Basic nutritional investigation

A gel-based proteomic analysis of the effects of green tea polyphenols on ovariectomized rats

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**A B S T R A C T**

Objective: Our recent study demonstrated the protective action of green tea polyphenols (GTPs) against bone loss in ovariectomized (OVX) rats through their antioxidant capacities to scavenge reactive oxygen species. The objective of the present study was to evaluate the alterations of liver protein profiles in estrogen-deficient middle-aged rats after GTP treatment by a gel-based proteomic approach. This may lead to understanding the mechanisms of GTPs in promoting bone health.

Methods: Liver samples were obtained from 14-mo-old female OVX rats treated with no GTPs (OVX) or 0.5% (w/v) GTPs (OVX + GTP) in drinking water for 16 wk (n = 10/group). Two-dimensional difference gel electrophoresis combined with mass spectrometry was used to compare the liver protein profiles of pooled samples from the OVX and OVX + GTP groups. Liver proteins were labeled in duplicate by reversing the fluorescent dyes.

Results: Approximately 800 protein spots were detected. The expression levels of superoxide dismutase-1 and adenosine triphosphate synthase were 2.0-fold and 1.5-fold higher in the OVX + GTP group versus the OVX group, respectively, whereas the expression level of catechol-O-methyltransferase was 1.5-fold lower in the OVX + GTP group versus the OVX group. The changes of superoxide dismutase-1 and catechol-O-methyltransferase in individual liver samples were confirmed by western blots.

Conclusion: Our data provide further evidence for the antioxidant role of GTPs by increasing superoxide dismutase-1 and adenosine triphosphate synthase and the estrogen-associated effect of GTPs by decreasing catechol-O-methyltransferase.

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Introduction

Osteoporosis is a systemic skeletal disorder characterized by low bone mass and structural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture [1]. Osteoporosis and related fractures represent a significant and growing public health concern worldwide. The prevalence of osteoporosis-related fractures is expected to increase due to the rise in aging populations [2]. Approximately half of women older than 50 y are expected to have an osteoporosis-related fracture during their lifetime [1,2]. Osteoporosis usually progresses asymptptomatically, thus few people are diagnosed before a fracture occurs. Therefore, an appropriate intervention in individuals at high risk is important [3].

Green tea polyphenols (GTPs), commonly known as flavans or catechins, include primarily (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin, (-)-epicatechin-3-gallate, and (-)-epicatechin [4]. GTP has been demonstrated to have beneficial health effects by decreasing oxidative damage [5,6] and anti-inflammation [7]. For example, the most widely recognized properties of GTPs are their antioxidant capacities arising by chelating redox-active transition metal ions, interrupting chain oxidation reactions, providing hydrogen atoms, and serving as acceptors of free radicals [8,9]. Oxidative stress has been shown to be a pivotal pathogenic factor for age-related bone loss in animal models through its involvement in bone formation and resorption [10,11]. Estrogen
deficiency occurring after menopause is another well-known cause of bone loss [12,13]. Our previous studies showed that ovariectomy in middle-age female rats could decrease antioxidan
capacity and increase bone loss in middle-age rats. In addition, we showed that liver glutathione peroxidase activity, serum estradiol,
and bone mineral density were lower, whereas urinary 8-hydroxy-
2'-deoxyguanosine and calcium were higher in ovariectomized
(O VX) rats compared with intact animals [10]. These data coupled
with previous research suggest that aging-induced bone loss in
OVX middle-aged female rats could represent a valid model to
study osteoporosis for postmenopausal women [14]. Furthermore,
GTPs alleviated deterioration of bone microarchitecture by
enhancing antioxidan
capability and bone formation, suppressing oxidative damage and bone resorption, and modulating endo-
cortical and cancellous bone compartments [10,15].

Two-dimensional (2D) gel elec
phoresis with mass spectrometry (MS) is considered a powerful proteomic
approach by simultaneously resolving hundreds to thousands of
proteins on a single gel [16,17]. The 2D-difference gel elec
phoresis (2D-DIGE) dramatically improves the capabilities of
traditional 2D elec
phoresis for reliability and reproducibility
of proteome comparison and the identification and quantiﬁca-
tion of low-abundance protein [18,19]. In the present study, we
extended our previous study to investigate the possible mecha

isms of GTP protective effects on bone health by comparing the
liver protein pro
ciles of the established OVX rat without (OVX) or
with GTP treatment (OVX + GTP) using 2D-DIGE.

Materials and methods

Animals and GTP treatments

Virgin 14-mo-old F344 × BN F1/NIA rats with ovariectomy were purchased
from the National Institute on Aging (Bethesda, MD, USA). Upon arriving, rats
were acclimated for 7 d to a powdered AIN-93M diet (DYETS, Bethlehem, PA,
USA) and distilled water ad libitum. Twenty OVX rats were randomly assigned to
the untreated control group that received no GTP supplement (OVX, n = 10) and the
treated group that received 0.5% GTP (wt/vol) in distilled drinking water
(OVX + GTP, n = 10). Rats were housed in individual stainless-steel cages under
a controlled temperature of 21 ± 2°C with a 12-h light–dark cycle. All rats were
fed a powdered AIN-93M diet during the 16-wk feeding period. GTP was
purchased from Shili Natural Product Company, Inc., a US–China joint venture
(Guilin, Guangxi, China), with a purity higher than 98.5%. According to analyses
with high-performance liquid chromatography with electrochemical detection
and high-performance liquid chromatography with ultraviolet, every 1000 mg of
GTP contained 480 mg of EGCg, 160 mg of (-)-epicatechin-3-gallate, 60 mg of
(-)-epicatechin, 103 mg of (-)-epigallocatechin, and 30 mg of catechin.
All procedures were approved by the Texas Tech University Health Sciences Center
institutional animal care and use committee.

Sample preparation

After animals were anesthetized and euthanized, livers were harvested,
rapidly frozen in liquid nitrogen, and stored at –80°C until further use. The indi-
vidual liver tissues was resuspended in standard cell lysis buffer containing 30 mM
Tris-HCl (Bio-Rad, Hercules, CA, USA), 2 M thiourea (Bio-Rad), 7 M urea (Bio-Rad),
and 4% (w/v) 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate
(Bio-Rad) at 10 μg/mL of weight. The samples were homogenized by soni-
cation on ice for 30 s with a sonic dismembrator (model 100, Fisher, Pittsburgh,
PA, USA). Protein concentrations were measured using the Bradford protein assay
(Bio-Rad).

2D-difference gel elec
phoresis

An aliquot of 500 μg of protein from individual samples of the OVX or
OVX + GTP group was added together to form two pooled samples. The pooled
samples from the OVX and OVX + GTP groups were run in duplicate by reverse
labeling with N-hydroxyyscuscimide ester Cy3 and Cy5 dyes (GE Healthcare, Piscataway, NJ, USA). The internal standard (a mixture of equal
amounts of OVX pooled samples and OVX + GTP pooled samples) was labeled with N-hydroxy-
yscuscimide ester Cy2 dye according to the procedure as described previously
[20]. Each Cydye was fi
rst diluted in 400 pmol/μL with N,N-dimethylformamide
(Sigma, St. Louis, MO, USA) on ice. An aliquot of 1 μL of diluted Cy2, Cy3, or Cy5 was
then added into microtubes containing 50 μg of protein. After centrifugation,
the mixture was incubated on ice for 30 min without light exposure. Thereafter, 1 μL of
10 mM l-lysine (Sigma) was added to stop the reaction by mixing and keeping on ice
for 10 min in the dark. Samples labeled with Cy2, Cy3, and Cy5 (containing 130 μg of
proteins) were mixed and adjusted to 300 μL by 2D rehydration buffer
(Bio-Rad) containing 8 M urea, 0.5% 3-(3-cholamidopropyl) dimethylamino-
1-propanesulfonate, 10 mM dithiothreitol, 0.5% bio-lytes ampholyte, and 0.1 mg/mL
of bromophenol blue. After overnight rehydration with 17 cm of immobilized
linear pH 3 to 10 gradient strips (Bio-Rad), isoelectric focusing was conducted
with 250 V for 20 min and was gradually increased to 10000 V within 2.5 h and kept at
10 000 V for a total of 50 000 voltage hours. Immobilized linear pH 3 to 10 gradient
strips were subsequently equilibrated with equilibration buffer I (6 M urea, 2% sodium
dodecyl sulfate [SDS], 375 mM Tris-HCl[pH 8.8], 20% glycerol, and 130 mM
dithiothreitol) and buffer II (6 M urea, 2% SDS, 375 mM Tris-HCl, 20% glycerol,
and 135 mM iodoacetamide). Proteins were finally separated with 12% SDS-
polyacrylamide gel elec
phoresis and visualized by Typhoon Trio Imager (GE Healthcare) with three excitation wavelengths of 488, 532, and 633 nm for Cy2, Cy3,
and Cy5, respectively.

In-gel digestion and MS identiﬁcation

Selected spots were harvested from the 2D gels stained by Coomassie Bril-
liant Blue R250 (Bio-Rad). Gel plugs were sequentially desiccated with acetonite-
trile, reduced with 10 mM dithiothreitol at 56°C, and alkylated with 55 mM iodoacetamide without light exposure. Samples were trypsinized overnight at 37°C (Promega sequencing grade-modiﬁed trypsin, Promega, Madison, WI, USA), and the supernatants were dried with a vacuum centrifuge (Labconco Corpora-
tion, Kansas, MO, USA). Peptides were eluted with a matrix (x-cyano hydroyx-
cinnamic acid: Sigma) prepared in 50% acetonitrile and 0.1% trifuluoroacetic acid
(Pierce, Rockford, IL, USA) and were analyzed by a matrix-assisted laser
desorption/ionization time-of-ﬂight mass spectrometry (4800 Plus MALDI TOF TOF Analyzer, Applied Biosys-
tem, Foster City, CA, USA). Peptide masses were located by the Mascot search engine (Matrix Science, London, UK).

Western blot

Thirty micrograms of protein from each of the pooled samples and also from
each individual samples from the OVX or OVX + GTP groups were separated by
12% SDS-polyacrylamide gel elec
phoresis. These were then transferred to
polyvinylidene ﬂuoride membranes and incubated overnight at 4°C in blocking
buffer containing 3% milk in 1× phosphate buffered saline with 0.1% Tween 20.
Thereafter, the membranes were incubated for 1 h with primary antibodies
against superoxide dismutase-1 [SOD1] or anti--catalase O-methyltransferase
[COMT]; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1,000 dilution and
then incubated at 1 h with horseradish peroxidase–conjugated donkey anti-
rabbit immunoglobulin G (Santa Cruz Biotechnology) at a dilution of 1:2,000 at
room temperature. After a brief incubation with enhanced chemiluminescence
reagents (GE Healthcare), the signals on membranes were exposed to Hyperfilm
(GE Healthcare) by autoradiography and analyzed by Quantity One software
(Bio-Rad). α-Tubulin was used as a loading control.

Statistical analysis

Images from 2D-DIGE were analyzed by DeCyder 6.5 software (GE Health-
care). Protein spots with MS-identifiable abundance were considered signiﬁcant
based on the criteria of greater than 1.5-fold changes in both duplicated gels. The
Mann-Whitney test was used to compare densitometric intensity of individual
sample between the OVX and OVX + GTP groups. All analyses were performed
using SSPI 13.0 software (SPSS, Inc., Chicago, IL, USA) and differences with
P < 0.05 were considered statistically signiﬁcant.

Results

2D-DIGE analysis

Approximately 800 spots were identiﬁed in the pooled liver
samples from the OVX and OVX + GTP groups. A representative
2D-DIGE gel is shown in Figure 1. Duplicate DIGE gels with
reverse label were run and showed more than 95% between-gel
reproducibility. Among all matched spots, a total of five spots
showed at least 1.5-fold changes in the OVX + GTP group compared with the
OVX group, including one spot with 2.0-fold increase, one spot with 1.7-fold increase, two spots with 1.5-fold increase, and one spot with 1.5-fold decrease (Fig. 1).

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Protein identification by MALDI-TOF/TOF-MS

All five spots with significant changes were identified by MS. The spot with 2.0-fold increase in the OVX + GTP group versus the OVX group was identified as copper/zinc SOD1 (a total score of 229 and six matched peptides with 44% sequence coverage; Fig. 2A). The single spot with 1.7-fold increase in the OVX + GTP group versus the OVX group was identified as subunit α of adenosine triphosphate (ATP) synthase (a total score of 388 and 18 matched peptides with 43% sequence coverage). The two spots with 1.5-fold increase in the OVX + GTP group versus the OVX group were identified as ATP synthase subunit β (a total score of 134 and four matched peptides with 48% sequence coverage; and a total score of 137 and three matched peptides with 30% sequence coverage). The single spot with 1.5-fold decrease in the OVX + GTP group versus the OVX group was identified as COMT (a total score of 340 and nine matched peptides with 70% sequence coverage; Fig. 2B).

Western blot analysis of SOD1 and COMT

To validate the results of 2D-DIGE, the protein expression levels of SOD1 and COMT in pooled samples and each of the 10 individual samples from the OVX and the OVX + GTP groups were analyzed by western blot (Fig. 3). The expression levels of α-tubulin showed a similar amount of protein loading. The expression of SOD1 in the pooled OVX + GTP group was a 1.3-fold increase, whereas the expression of COMT was a 1.4-fold decrease compared with the pooled OVX group. The measurements of densitometric intensity (mean ± standard deviation) were 422.0 ± 25.8 (range 394.2–477.0) in the OVX samples and 505.0 ± 72.3 (range 367.6–583.6) in the OVX + GTP samples for SOD1 (P = 0.010) and 89.1 ± 18.6 (range 59.4–124.9) in the OVX samples and 63.9 ± 23.9 (range 28.9–119.3) in the OVX + GTP samples for COMT (P = 0.013).

Discussion

Osteoporosis is an age-dependent degenerative bone disease and represents a common public health problem particularly for postmenopausal women [21,22]. Development of feasible and valuable prevention strategies are of major importance in minimizing the burden resulting from this disease [21]. The protective effect of tea consumption on bone has been observed in epidemiologic studies and in vivo experimental studies [10,15,23,24]. For instance, our previous studies demonstrated that aging-induced bone loss in OVX middle-aged female rats represents a well-established model for studying estrogen-deficiency induced deterioration of bone microarchitecture in postmenopausal women. In addition, GTP enabled protection of bone loss in this rat model by suppressing bone resorption and modulating endocortical and cancellous bone compartments [10,15]. In the present study, the GTP protective effects on the middle-aged female OVX rats were further studied by investigating the liver protein profiles using 2D-DIGE combined with MS. SOD1, ATP synthase, and COMT showed significant protein expression changes in the OVX + GTP group compared with the OVX group. These data were further confirmed by using western blot analysis.

Reactive oxygen species (ROS) play dual roles in bone metabolism. Under physiologic conditions, the production of ROS facilitates bone remodeling by accelerating destruction of calcified tissue. However, under pathologic conditions, the increases in ROS production and osteoclastic activity are
of O₂ [29,30]. The main function of SOD1 is to catalyze the conversion and is widely distributed in the organelles of mammalian cells contributes to approximately 70% to 80% of cellular SOD activity proteins containing a highly conserved pair of cysteines, [28]. SOD1, one of the most abundant disulfide containing antioxidant enzymes that play crucial roles in scavenging ROS.

Superoxide dismutases, including SOD1, manganese SOD (SOD2), and extracellular SOD (SOD3), are major endogenous antioxidant enzymes that play crucial roles in scavenging ROS [28]. SOD1, one of the most abundant disulfide-containing proteins containing a highly conserved pair of cysteines, contributes to approximately 70% to 80% of cellular SOD activity and is widely distributed in the organelles of mammalian cells [29,30]. The main function of SOD1 is to catalyze the conversion of O₂ to O₂⁻ and H₂O₂, which is in turn removed by glutathione peroxidase or catalase [31]. A recent study showed that SOD1 knockout mice (SOD1⁻/⁻) had elevated oxidative stress and significantly decreased bone mineral density compared with SOD1⁺/+ mice [27]. An increased expression level of liver SOD1 in GTP-supplemented rats was observed in the present study. Our previous study also showed decreased urinary 8-hydroxy-2'-deoxyguanosine and increased liver glutathione peroxidase in OVX rats after GTP treatment [10,15]. These results corroborate the osteoprotective role of GTP by increasing antioxidant capacity and/or decreasing oxidative stress damage. Our observations that supplementation of GTP in the drinking water to OVX rats enhanced SOD1 activities is consistent with a report by Meng et al. [32], which showed that EGCG increased SOD1, SOD2, and glutathione peroxidase gene expression and enzyme activities in the aging process of human fibroblasts.

Defective activity or decreased expression of SOD1 has also been identified in a number of other human diseases such as aging, amyotrophic lateral sclerosis, Parkinson’s disease, and cancers [33,34]. Conversely, increased levels of SOD1 can protect motor neurons by reducing oxidative stress [35], and overexpression of SOD1 could also protect the heart from postischemic injury and mitigate spontaneous intracerebral hemorrhage during hypertension [31,36]. Therefore, the increased expression level of SOD1 after GTP treatment could lead to the protection against a variety of chronic diseases besides osteoporosis.

In the present study, we also demonstrated the upregulation of ATP synthase after GTP treatment in OVX rats. These data provide more evidence of GTP’s protective effect in decreasing oxidative damage. Such findings agree with those of others [37,38]. Roy et al. [37] reported that an inhibition of ATP synthase activity significantly stimulated the production of mitochondrial ROS. In addition, Wang et al. [38] found that ATP synthase subunit α showed protective and therapeutic roles in primary cardiac myocytes of iron overloaded rats by suppressing ROS production.

Estrogen deficiency occurring after menopause is the most frequent cause of bone loss, which is due to a complex interplay of hormones and cytokines [12]. Estrogen is a potent antiresorptive agent with direct effects in promoting the development and proliferation of osteoblasts, inhibiting apoptosis of osteoblasts and osteocytes, increasing apoptosis and suppressing activity of osteoclast, and decreasing the production of the

Fig. 2. Tandem mass spectrometry identification of copper/zinc superoxide dismutase type 1 (A) and catechol O-methyltransferase (B). The mass spectrometric/mass spectrometric fragmentation spectra (obtained after trypsin digestion) of VISLSGEHSLGIR for copper/zinc superoxide dismutase type 1 and KGTVLLADNVIVPGTPDFLAYVR for catechol O-methyltransferase are shown. The resultant mass spectrometric/mass spectrometric data were identified using Mascot.

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receptor activator of the nuclear factor-κB ligand [39]. Moreover, estrogen is a phenolic compound sharing similarities with the structure of lipophilic antioxidants, suggesting the capability of estrogen to detoxify accumulated ROS [40]. Therefore, prevention of estrogen deficiency is useful to protect postmenopausal women from estrogen-deficiency induced osteoporosis. Although GTP did not reverse the decreasing serum estradiol level in OVX rats in our previous report [10], we have found for the first time in the present study that the expression of COMT, an estrogen-degrading enzyme, was significantly downregulated in the GTP-supplemented OVX rats compared with the OVX-only rats. COMT inactivates catechol estrogens by catalyzing the methylation of catechol estrogens to methoxy estrogens through phase II metabolic inactivation [41]. In line with our findings, cytosolic COMT could catalyze the rapid O-methylation of EGCG and (−)-epigallocatechin and be further potently inhibited by EGCG and its metabolites [42, 43]. Therefore, the decreased COMT expression observed in OVX rats supplemented with GTP in drinking water would inhibit the inactivation of estrogen and it may eventually contribute to a protective role in bone loss.

The COMT genotype has been reported to influence bone through endogenous sex steroids in postmenopausal women [44]. The low activity genotype of COMT could protect against myocardial infarction, partly through the estrogen mechanism [45]. Furthermore, the COMT inhibitors, tolcapone and entacapone, were approved as adjunct therapies for Parkinson’s disease [46], and ECGG has been shown as a potent COMT inhibitor to be beneficial in treating Parkinson’s disease [47]. Therefore, besides the importance in bone health through estrogen mechanism, COMT inhibition by GTP treatment could provide further evidence for GTP’s protective effects in other human diseases such as Parkinson’s disease.

Taken together, the proteomic approach combining 2D-DIGE and MS is an efficient strategy to identify differential expression of multiple proteins in the liver samples of OVX rats supplemented with GTP. Based on this proteomic analysis, the expression levels of SOD1, ATP synthase, and COMT were altered in the GTP-supplemented OVX rats compared with those in the OVX-only rats. SOD1 and ATP synthase play fundamental antioxidant roles in the detoxification of ROS. COMT is an estrogen-degrading enzyme involved in phase II metabolic inactivation of catechol estrogens. Our data provide evidence for antioxidant and estrogen-like roles for GTP. However, the gel-based approach has low resolution for hydrophobic proteins, low-abundance proteins, low- or high-molecular-weight proteins, and proteins at extreme pH. Other important protein fingerprints need to be investigated using additional complementary proteomic methods. In addition, future studies will be warranted to confirm these findings in human clinical studies.

Conclusion

It was shown that the expression levels of SOD1 and ATP synthase were increased, whereas that of COMT was decreased in the OVX + GTP group versus the OVX group, providing further evidence for the antioxidant and estrogen-associated effects of GTP.

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