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Genomic responses to herbal extracts: lessons from *in vitro* and *in vivo* studies with an extract of *Ginkgo biloba*

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Abstract

Do herbal extracts offer effective dietary supplements to prevent deregulation of the transcriptome? Can they normalize deregulated transcriptomes of chronic human diseases? Are the effects of herbal extracts targeted to specific molecular pathways in tissue-specific manner? Are the effects of herbal supplements reversible? These questions pose important challenges to the fields of molecular nutrition and medicine, which are committed to understanding the molecular basis of physiology during health and disease. Transcription of the molecular information encoded in the deoxynucleotide sequences of DNA to the nucleotide sequences of RNA play a vital, causative, role in the coordinated adaptation of the organism to its changing environment and its nutritional needs. Pathogenesis is a manifestation of defects in transcription of the genome. Herbal extracts may target these obligatory processes. Increased availability of tools for quantitative and comprehensive analysis of messenger RNAs offer powerful means to understand and identify changes in these fundamental processes. Studies with the extract of *Ginkgo biloba* leaves show that the extract affects transcription of functionally diverse groups of genes *in vitro* and *in vivo*. The observations offer molecular evidence for bioactivity of the extract and offer an analytical strategy to define and predict physiological effects of complex mixtures of phytochemicals.

Keywords: Oligonucleotide arrays; Micronutrients; Public health

1. The challenge

Recent epidemiological surveys to determine the intake of dietary herbal supplements to maintain good health and counteract chronic diseases are remarkable. If the data from regional surveys [1,2] are extrapolated to country wide dietary supplementation of herbal extracts then an estimated 50% of the population in the United States may ingest some herbal supplements as remedies for acute and chronic illnesses. This increased consumption of diverse families of herbal supplements by an increasingly broader spectrum of human population poses a major challenge to public health. Herbal supplements may provide cost effective remedies with low toxicities for the treatment and prevention of human diseases. However, poor understanding of the comprehensive chemical composition of the herbs and a lack of comprehensive and quantitative understanding of their actions *in vivo* in normal physiology are of major concerns. These concerns are further compounded by their potential adverse actions during disease states, often triggered by environmental factors such as pollutants and chronic stress.

More than two decades ago Arthur Kornberg, an early "vitamin hunter," and later a winner of the Nobel Prize for medicine for the discovery of DNA polymerase noted that the science of nutrition was on the decline. This was because, "Compared with the classical deficiency diseases, the residual problems of animal and human nutrition are elusive and their scientific pursuit far more difficult, time consuming, and unglamorous." These observations resonate today [3]. The investigations into the potential nutritional effects of herbal extracts must not be allowed to fall victim to such criticisms. Systematic, comprehensive and quantitative understanding of the physiological effects of complex mixtures of plant extracts can be obtained by objective and critical applications of the tools of functional genomics.

2. Composition of herbal extracts

The comprehensive and quantitative composition of most herbal extracts is unknown. Although many extracts

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are marketed as standardized, only specific markers are used as the indicators of standardization such as flavonoid glycosides or ginkgolides for *Ginkgo biloba* extract. Many minor components with potential biological effects remain neglected from quantitative analysis.

The extract of *G. biloba* leaves contains natural organic molecules that have the potential to affect the functions of signal transduction pathways. For example, flavonoid glycosides (~25% of the extract) may modulate the activities of protein kinases [4,5]. These actions may be mediated by its effects on interactions at the membrane interface through specific receptors such as those for cytokines. Alternatively they may directly affect the activities of the intracellular proteins. The terpenoid lactones (6%) of the extract may also affect signal transduction pathways through interactions of GTP binding proteins with effectors in membranes or the cytosolic compartments [6]. However, analytical tools for defining these complex interactions are time consuming and laborious to perform. These are complex interactions whose systematic investigations are important because they play vital role in cellular proliferation and apoptosis. Deregulation of cellular homeostasis is attendant in all chronic diseases. The application of tools for functional analysis of genomic responses by simultaneous and quantitative analysis of messenger RNAs provide effective means to define bioactivities of plant extracts.

3. Effects of *G. biloba* extract in humans and rodents

Prevention of neurological decline by dietary supplementation with a standardized extract from leaves of *G. biloba* offers an attractive practice to prevent or treat neurological diseases. The observations from early investigations indicated that the extract improves blood circulation [7,8] in the brain and may also affect that in peripheral regions such as finger tips [9]. Results from a limited number of clinical trials in humans offer encouraging data for the beneficial effects [10,11] but some clinical trials have reported the lack of any clinically beneficial outcome [12,13]. In rare cases, the dietary supplementation of the extract may cause life threatening complications [14–16]. These observations demand a better understanding of the spectrum of effects of this widely used herbal extract under normal physiology and under disease states.

In rodents, dietary supplementation of *G. biloba* extract has been shown to provide neuroprotective effects in models of human stroke [17,18]. In addition, improved behavioral repertoires such as learning and memory have also been documented in rodents whose diets were supplemented with the extract of *G. biloba* leaves (reviewed in [19,20]). These observations suggest that the extract is bioactive *in vivo*. However, the molecular targets through which the extract acts remain poorly defined.

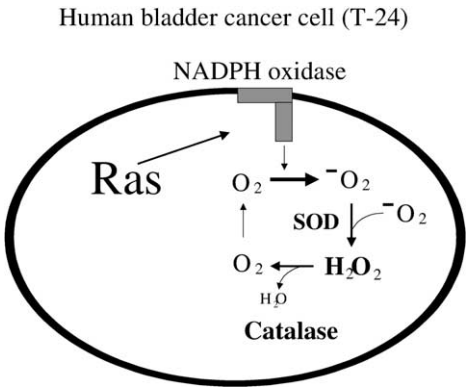

Fig. 1. Cyclical formation of reactive oxygen species in cells with constitutively active, GTP regulated protein, Ras. The enzymes essential for the cyclical formation of reactive oxygen species, NADPH oxidase, superoxide dismutase and catalase are ubiquitous. Unregulated activation of NADPH oxidase such as that initiated by Ha-Ras will initiate "perpetual" increase in the formation of reactive oxygen species.

4. Molecular targets of *G. biloba in vitro*

The spectrum of functional classes of mRNAs affected by an extract of *G. biloba* leaves in a human bladder cancer cell line was determined using the GeneChip assay [21]. Human bladder cancer cells (T-24) have constitutively active, oncogenic G-protein, Ha-Ras [22]. Activation of Ras results in increased production of reactive oxygen species by NADPH oxidase [23], a ubiquitous enzyme complex. Hence, cells with constitutively active Ras would be expected to be under "oxidative stress" (Fig. 1).

GeneChip analysis of the human bladder cancer cells under basal conditions revealed increased expression of several genes that are activated by reactive oxygen species [21]. These included mRNAs encoding metallothionein and ferritin L chains. In addition the analysis revealed elevated expression of several genes that are associated with malignant growth. These included mRNAs for Wilms Tumor related protein, calcium binding proteins, v-fos transformation effector protein and several ribosomal proteins.

Incubation of human bladder cancer cells for a period of 72 hr with the extract of *G. biloba* leaves unraveled time-dependent changes in the abundance of mRNAs suggesting bioactivity of the extract in the human cancer cells. The GeneChip assay detected ~2000 distinct mRNAs showing that only a small fraction of the human trancriptome can be analyzed. Differential analysis of the affected genes (>2-fold change) showed that only a small fraction of the genes were affected by the extract (Fig. 2). This suggested that the action of the extract is specific on the cell's genome. Classification of the affected genes into either up- or down-regulated genes showed that there was a net activation in the transcription of the cell's genome by the herbal extract (Fig. 2).

Functional classification of the *G. biloba* sensitive genes enabled grouping of the genes into several distinct classes such as transcription factors, heat shock and antioxidant transcripts, mitochondrial, cell cycle and transcripts for

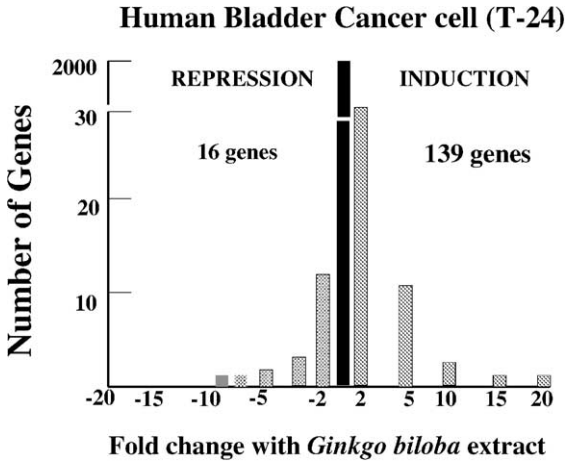

Fig. 2. Net induction of transcription of the cancer cell genome by an herbal extract of *Ginkgo biloba* leaves. Human bladder cancer cells were incubated with the herbal extract for 72 hr. Messenger RNA profiles of control and treated cells were analyzed by the GeneChip assay [21]. Differential analysis of the data from extract treated and untreated cells are displayed as groups of genes that are either induced or repressed. Most of the genes were unaffected by the extract. Some genes were activated by 20-fold while a few genes were repressed by more than 5-fold.

DNA repair enzymes [21].The induction of several transcripts encoding mitochondrial proteins was remarkable because it suggested an activation of selected mitochondrial genes in the nucleus. These are illustrated in Fig. 3 below. The implication of such observations is that the extract induces ''mitochondrial differentiation''; the biochemical composition of mitochondria from untreated cells is different from that of *G. biloba* treated cells.

5. Molecular effects of *G. biloba in vivo*

Analysis of the expression of 12,000 mouse genes in cortex and hippocampus showed that the orally administered extract was centrally active [24]. The expression of ~43 genes and ~13 genes was up-regulated by at least 2-fold in cortex and hippocampus, respectively. A small number of genes such as those encoding growth hormone, prolactin, serum albumin and serine protease inhibitor were activated in both the brain regions. These observations are remarkable for three reasons. First, they provide molecular evidence for the action of a dietary supplement in the brain. Second, there is a differential effect in the two brain regions that were selected for gene expression analysis. Third, the extract increased the abundance of the affected mRNAs suggesting increase in transcription although we cannot exclude the possibility of increased stability of mRNAs.

Gene expression analysis also showed differential effects in cortex and hippocampus (Fig. 4). The heterogeneity in the responsiveness of the two neuroanatomical regions raises an important issue of targeting specific brain regions by dietary supplementation for a desired molecular outcome and its effects on physiological, pathophysiological and behavioral outcomes. Functional classification of the affected genes showed that *G. biloba* extract affected the expression of genes encoding transcription factors, ion channels, growth factors and neuromodulators, synaptic vesicles and transport, cell surface, and protein kinases and phosphatases.

A large (12-fold) induction in the expression of transthyretin in hippocampus but not in cortex is noteworthy. Defects in hippocampal functions have been described in Alzheimer's disease [25–27]. Transthyretin plays a role in the formation of amyloid products present in degenerating brain [28] and the activation of transthyretin gene expression by *G. biloba* extract may regulate this process *in vivo*. The mechanisms by which transthyretin may affect the formation of neuronal amyloid is poorly understood. However, a role for thyroxine may be implicated because it binds to transthyretin and affects neuronal growth and development [29]. A gene related to that of transthyretin, serum albumin gene with LINE-1 repeat, was induced (8-fold) in cortex and to a lesser magnitude (3-fold) in

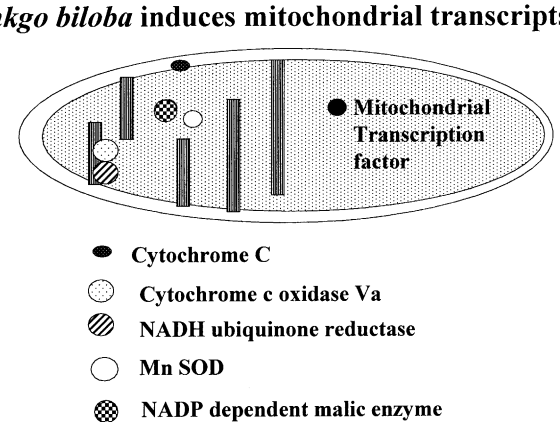

Fig. 3. GeneChip analyses detected increased expression of a number of mRNAs encoding mitochondrial proteins. Only some of the mitochondrial transcripts were induced while most were unaffected suggesting a specific effect of *Ginkgo biloba* extract on the transcription some of nuclear encoded mitochondrial transcripts.

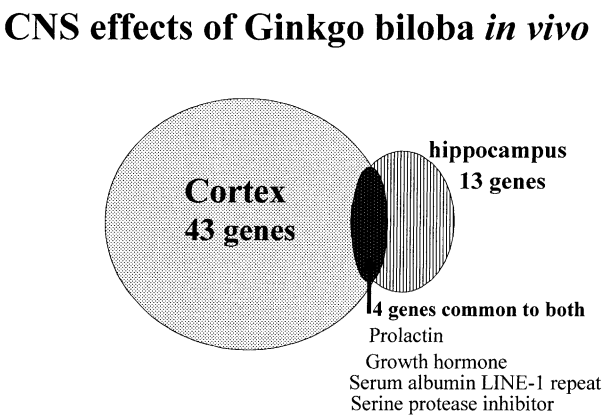

Fig. 4. Differential effects of *G. biloba* extract on brain regions.

hippocampus. These observations emphasize the differential effects the herbal extract in the brain.

Dietary supplementation with *G. biloba* extract also activated the transcription of genes that encode peptides important in cellular growth and function. These included growth hormone, prolactin, oxytosin neurophysin 1, placental growth factor, brain derived neurotrophic factor, platelet derived growth factor and insulin-like growth factor receptor 1. Also noteworthy was the simultaneous induction of the genes for oligodendrocyte basic protein and proteolipid protein, both of which are essential for the biogenesis and assembly of myelin [30,31]. Collectively, these observations provide some of the potential molecular targets through which the extract of *G. biloba* confers neuroprotective effects observed in rodent models of human stroke [18,32].

Ion channels play a vital role in the complex processes of learning and memory [33,34] which recruit multiple cellular types and molecular pathways. The expression of several genes encoding proteins of ion channels was induced in the brain cortex and hippocampus after 30 days of dietary supplementation [24]. These included the genes for chloride channel protein 3, calcium activated potassium channel, alpha subunit of GABA-A receptor, alpha-2 subunit of glutamate receptor, and type VII alpha polypeptide of voltage gated sodium channel. The induction of the ion channel genes was accompanied by the activation of several genes encoding proteins in the signal transduction pathways. These included calmodulin 3, neuronal tyrosine threonine phosphatase 1, the beta subunit of phosphatidylinositol 4-phosphate 5-kinase, cAMP-dependent regulatory subunit of protein kinase, magnesium-dependent protein phosphatase 1B, protein tyrosine phosphatase IF2P, protein tyrosine phosphatase-1, serine/arginine-rich protein-specific kinase 2, Janus N-terminal kinase 2, Gz subunit of GTP binding protein and epsilon subunit of protein kinase C.

6. Conclusions and future goals

Coordinated changes in the expression of the diverse but functionally related families of genes by *G. biloba* extract *in vivo* and *in vitro* show that the extract is bioactive and these changes identify the molecular processes targeted by the extract. The *in vivo* effects of the extract suggest the potential of *G. biloba* extract to alter neuronal and synaptic plasticity. These processes play vital roles in the functions of the central nervous system. The dynamics of the interactions between the various proteins encoded by the mRNAs induced by *G. biloba* extract are unknown. Even more difficult to predict are the behavioral outcomes of such molecular modifications. Future studies that combine the tools of functional genomics and behavioral analysis to study the effects of herbal extracts such as those of *G. biloba* leaves are essential for critical and objective analysis of the *in vivo* effects of the dietary antioxidant supplement.

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