Phytoestrogen exposure elevates PTEN levels

Kristin A. Waite1,†, Michelle R. Sinden1,† and Charis Eng1,2,*

1Clinical Cancer Genetics Program, Human Cancer Genetics Program, Comprehensive Cancer Center, Division of Human Genetics, Department of Internal Medicine and Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, OH 43210, USA and 2Cancer Research UK Human Cancer Genetics Research Group, University of Cambridge, Cambridge CB2 1XZ, UK

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Epidemiological data suggest that consumption of phytoestrogens can be protective against the development of breast cancer. It may be logical to postulate that phytoestrogens may regulate proteins that control cellular division, such as the tumor suppressor PTEN. Germline, and more significantly, somatic PTEN mutations have been observed in a broad range of human cancers, especially those of the breast. Active PTEN results in decreased phosphorylation of Akt and MAPK, the up-regulation of p27 and down-regulation of cyclin D1 protein levels resulting in decreased proliferation and an increase in apoptosis. We hypothesized that phytoestrogen exposure regulates PTEN protein expression in the breast cancer cell line, MCF-7. When MCF-7 cells were stimulated with resveratrol, quercetin or genistein, there was an increase in PTEN protein levels. Concomitantly, phytoestrogen stimulation resulted in decreased Akt phosphorylation and an increase in p27 protein levels, indicating active PTEN lipid phosphatase activity. In contrast, we found that MAPK phosphorylation and cyclin D1 levels, which are regulated by PTEN’s protein phosphatase activity, were not altered. Using semi-quantitative RT–PCR, we found that mRNA levels were slightly increased in cells stimulated by phytoestrogens, suggesting that the mechanism for increased PTEN protein expression is dependent upon transcription. Concurrently, our data provide evidence that a mechanism for phytoestrogens’ protective nature is partially through increased PTEN expression. More importantly, it provides a novel target for the regulation of PTEN expression and suggests that dietary changes may be adjunctive to traditional preventive and therapeutic strategies against breast cancer.

INTRODUCTION

The tumor suppressor gene PTEN, localized to 10q23.3, has been implicated in the pathogenesis of a variety of human cancers, including the inherited hamartoma–neoplasia syndromes, Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome and Proteus and Proteus-like syndromes (1). Cowden syndrome is an under-diagnosed syndrome with high risk of breast (28–50% in a lifetime) and thyroid (10%) cancers (2,3). We have found that germline mutations of PTEN cause 85% of Cowden syndrome cases and there is a genotype–phenotype correlation with breast cancer (4,5). More importantly, PTEN has been shown to play a role in sporadic cancers, including those of the breast. Since its discovery in 1997, abundant data show that PTEN, the protein product of PTEN, is a tumor suppressor in vitro and in vivo.

In vitro, recombinant PTEN has been shown to dephosphorylate protein substrates on serine, threonine and tyrosine residues (6). In vivo, PTEN has been demonstrated to lie upstream of cellular signaling cascades [(1) and references therein]. For example, it has been shown to dephosphorylate focal adhesion kinase (FAK), which inhibits cell spreading and migration. PTEN has also been shown to dephosphorylate lipid substrates, specifically phosphatidylinositol(3,4,5)triphosphate (PI(3,4,5)P3) yielding phosphatidylinositol(4,5)biphosphate (PI(4,5)P2). Consequently, PTEN is an antagonist of P13K, the enzyme that phosphorylates PI(4,5)P2. Given that PI(3,4,5)P3 is required for Akt recruitment to the plasma membrane and its subsequent activation, PTEN inhibits the pro-proliferative Akt-dependent pathways (1). Accordingly, proper PTEN function leads to decreased phosphorylated-Akt (P-Akt) levels and apoptosis occurs. In contrast, absent

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or dysfunctional PTEN leads to high levels of P-Akt, which is pro-proliferative. PTEN also regulates cell survival by coordinating the cell cycle. In addition, PTEN coordinates G1 arrest through up-regulation of p27 and concomitant down-regulation of cyclin D1 (7) as well as regulating the activation of the MAPK pathway.

PTEN appears to be constitutively active in vivo, suggesting that modulation of its activity is through regulation of both transcription and protein degradation. Activated PPARγ (8), p53 (9) and EGR-1(10) have been shown to up-regulate PTEN transcription. Factors that may down-regulate transcription are yet to be identified. Protein stability has been shown to be regulated in both a phosphorylation-dependent and -independent manner (11,12), and modes of PTEN protein regulation are still to be elucidated.

Epidemiological data suggest that the consumption of moderate levels of soy, red wine and a diet rich in vegetables and fruits can be protective against breast cancer (13,14). Previous research has shown that an important component of these foods is the phytoestrogens. However, how they elicit this effect is not completely known. Recently, there has been an emphasis on the elucidation of the interaction between phytoestrogens and regulatory proteins such as those encoded by tumor suppressor genes and the cellular signaling cascades that regulate gene expression/activation. To date, there has not been direct evidence for phytochemicals interacting with PTEN. However, phytoestrogen exposure leads to cellular effects identical to those mediated by proper PTEN function and promotes apoptosis via a decrease in P-Akt levels. Also similar to PTEN, phytoestrogens play a role in regulating cell cycle and apoptosis such as MAPK (15–17) and cell cycle arrest (18). These similar cellular effects mediated by PTEN and phytoestrogens could be explained by independent mechanisms but it is much more plausible that one or more are the result of an interaction between PTEN and the phytoestrogens. We therefore investigated the effect of phytoestrogens on PTEN protein levels and activity.

RESULTS
Phytoestrogen stimulation increases PTEN protein levels
We investigated the ability of three phytoestrogens, genistein, resveratrol and quercetin, to regulate PTEN protein levels in the MCF-7 breast cancer cell line. Genistein, a soybean isoflavone, has been characterized as the most likely factor contributing to the putative breast- and prostate-cancer-preventative activity of soy (14). Resveratrol, an antibiotic produced in the skin of grapes (Vitis vinifera) as a defense against bacterial or fungal infection, is the most likely cause of the ‘French paradox’, which is the link between the consumption of red wine and the reduction of mortality rates from cardiovascular diseases and certain cancers (19,20). Finally, quercetin is the archetypal dietary flavonoid and is ubiquitous in fruits and vegetables, with significant amounts occurring in onions, tomatoes and apples and in wine. These compounds were selected due to the increased consumption in the United States of soy-based products, red wine and fruits and vegetables, according to dietary suggestions. Furthermore, as these compounds mimic the actions of estrogens, information obtained from these phytoestrogens will have a greater impact on women’s health than other phytochemicals.

MCF-7 cells were stimulated with each of these three phytoestrogens. We found that PTEN protein levels increased in response to each of these compounds, resveratrol, genistein and quercetin (Fig. 1). Stimulation with genistein resulted in an increase in PTEN protein levels, ~3-fold, at as little as 0.1 nM. An increase in PTEN protein was also observed at 0.1 and 1.0 μM. Interestingly, we found that there was a reproducible decrease in the ability of genistein to induce PTEN protein at 1 nM (Fig. 1). Like genistein, resveratrol and quercetin stimulation also resulted in an increase in PTEN protein levels. Resveratrol increased PTEN protein levels at 0.1 nM and was maximal at 100 nM (Fig. 1). Interestingly, like genistein, we observed a reproducible decrease in the ability of resveratrol to increase PTEN protein levels at 10 nM (~1.8-fold increase). An increase in PTEN protein expression was observed at 0.1 nM quercetin. We found that all three phytoestrogens stimulated PTEN protein expression in a time-dependent manner and that maximal stimulation occurred after 48 h of stimulation (data not shown).

Phytoestrogen stimulation decreases P-Akt levels
It has been well documented that PTEN antagonizes the PI3K pathway via its lipid phosphatase activity (1,21). When PTEN protein levels increase and are functional, PI(3,4,5)P3 levels decrease which results in a decrease in the levels of phosphorylated-Akt (P-Akt). However, it has also been shown that PTEN induction does not always correlate with an increase in PTEN activity (12). Based upon this, we investigated the ability of genistein, resveratrol and quercetin to decrease P-Akt levels at concentrations that induced PTEN protein expression. Exposure to all three compounds resulted in a decrease in P-Akt levels, albeit to differing degrees, at concentrations that resulted in an increase in PTEN protein (Fig. 2A). Resveratrol stimulation resulted in the most apparent decrease in P-Akt levels followed by quercetin and genistein (Fig. 2A).

Phytoestrogen stimulation increases p27Kip1 protein levels
We have previously shown that PTEN expression can result in an increase in p27 protein levels and that this increase in p27 expression is due to PTEN’s lipid phosphatase activity (7). Therefore, like P-Akt, we sought to determine whether the increase in PTEN protein expression observed with phytoestrogen stimulation resulted in an up-regulation of p27 protein. Figure 2B demonstrates that stimulation by each of the three phytoestrogens, at concentrations that produced an increase in PTEN protein expression, resulted in an increase in p27 protein levels. Stimulation of MCF-7 cells for 48 h with genistein, quercetin or resveratrol resulted in an increase in p27 protein levels. Genistein stimulation resulted in the
highest increase in p27 protein levels, followed by quercetin and resveratrol both of which had similar stimulatory effects. These data, taken together with the results aforementioned, suggest that phytoestrogen stimulation results in an increase in PTEN protein levels with concomitant downstream effects that are due to PTEN’s lipid phosphatase activity.

Phytoestrogen stimulation does not affect P-MAPK or cyclin D1 levels

PTEN is a dual specificity phosphatase having both lipid and protein phosphatase activities. We have previously shown that functional PTEN decreases cyclin D1 levels via its protein phosphatase activity (7). In addition, we have shown that the protein phosphatase activity of PTEN is involved in down-regulating the activity of p44/p42 MAPK (22). The down-regulation of p44/p42 MAPK can be monitored by observing a decrease in MAPK phosphorylation by western blot. Because of this dual activity, we chose to investigate the effect of phytoestrogen stimulation on downstream targets of PTEN’s protein phosphatase activity, namely, cyclin D1 protein levels and MAPK phosphorylation.

When the effect of phytoestrogen stimulation on cyclin D1 levels was examined, we found that there was little effect on cyclin D1 levels when stimulated at concentrations that induced PTEN protein. As shown in Figure 3A, stimulation with genistein results in a slight increase in cyclin D1 levels, where we would expect a decrease due to the increase in PTEN protein levels. In contrast to unstimulated cells, cyclin D1 levels remained unchanged in cells stimulated with either resveratrol or quercetin (Fig. 3A). Similarly, when we examined MAPK phosphorylation, we found that there was little effect after phytoestrogen stimulation. MAPK phosphorylation levels also remained unchanged when stimulated with genistein, quercetin or resveratrol at levels that induced PTEN protein expression (Fig. 3B). These observations suggest that although these phytoestrogens can increase PTEN protein, whose lipid phosphatase activity results in intact Akt-p27 downstream signaling, they may modulate the protein phosphatase activity of PTEN, although other explanations are also equally plausible.

Phytoestrogen stimulation results in a slight increase in PTEN mRNA production

PTEN protein levels can be regulated by both transcription and degradative mechanisms. As phytoestrogens have been shown to increase transcriptional activity, we investigated the effect of these phytoestrogens on PTEN’s mRNA levels using semi-quantitative RT–PCR (Fig. 4). Stimulation of MCF-7 cells with genistein, quercetin or resveratrol results in a slight increase in mRNA levels (Fig. 4). Stimulation with genistein, which resulted in the highest increase in PTEN protein levels, resulted in a slight, but reproducible, increase in mRNA levels by \(1.3\)-fold \((P = 0.02)\). Quercetin stimulation also resulted in a \(1.3\)-fold increase in PTEN mRNA, whereas resveratrol stimulation resulted in a \(1.2\)-fold increase (both \(P = 0.02\)). This suggests that phytoestrogen stimulation increases PTEN transcription.

DISCUSSION

More than two-thirds of human cancers have been postulated to be preventable by modifying lifestyle. It has been reported that 10–70% (average 35%) of human cancer mortality is attributable to diet alone (23). According to more than 250 population-based studies, including case–control and cohort studies, individuals who consume about five servings of fruit and vegetables a day have approximately half the risk of developing cancer when compared with those who eat fewer than two servings (14). In addition, recent studies have shown that an increased consumption of soy during childhood
is associated with a decreased breast cancer risk (24–27) and the moderate partaking of red wine can be protective against breast cancer (20). The biochemical basis for this protective action is not completely understood, but we do know that compounds from these dietary sources, the phytoestrogens, play an important role.

We provide evidence here that one of the biochemical mechanisms for the protective effects of phytoestrogens may be regulation of PTEN protein levels as well as function. First, we have demonstrated that phytoestrogen exposure results in an increase in PTEN protein and subsequent increase in cellular responses that are downstream of PTEN’s lipid phosphatase activity (Akt phosphorylation and p27 protein levels). Interestingly, we found that although PTEN protein was increased, we did not observe an increase in cellular responses that are downstream of PTEN’s protein phosphatase activity (cyclin D1 protein levels and MAPK phosphorylation). This suggests that phytoestrogens may also regulate PTEN’s phosphatase activity or that PTEN may modulate the effects of phytoestrogens; however, this remains to be determined and is beyond the current scope of this study.

Although a direct interaction between PTEN and phytoestrogens has not been identified, phytoestrogens have been documented to affect cellular processes much in the same manner as PTEN, suggesting that there is a connection. Phytoestrogens, like PTEN, have been shown to regulate the PI3K pathway. Quercetin and genistein are known inhibitors of the PI3K pathway (28,29) and have been shown to inhibit Akt signaling pathways (30), while similar effects have been demonstrated in MCF-7 cells when high concentrations (>50 μM) of resveratrol were used (31). Our data suggest that a mechanism of quercetin and genistein inhibition of Akt signaling and the PI3K pathway is through the up-regulation of PTEN, an antagonist to the PI3K/Akt pathway. We show a response to resveratrol at lower levels than previously observed. Previous studies have used non-synchronized cells. In our hands, resveratrol did not increase PTEN levels or inhibit phosphorylation of Akt in cells that were not synchronized. Regulation of PI3K/Akt pathway by PTEN is via its lipid phosphatase activity. The lipid phosphatase activity of PTEN has also been shown to increase p27 protein levels. We show here that stimulation with phytoestrogens also results in an increase in p27 levels. Thus, phytoestrogen exposure leads to cellular effects identical to those mediated by proper PTEN function, and more specifically the lipid phosphatase activity of PTEN, and promotes apoptosis via a decrease in P-Akt levels and subsequent cellular events.

As is the case with PTEN, regulation of the MAPK pathway by phytoestrogens is important in the mediation of cellular growth arrest. Resveratrol, quercetin and genistein have all been shown to inhibit signaling kinases such as MAPK (15,16,32). As the documented effects of phytoestrogens on p44/p42 MAPK and cyclin D1 are identical to that of PTEN, we had anticipated an augmented decrease in levels of these two signaling factors along with the increased levels of PTEN protein with phytoestrogen stimulation. This, as the results have shown, was not the case. Cyclin D1 and MAPK phosphorylation levels were apparently affected very little, if at all, by the addition of phytoestrogens and subsequent increase in PTEN protein to our system. Interestingly, these cellular events, which did not exhibit expected results based on previous research and knowledge of PTEN and phytoestrogens, are downstream of PTEN’s protein phosphatase activity. This may suggest that higher levels of PTEN are required for cellular responses due to its protein phosphatase activity to be observed, particularly when compared with its lipid phosphatase activity. It is also possible that phytoestrogen stimulation results in changes in subcellular localization of PTEN, moving PTEN closer to lipid substrates and away from protein phosphatase substrates. It is becoming clear that PTEN’s subcellular localization may play an important role in its ability to regulate cell cycle (33). Indeed, cytosolic PTEN seems to be important in breast and thyroid tumors (34,35) (C. Eng, unpublished data). Upon examination of PTEN’s subcellular localization, we found that there was no...
change in subcellular localization in response to phytoestrogen stimulation (data not shown), suggesting that alterations in subcellular localization are not responsible for the lack of PTEN’s protein phosphatase activity. It is possible that phytoestrogen stimulation results in a conformational change in PTEN, which inhibits its ability to bind to protein substrates. This and other mechanisms remain to be investigated and are beyond the scope of this paper.

PTEN is thought to be constitutively active, in vivo. Thus, regulation of PTEN levels via transcription, translation and post-translational means remains to be investigated in depth. We show here that phytoestrogen stimulation results in a slight, but reproducible, increase in PTEN mRNA. Activated PPAR\(\gamma\) (8), p53 (9) and EGR-1(10) have been shown to up-regulate PTEN transcription. Thus, it is plausible that phytoestrogen exposure results in the activation of one of these transcription factors, although this remains to be determined. Perhaps, the most attractive target for phytoestrogen with regard to PTEN transcription is PPAR\(\gamma\). Both genistein and quercetin have been shown to have an effect on PPAR\(\gamma\) activation. In addition, PPAR\(\gamma\) heterodimerizes with RXR for optimal transcriptional activation. It is possible that phytoestrogens activate RXR by mimicking estrogen, although this remains to be seen. These phytoestrogens may also inactivate transcription factors that down-regulate PTEN; however, these factors are yet to be identified.

The slight, yet statistically significant, increase in PTEN mRNA after phytoestrogen stimulation may suggest that although transcriptional regulation plays a role in the increase in PTEN, there may be other mechanisms involved. We already know that the level of PTEN protein can also be regulated by its stability. The phosphorylation status of PTEN plays an important role in regulating PTEN by influencing protein levels. The C-terminal tail also contains several phosphorylation sites located in the last 50 amino acids. Although this region or ‘tail’ of PTEN has been shown to be unnecessary for phosphatase activity and cell growth suppression (36), it is critical for protein stability. Protein stability is dependent upon the phosphorylation of Ser380, Thr382 and Thr383. Mutations of these sites reduce both the protein half-life and the PTEN protein levels (36). We found that the phosphorylation level of PTEN, by western blot, after phytoestrogen stimulation was unchanged (data not shown), indicating that the increase in PTEN protein is not due to changes in PTEN’s phosphorylation levels. We have recently shown that PTEN protein in MCF-7 cells can be increased by exposure to bone morphogenic protein (12), suggesting that there are other regulatory pathways of protein stability that are protein phosphorylation-independent, e.g. involving the ubiquitin conjugating enzymes UbcH7 and Ubc9 (12). We found that when cells were stimulated with phytoestrogens, the amount of PTEN associated with either UbcH7 or Ubc9 did not change. This suggests that the increase in PTEN protein is not due to inhibition of protein degradation via the ubiquitin pathway.

We provide evidence that a mechanism for phytoestrogens’ protective nature is partially through increased PTEN expression and subsequent lipid phosphatase activity. Increased PTEN protein expression may provide a balance to proliferative effects that phytoestrogen exposure may have. This may be an important event to examine in patients that harbor germline PTEN mutations, in that over-expression of non-functional PTEN, induced by phytoestrogens may not have a beneficial effect, but in fact may be detrimental. This is also germane to women who already have sporadic breast cancers with intragenic somatic PTEN mutations. In contrast, phytoestrogens may be helpful in increasing PTEN protein levels in tumors with hemizygous deletion of PTEN. Thus, PTEN mutation status of tumors may help personalize the decision whether phytoestrogen exposure is beneficial or detrimental. Equally important, our data suggest a novel target for the regulation of PTEN expression and function and that dietary changes may be adjunctive to traditional preventive and therapeutic strategies against breast cancer.

MATERIALS AND METHODS

Materials

Genistein was obtained from LC Laboratories Inc. (Woburn, MA, USA), quercetin dihydrate from EMD Bioscience (San Diego, CA, USA) and resveratrol was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies were obtained from Cascade Biosciences (PTEN 6H2.1), Cell Signaling (P-p44/42, P-AKT S473, AKT and actin; Beverly, MA, USA) and BD Biosciences (Cyclin D1 and p21\(^{kip1}\); Palo Alto, CA, USA). DMEM cell culture medium was obtained from Gibco-BRL (Rockville, MD, USA). Charcoal-treated FBS and phenol-free culture media MEMB were obtained from Hyclone and Cambrex Bio Science (Logan, UT, USA and Walkersville, MD, USA). M-PER Mammalian Protein Extraction Reagent was obtained from Pierce Biotechnology Inc. (Rockford, IL, USA). All other reagents were purchased from standard commercial sources.

Cell culture and stimulation

The MCF-7 breast cancer cell line was maintained at 37°C with 5% carbon dioxide in DMEM containing 10% FBS and 100 U/ml each penicillin and streptomycin (7). Sub-confluent cells were washed with PBS, trypsinized and plated at 1.0 \(\times 10^6\) cells/ml in phenol-free MEMB containing 10% charcoal-treated FBS and 100 U/ml of penicillin and streptomycin (37). The medium was augmented with hydroxyurea (3 \(\mu\)M) for 16–18 h to synchronize the cells. Cells were released from HU arrest by washing with PBS and replacement of media. Genistein, quercetin and resveratrol were added for a final concentration range of 1.0 \(\mu\)M to 10 \(\mu\)M for 48 h. Phytoestrogens were diluted in DMSO prior to use.

Protein extraction

After stimulation, cells were washed with PBS and lysed with M-PER lysis buffer containing 0.75 \(\mu\)M PMSF; 0.5 mg/ml benzamidine hydrochloride; 2 \(\mu\)M/ml leupeptin; 2 \(\mu\)M/ml aprotinin; 2 \(\mu\)M/ml pepstatin; 10 \(\mu\)M \(\beta\)-glycerophosphate; 0.2 \(\mu\)M NaOV and 25 \(\mu\)M NaF. Cells were incubated at room temperature with lysis buffer for 1 min before harvesting. Samples were then centrifuged at 16,000 g for 10 min at 4°C to remove cellular debris. The resulting supernatant was
stored for further analysis at −80°C. For nuclear and cytosolic protein isolation, cells were subjected to nuclear isolation protocol using the Pierce NE-PER system (Pierce Biotechnology, Inc., Rockford, IL, USA). The following inhibitors were added to the CERI buffer at concentrations recommended by the manufacturer: 0.75 mM PMSF, 0.5 mg/ml benzamidine, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 mM NaOV and 0.25 mM NaF. Inhibitors were also added to the NER buffer provided at the following concentrations: 2 mM PMSF, 0.5 mg/ml benzamidine, 2 μg/ml leupeptin and 2 μg/ml aprotinin. The isolated nuclear and cytoplasmic fractions were stored for further analysis at −80°C. Protein concentrations for all samples were determined using the bicotinic method (38) with BSA from Pierce Biotechnology as a standard.

Western blot analysis
Proteins (30 μg) were fractionated on a 13% SDS–PAGE gel, according to the Laemmli system (39) and electrophoretically transferred onto nitrocellulose (BioRad Corp.) (40). Equal loading of lanes was confirmed by staining with Ponceau S solution (Pierce Biotechnology Inc.) before hybridization and by analysis of actin protein levels by western blot. Nitrocellulose blots were blocked with 5% milk in TBS-T (10 mM Tris pH = 7.0, 100 mM NaCl and 0.1% Tween) for 1 h at RT. Blots were probed with primary antibody at a 1:1000 dilution for 2 h at RT and then washed with TBS-T for 1 h. The appropriate horseradish peroxidase-conjugated secondary antibody (Promega; Madison, WI, USA), at a 1:2500 dilution, was incubated with the nitrocellulose for 1 h at room temperature or overnight at 4°C. The blots were then washed with TBS-T for 1 h. Antibody complexes were detected with enhanced chemiluminescence reagent by Amersham Pharmacia Corp. (Piscataway, NJ, USA) and autoradiography according to manufacturer’s directions. The resultant autoradiographs were then quantitated using NIHImager densitometry software.

RT-PCR
Cells were stimulated as described earlier and RNA was extracted as follows. After stimulation, cells were released from the dish by use of trypsin and subsequently washed three times with PBS through centrifugation. RNA was extracted from the cells following the QIAGEN RNAeasy Mini Protocol for isolation of total RNA from animal cells and then converted to cDNA by Superscript II Reverse Transcriptase (SSIRT; Invitrogen Life Sciences). PCR was performed on the cDNA using primers specific to exon 1 and exon 9 of PTEN (5′-TCAAGAGGATGGATTGCATT-3′ and 5′-TGAAGTACAGCTTACCTTAAA-3′) obtained from Integrated DNA Technologies, Inc. Primers were allowed to anneal at 58°C for 30 s. The products from the PCR were run on a 1% agarose gel containing ethidium bromide and visualized under a UV light. RNA was quantified by densitometry with normalization to β-actin.

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Conflict of Interest statement. None declared.

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