enzymes, various xenobiotic metabolizing enzymes, antioxidants, and biosynthetic enzymes of the glutathione and glucuronidation conjugation pathways. This study expanded the horizon of Nrf2-regulated genes, highlighted the cross-talk between various metabolic pathways, and identified downstream mediators for chemoprevention by sulforaphane.

Recently, we used oligonucleotide DNA microarrays (4967 oligos) to assess gene changes that are modulated by sulforaphane in in vivo rat livers (unpublished data). The most robust cluster of genes is the metallothionein-like genes (MT-1/2 and MT-1a), which increased up to more than 10-fold by 2 to 4 h after sulforaphane dosing. The second cluster of genes that are induced by sulforaphane included GST-A3, aflatoxin B1 aldehyde reductase, and aldehyde oxidase. These genes most likely are modulated by the ARE/Nrf2 signaling pathway. Some of the downregulated genes include mitochondrial genes cytochrome-c oxidase, subunits II, III, adenosine triphosphate synthase F0 and nicotinamide adenine dinucleotide dehydrogenase; cell cycle related genes, cyclin D1, check point kinase Bub1; phosphodiesterase gene (CaM-PDE); and transcription factors NF-A1, NF-X1, and HNF-3β. The elucidation of this global gene expression profile elicited by sulforaphane may yield further insights into their chemopreventive functions.

These studies indicate that gene expression profiles can be used as mechanistic tools and possible biomarkers to better understand how nutrition influences metabolic pathways and homeostatic control and how this regulation is potentially perturbed in the development of cancer.

DISCUSSION

Our studies with various chemopreventive agents including green tea polyphenol (EGCG and [−]-epigallocatechin), ITOs (PEITC, and sulforaphane), and phenolic antioxidants (BHA and tBHQ) have provided some insights into the signal transduction pathways induced by these compounds. It appears that, at low concentrations, some of these compounds (PEITC and BHA) may activate the MAPK pathway (ERK, JNK, and/or p38), which may lead to the induction of phase II detoxifying enzyme GST and QR, resulting in protection and/or survival mechanisms. At high concentrations, they will also activate the MAPK pathway; however, in addition, the caspase pathway will be activated, which will lead to apoptotic cell death. Indeed, in our most recent study on sulforaphane in ARE-LUC stably transfected HepG2 cells, 35 μM of sulforaphane maximally induced ARE-LUC reporter gene activity without inducing significant cell death. However, when sulforaphane concentration was increased up to 50 μM, the ARE-LUC reporter gene activity was totally diminished and the caspase-3 activity was dramatically induced, leading to significant cell death.[32] This concentration-dependent cellular response phenomenon may in part explain the beneficial pharmacologic effects observed in animals after administration of low doses of BHA, in

contrast to its undesirable toxicological responses after very high doses.[81,82]

Several studies have shown that phenolic compounds such as apigenin, silymarin, baicailein, resveratrol, EGCG, and theaflavin can inhibit signaling kinases including MAPK and block AP-1–mediated gene expression. The exact mechanisms as to how these compounds inhibit kinase activation are not known, although direct inhibition of upstream kinases such as c-Src kinase may contribute to the inhibition as seen with resveratrol. Future studies of the inhibitory mechanisms induced by other phenolic compounds may yield insights. It is tempting to speculate that, in proliferating or stimulated cells, these chemopreventive compounds may block proliferation by inhibiting signal transduction kinases, whereas in non-proliferating or quiescent cells, some of these compounds may activate these signaling kinases leading to gene expression of cellular defensive enzymes such as phase II detoxifying enzymes. In contrast, the inhibition or activation of these signaling pathways could depend on cell line and on the concentrations/doses and types of compounds; future in vivo studies will help to address these issues.

It is interesting to observe the differences in the kinetics of induction of caspase activities and, consequently, apoptosis between the different chemopreventive compounds. We observed at least two groups of apoptotic-inducing chemopreventive agents based on their kinetics of activation of caspase activities and thereby apoptosis. The first group of compounds, including ITCs PEITC and the phenolic antioxidants BHA, appear to induce the rapid pathway connecting mitochondria, cytochrome-c release, and caspase activation leading to apoptosis, and the kinetics of these events occur rather quickly, within 2 to 4 h. In contrast, for the second group of compounds such as EGCG, quercetin, and chrysin (in addition to tamoxifen and benzo[a]pyrene), stimulation of caspase activities and induction of apoptosis occur quite late at 12 h after treatment. This suggests that transcription and/or nuclear events may contribute to this delayed type of apoptotic cell death. Understanding the mechanisms of induction of caspases by this latter group of compounds will help to address this kinetic difference.

Several studies have been performed on the gene expression profiles induced by dietary chemopreventive compounds. In addition to the known genes and the known signaling pathways, which were expected to be regulated by these compounds, some new and novel genes were identified, such as Nrf2-dependent genes, and transcription factors, which were up- or downregulated by these compounds, and some other genes regulating cell migration and cell volume control, which may or may not be related to the chemopreventive properties of these compounds. Future studies on the functions of these genes in in vitro cell culture coupled with in vivo transgenic animals will provide some insights into the biological relevance of these genes in their cancer chemopreventive effects. In short, nutrigenomics may provide new and novel insights into the chemopreventive functions of our dietary components.

In conclusion, studies of cellular signal transduction events elicited by cancer chemopreventive compounds may yield important insights into the induction of various genes including the phase II detoxifying enzymes and other cellular defensive enzymes, which potentially may result in the homeostatic cell survival or protective response. In contrast, the activation of the cell death/apoptotic proteins such as the caspases, which result in cell death, potentially may be beneficial if this occurs in preneoplastic or tumor cells, but may result in cytotoxicity when it occurs in normal cells. Further, the activation of MAPK pathways (in particular JNK) may lead to cell survival or cell death, depending on the stimuli and the concentrations of the compounds. These concentration ranges between the activation of defensive enzymes and cell survival, and between caspases and cell death, exhibited by phenolic compounds and ITCs, respectively, in mammalian cells, may potentially reflect their respective therapeutic windows in vivo. Consequently, the studies of these and other signaling pathways with the emergence of nutrigenomics will advance our knowledge and understanding of the efficacy and safety of many natural chemopreventive compounds found in our diet, some of which may become therapeutic drugs of the future.

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