Altered Carcinogenesis and Proteome in Mammary Glands of Rats after Prepubertal Exposures to the Hormonally Active Chemicals Bisphenol A and Genistein

Angela M. Betancourt, Jun Wang, Sarah Jenkins, Jim Mobley, Jose Russo, and Coral A. Lamartiniere

The Journal of Nutrition

Abstract

Through our diet, we are exposed to numerous natural and man-made chemicals, including polyphenols with hormone-like properties. The most abundant hormonally active polyphenols are characterized as weak estrogens. These chemicals are hypothesized to interfere with signaling pathways involved in important diseases such as breast cancer, which in most cases is initially estrogen dependent. Two such chemicals are bisphenol A (BPA), a plasticizer, and genistein, a component of soy. In spite of both possessing estrogenic properties, BPA and genistein yield different health outcomes. The exposure of rats during the prepubertal period to BPA increases the susceptibility of adult animals for mammary cancer development, whereas genistein decreases this susceptibility in a chemically induced model. Because both BPA and genistein possess estrogenic properties, it is certainly plausible that additional mechanisms are affected by these chemicals. Hence, it was our goal to investigate at the protein level how exposure to these 2 chemicals can contribute to mammary cancer causation as opposed to cancer chemoprevention. Using 2-dimensional gel electrophoresis followed by MS analysis, we identified differentially regulated proteins from the mammary glands of rats prepubertally exposed to BPA and genistein. Following protein identification, we used immunoblotting techniques to validate the identity and regulation of these proteins and to identify downstream signaling proteins. Our studies highlight the importance of proteomics technology in elucidating signaling pathways altered by exposure to hormonally active chemicals and its potential value in identifying biomarkers for mammary cancer.

Introduction

Through diet and the environment, humans are exposed to both beneficial and harmful hormonally active compounds. Recent

1 Published in a supplement to The Journal of Nutrition. Presented at the 2010 American Institute for Cancer Research Annual Conference held in Washington, DC, October 21–22, 2010. The conference was organized by the American Institute for Cancer Research. This work was supported by an Intergovernmental Personnel Act from the Nutritional Sciences Research Group, Division of Cancer Prevention, National Cancer Institute, NIH to Donato F. Romagnolo, University of Arizona, Tucson. The views expressed in this publication are those of the authors and do not necessarily represent the official views of the sponsors or the publisher, Editor, or Editorial Board of The Journal of Nutrition. The supplement coordinator for this supplement was Donato F. Romagnolo, University of Arizona, Tucson. Supplement Coordinator disclosures: D. F. Romagnolo, no conflicts of interest. The supplement is the responsibility of the Guest Editor to whom the Editor of The Journal of Nutrition has delegated supervision of both technical conformity to the published regulations of The Journal of Nutrition and general oversight of the scientific merit of each article. The Guest Editor for this supplement was Harry D. Dawson, ARS/USDA. Guest Editor disclosure: H. D. Dawson, no conflicts of interest. Publication costs for this supplement were defrayed in part by the payment of page charges. This publication must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact. The opinions expressed in this publication are those of the authors and are not attributable to the sponsors or the publisher, Editor, or Editorial Board of The Journal of Nutrition.

2 Supported by the Breast Cancer and the Environment Research Centers grant U01ES/CA0012717 and Genes, Environment and Health Initiative grant 1U01ES016003 from the National Institute of Environmental Health Sciences (NIEHS), National Cancer Institute (NCI), NIH, Department of Health and Human Services. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS, NCI, or NIH. S.J. was supported by a Department of Defense Breast Cancer Program Traineeship Award (W81XWH-08-0777) and through a postdoctoral fellowship from the National Cancer Institute Cancer Prevention and Control Training Program (R25 CA07888-22).

3 Author disclosures: A. M. Betancourt, J. Wang, S. Jenkins, J. Mobley, J. Russo, and C. A. Lamartiniere, no conflicts of interest.
women, delayed first full-term pregnancy, late menopause, or early menarche (2–4). Consequently, exposure to both endogenous estrogen and estrogendriven chemicals could augment the risk for breast cancer.

Evidence for the role of estrogenic compounds as mediators of breast cancer risk was provided by studies showing that prenatal exposure to the synthetic estrogen diethylstilbestrol increased the incidence of breast cancer in women that were ≥40 y of age (5) and increased mammary gland tumorigenesis during adulthood in rodents (6). However, the effects of exposure to estrogenic chemicals commonly found in the environment on the biology of the mammary gland remain elusive. Such is the case of the plasticizer bisphenol A (BPA) and the isoflavone genistein. The exposure of female rats during the early prepubertal period to BPA decreased the susceptibility of the mammary gland to carcinogenesis (7,8), whereas exposure during the same period to BPA increased the susceptibility to chemically induced mammary cancer (9). Although both BPA and genistein possess estrogenic properties, these chemicals appear to influence mammary cancer risk via unknown, yet different, mechanisms.

Human Exposures to BPA and Genistein

BPA

BPA is a chemical used in many settings, especially in the manufacturing of polycarbonate plastics and resins, which are commonly used in the packaging of canned foods and polycarbonate beverage bottles. BPA can be ingested by humans as it leaches from the lining of tin cans into foods, from polycarbonate bottles into their contents (10,11), and from dental sealants into saliva under normal conditions of use (12,13). Factors such as elevated temperature and extreme pH increase the leaching of BPA from food containers (12,14). Oral exposure is considered the main route of human exposure to BPA due to the leaching of BPA from food containers into food products. Evidence of the widespread human exposure to BPA is provided by recent studies showing that an estimated 95% of Americans tested, including young girls, showed detectable concentrations of BPA in their urine (15,16). It is estimated that human exposure to BPA ranges from 0.05 to 10 μg BPA/kg body weight (BW) per day (17–20). The metabolism of BPA occurs mainly in the liver where most of the absorbed BPA is conjugated by phase II enzymes to BPA-glucuronide. However, the biological effects of BPA are attributed to free BPA. Although elimination of absorbed BPA occurs within 24 h after oral exposure (21,22), it is likely that humans are exposed to BPA on a daily basis considering that foods and water are the main routes of exposure. This chronic nature of human exposure to BPA has been overlooked in most animal studies.

Genistein

Genistein is a phytoestrogen found in many food products, especially soy-based foods such as tofu, soy milk, soy infant formula, and in some over-the-counter dietary supplements. Exposure to genistein occurs primarily through dietary intake of beverages and foods containing fruits, herbs, and vegetables. Humans ingest genistein mainly as genistin (genistin bound to a sugar molecule). The sugar molecule is released during digestion in the stomach and intestine. Similarly to BPA, genistein is also metabolized and conjugated in the liver and some studies report that genistin can be conjugated in the intestinal wall. However, the pharmacokinetics of genistein in humans is complex and not well understood. Dietary exposure to genistein can reach up to 1 mg/kg BW in adults and up to 10-fold higher levels in infants fed milk formulas containing soy (23). The level of genistein exposure in Asian populations consuming a soy-rich diet has been reported to range from ~1 to 30 mg/d, or ~0.02–0.55 mg/(kg BW·d), and considerably less in Western populations (24).

Effects of BPA and genistein in rodent models

Mammary gland tumorigenesis

Exposure of rodents to BPA during the prenatal or prepubertal periods has been reported to induce alterations in the mammary gland architecture manifested in adulthood. Mice exposed in utero to 250 μg BPA/kg BW via osmic pumps had a significantly greater number of ductal and alveolar structures relative to the control group on postnatal day (PND) 180 (13). Similarly, the prenatal exposure of CD-1 mice to 2.5, 25, 50, and 1000 μg BPA/(kg BW·d)induced ductal hyperplasia on PND50 and PND95 (25).

One potential drawback of s.c. administration is that it does not mimic the typical exposure to endocrine disruptors in humans, for whom oral exposure is the most common route for BPA and genistein. The route of administration can determine metabolism, disposition, and internal dose. For this reason, we choose to investigate in a rodent model the effects of early oral exposure to BPA and genistein on mammary cancer development, mammary gland morphology, and cell proliferation. The prepubertal period was chosen, because the mammary tissue undergoes extensive development during this period.

For early postnatal BPA exposure studies, lactating dams were administered either a low- or high-BPA dosage (25 and 250 μg BPA/kg BW, respectively) and controls were treated with an equivalent volume of the vehicle, sesame oil. The low dose was one-half of the daily tolerable dose of 50 μg BPA/(kg BW·d) established by United States Environmental Protection Agency (26). The high dose [25 μg BPA/(kg BW·d)] was >200-fold less than the lowest observable adverse effects level of 50 μg/(kg BW·d). Based on a study by Snyder et al. (27) and accounting for fetal metabolism, disposition, lactation, and number of offspring, we estimated that <0.01% of the dose administered to the lactating dam was transferred to the offspring. Therefore, offspring were exposed to ~2.5 ng BPA/(kg BW·d) for the low dose and 25 ng BPA/(kg BW·d) for the high dose.

For genistein, low and high doses were selected (25 and 250 mg genistein/kg diet, respectively). These dietary levels resulted in serum concentrations in 21-d-old rats of 86 and 726 nmol/L, respectively (7), which approximated levels found in Japanese populations eating a traditional diet high in soy (276 nmol/L) (28).

To determine if BPA and genistein could alter mammary cancer development, we used a rat mammary tumor model (29). In this model, the highest incidence of mammary carcinomas in Sprague Dawley rats is obtained when exposure to the carcinogen dimethylbenz[a]anthracene (DMBA) occurs between 30 and 55 d of age (30–31). During this period of postnatal development, the mammary gland possesses a considerable number of terminal end buds (TEB), which are targeted by chemical carcinogenesis due to the high mitotic index and undifferentiated state (32). In our experiments, female offspring prepubertally exposed to either BPA or genistein received a single dose of DMBA on PND50. Rats were subsequently palpated for mammary tumors. Lactational exposure to BPA (250 μg/kg BW) significantly increased the number of DMBA-induced mammary tumors (33).

Abbreviations used: BPA, bisphenol A; BW, body weight; DMBA, dimethylbenz[a]anthracene; PND, postnatal day; TEB, terminal end bud.
mammary tumors compared with the sesame oil-treated group (9). On the other hand, prepubertal exposure to 250 mg genistein/kg diet significantly reduced the number of DMBA-induced mammary tumors compared with the controls (7,8).

Mammary gland morphology and cell proliferation
We found that prepubertal genistein exposure reduced the number of TEB in 50-d-old female rats (7,8,33,34). However, we did not detect significant changes in mammary gland morphology in 50-d-old rats prepubertally exposed to BPA. Because cell proliferation is a key cellular process involved in the pathogenesis of tumor formation, we investigated if BPA and genistein exerted differential effects on cell proliferation. We measured the nuclear protein Ki-67, a cellular marker for proliferation. Cell proliferation in the TEB of 50-d-old rats was significantly increased by 23% in female offspring lactationally exposed to the high-BPA dosage (9). Similarly, perinatal exposure of CD-1 mice to BPA has been reported to increase the number of TEB on PND30 (35) and prenatal exposure to BPA was shown to induce preneoplastic lesions in Sprague Dawley and Wistar rats (9,36).

Conversely, prepubertal exposure to genistein decreased by 58% the rate of cell proliferation in the TEB of mammary glands.

### TABLE 1

<table>
<thead>
<tr>
<th>Protein identification</th>
<th>Fold change</th>
<th>ANOVA</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosylhomocysteinase</td>
<td>−1.4</td>
<td>0.017</td>
<td>NAD or NADH binding</td>
</tr>
<tr>
<td>α-1B-glycoprotein precursor (C44)</td>
<td>−1.9</td>
<td>0.008</td>
<td>Unknown</td>
</tr>
<tr>
<td>Annexin A2</td>
<td>1.2</td>
<td>0.088</td>
<td>Rab GTPase binding</td>
</tr>
<tr>
<td>apoA-1</td>
<td>1.3</td>
<td>0.048</td>
<td>Lipid binding/lipase inhibitor</td>
</tr>
<tr>
<td>Carbonyl reductase 3</td>
<td>2.1</td>
<td>0.002</td>
<td>Reductase activity</td>
</tr>
<tr>
<td>Carboxylesterase 3</td>
<td>−1.5</td>
<td>0.003</td>
<td>Hydrolase/serine esterase</td>
</tr>
<tr>
<td>Calponin-3</td>
<td>1.2</td>
<td>0.039</td>
<td>Protein binding/bridging</td>
</tr>
<tr>
<td>Glucose-6-phosphate 1-dehydrogenase</td>
<td>−1.2</td>
<td>0.036</td>
<td>Oxidoreductase</td>
</tr>
<tr>
<td>Glutathione S-transferase omega-1</td>
<td>1.3</td>
<td>0.020</td>
<td>Glutathione transferase activity</td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>1.2</td>
<td>0.055</td>
<td>NAD or NADH binding</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>1.4</td>
<td>0.009</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
<td>1.3</td>
<td>0.046</td>
<td>Metal ion binding</td>
</tr>
<tr>
<td>NADH dehydrogenase flavoprotein 2</td>
<td>1.4</td>
<td>0.008</td>
<td>Oxidoreductase</td>
</tr>
<tr>
<td>Stress-70 protein</td>
<td>1.4</td>
<td>0.038</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Selenium-binding protein 1</td>
<td>−1.3</td>
<td>0.056</td>
<td>Selenium binding</td>
</tr>
<tr>
<td>Tubulin β-2C chain</td>
<td>−1.3</td>
<td>0.016</td>
<td>GTP binding/GTPase activity</td>
</tr>
<tr>
<td>Transglutaminase 2</td>
<td>−1.6</td>
<td>0.030</td>
<td>Transglutaminase</td>
</tr>
<tr>
<td>GRP-78</td>
<td>1.5</td>
<td>0.041</td>
<td>Protein binding</td>
</tr>
</tbody>
</table>

1 Positive and negative fold changes in protein expression indicate up- and downregulation of protein expression, respectively. BPA, bisphenol A.

### TABLE 2

<table>
<thead>
<tr>
<th>Protein identification</th>
<th>Fold change</th>
<th>ANOVA</th>
<th>Molecular function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin A1</td>
<td>−1.5</td>
<td>&lt;0.001</td>
<td>Phospholipase A2 Inhibitor</td>
</tr>
<tr>
<td>Annexin A2</td>
<td>−1.3</td>
<td>0.001</td>
<td>Rab GTPase binding</td>
</tr>
<tr>
<td>apoA-1</td>
<td>1.2</td>
<td>0.001</td>
<td>Cholesterol transporter</td>
</tr>
<tr>
<td>Endoplasmic reticulum protein</td>
<td>1.4</td>
<td>0.014</td>
<td>Processing of secretory protein</td>
</tr>
<tr>
<td>Fetuin B</td>
<td>1.5</td>
<td>&lt;0.001</td>
<td>Cysteine-type endopeptidase inhibitor</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>1.3</td>
<td>0.013</td>
<td>Iron ion binding</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleo-protein H</td>
<td>1.5</td>
<td>0.006</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>Keratin, type I cytoskeletal 19</td>
<td>−1.4</td>
<td>&lt;0.001</td>
<td>Protein binding</td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 2</td>
<td>−2.0</td>
<td>&lt;0.001</td>
<td>Protein binding</td>
</tr>
<tr>
<td>Nonmuscle caldesmon</td>
<td>1.5</td>
<td>0.019</td>
<td>Muscle protein</td>
</tr>
<tr>
<td>Peroxiredoxin-2</td>
<td>1.2</td>
<td>&lt;0.001</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>PGK</td>
<td>−1.4</td>
<td>&lt;0.001</td>
<td>Kinase transferase</td>
</tr>
<tr>
<td>PDIA3</td>
<td>1.5</td>
<td>&lt;0.001</td>
<td>Isomerase</td>
</tr>
<tr>
<td>Stress-70 protein</td>
<td>1.5</td>
<td>0.014</td>
<td>Chaperone</td>
</tr>
<tr>
<td>Ubiquitin carboxyl-terminal hydrolase isozyme</td>
<td>−1.3</td>
<td>0.01</td>
<td>Hydrolase protease</td>
</tr>
<tr>
<td>Vitamin D-binding protein</td>
<td>2.0</td>
<td>0.004</td>
<td>Vitamin D binding</td>
</tr>
</tbody>
</table>

1 Positive and negative fold change in protein expression indicate up- and downregulation of protein expression, respectively. PDIA3, protein disulfide-isomerase A3; PGK, phosphoglycerate kinase.
of 50-d-old rats compared with the control group (37). It is important to consider that the effects on cell proliferation elicited by both BPA and genistein occurred on PND50 (30 d postexposure), suggesting the ability of these compounds to differentially induce delayed, long-term effects in the mammary gland.

Timing of exposure

Animal and human studies show that the timing of exposure to hormonally active chemicals appears to be a critical factor in determining the protective effects of genistein against carcinogenesis. Sprague-Dawley rats exposed to 250 mg genistein/kg diet from birth through PND21 had fewer chemically induced mammary tumors on PND50, but prenatal exposure to genistein did not protect against chemically induced tumors (8). Similarly, early exposure of pubertal girls (ages 13–15 y) to soy containing genistein has been reported to have a preventive effect on mammary cancer development during adulthood (38). On the other hand, oral prenatal exposure to BPA increased mammary cancer susceptibility in offspring and shifted the window of susceptibility for DMBA-induced tumorigenesis in the rat mammary gland from PND50 to PND100 (39).

Effects of BPA and genistein on the mammary proteome

Because the differential effects of BPA and genistein on cellular proliferation were observed on PND50, we utilized proteomic tools to investigate proteins and the pathways targeted by these compounds. Using 2-dimensional gel electrophoresis/MS, we discovered 18 and 16 proteins differentially regulated by BPA and genistein, respectively. The protein list, fold change, ANOVA, and a brief description of molecular function are listed in Table 1 for BPA and Table 2 for genistein. Annotation of the molecular function of proteins regulated by both compounds (Fig. 1C).

Interestingly, we found 4 common proteins that were affected by prepubertal exposure to BPA and genistein. Among these proteins, only annexin A2 was increased by BPA, whereas it was decreased by genistein (Table 3). Annexin A2 is a calcium-dependent, phospholipid-binding protein that mediates angiogenesis, cell proliferation, and differentiation, and enhances tumor growth and metastasis (40–43). Interestingly, annexin A2 is overexpressed in several cancers, including breast cancer, hepatocellular carcinoma, pancreatic adenocarcinoma, glioma, gastric carcinoma, primary colorectal cancer, lung cancer, and cervical squamous cell carcinoma (41–48). Because of the involvement of annexin A2 in processes directly related to tumorigenesis (angiogenesis, proliferation, and metastasis) and because the effects of BPA and genistein in tumorigenesis correlated well with the direction of change in annexin A2 expression, we validated these results by Western-blot analysis on a different set of mammary glands (37). As expected, BPA increased annexin A2 levels by 73%, whereas genistein decreased it by 67% (Fig. 2). Differential effects of BPA and

### Table 3

<table>
<thead>
<tr>
<th>Protein name</th>
<th>BPA</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin A2</td>
<td>Up</td>
<td>Down</td>
</tr>
<tr>
<td>apoA1</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>Up</td>
<td>Up</td>
</tr>
<tr>
<td>Stress-70 protein</td>
<td>Up</td>
<td>Up</td>
</tr>
</tbody>
</table>

1 BPA, bisphenol A.
genistein in annexin A2 expression could influence the tumorigenic response of the mammary gland to these compounds.

Proteomic analysis of signaling pathways influenced by BPA and genistein

Next, we sought to examine how BPA and genistein influenced cellular pathways involved in mammary tumorigenesis. Previous studies reported that in utero exposure to BPA in Sprague-Dawley rats affected both the extracellular-signal regulated kinase 1 and 2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (9). Similarly, Rowell et al. (49) reported that prepubertal exposure of Sprague-Dawley rats to genistein decreased vascular endothelial growth factor receptor 2 (VEGFR2) expression in the mammary gland. Activation of the receptor tyrosine kinase VEGFR2/fetal liver kinase 1 regulates angiogenesis and switches on the ERK1/2 and PI3K/AKT pathways. The ERK1/2 pathway regulates cell proliferation, whereas PI3K/AKT plays a role in cell survival and antiapoptotic signaling. Therefore, we hypothesized that prepubertal exposure to BPA may target VEGFR2 signaling. The results showed that BPA and genistein had opposing effects on expression of VEGFR2 (Fig. 2). The expression of phospho-AKT was stimulated by BPA, but not by genistein. These results suggested that VEGFR2-mediated pathways could be important in determining the adverse and chemopreventive effects of BPA and genistein in the rat mammary gland, respectively.

For BPA, we also validated by Western blotting the expression of selenium binding protein, glucose-regulated protein-78 (GRP-78), and heat shock protein-70 (HSP-70) (Fig. 3). BPA decreased the expression of selenium binding protein by 34% and increased GRP-78 and HSP-70 by 40 and 36%, respectively. Selenium binding protein covalently binds selenium and is expressed in a variety of tissues and cell lines (50). Its expression is markedly reduced in multiple tumor types compared with their corresponding normal tissues and its reduction has been associated with poor outcome in lung (51), ovarian (52), and colorectal (53) cancers and pleural mesothelioma (54). This is the first report to our knowledge of BPA influencing the expression of...
selenium binding protein. The potential of using selenium binding protein as a possible biomarker of BPA exposure should be explored.

The increased expressions of GRP-78 and HSP-70 in response to BPA are of interest, because GRP-78, a member of the HSP-70 family, is preferably required for cancer cell survival under pathologic conditions such as tumor progression and drug resistance (55). HSP-70 is a stress-induced protein that is overexpressed in response to hyperthermia, oxidative stress, and changes in GRP-78 expression (56). For genistein, we validated fetuin B and phosphoglycerate kinase (PGK) (37) (Fig. 4). Genistein increased fetuin B expression by 67% compared with control. Fetuin B possesses antiangiogenic properties and its overexpression in skin squamous carcinoma cells leads to suppression of tumor growth in nude mice (57). Therefore, fetuin B could be involved in the tumor-suppressing effects of genistein in the rat mammary gland. Genistein also decreased the levels of PGK by 54% compared with control (37). PGK is involved in the glycolytic pathway. Annexin A2 and PGK are components of the primer recognition complex that stimulates the activity of DNA polymerase (58,59). Therefore, decreased expression of annexin A2 and PGK could explain in part the reduced rate of cell proliferation observed in the mammary gland following treatment with genistein.

**Summary**

Prepubertal exposures to orally administered genistein and BPA resulted in a significant decrease and increase, respectively, in tumor multiplicity in the DMBRA-rat model of mammary carcinogenesis (7–9). Although genistein and BPA could have effects on numerous organs and systems of the body, our studies have focused on the mammary gland and endocrine system, with our data suggesting that the differential effects of BPA and genistein on the expression of annexin A2, VEGFR-2, and phospho-AKT could explain at least in part the opposing effects of these hormonally active compounds on cell proliferation and mammary carcinogenesis. The effects of BPA and genistein on these signaling pathways were observed 30 d postexposure, suggesting that dietary exposure during early postnatal development to these compounds could epigenetically reprogram the expression of genes involved in remodeling of the rat mammary gland (60–62). These studies emphasize the importance of proteomic technologies in elucidating the mechanisms of hormonally active chemicals in target organs such as the mammary gland. In addition, these technologies allow the identification of potential biomarkers of susceptibility such as annexin A2, selenium binding protein, GRP-78, HSP-70, fetuin B, and PGK.

**Future studies** will address how the combined exposure to both BPA and genistein may alter the expression of these biomarkers and their possible validation in human studies.

**Acknowledgments**

C.A.L. and J.R. designed the research; A.M.B., J.W., S.J., and J.M. carried out the research; and A.M.B. wrote the manuscript. All authors read, critiqued, and approved the final manuscript.

**Literature Cited**

17. European Food Safety Authority. Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food. EFSA J. 2006;428:1–75.


