Isolation of a vitamin E analog from a green barley leaf extract that stimulates release of prolactin and growth hormone from rat anterior pituitary cells in vitro

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We have previously reported that green barley leaf extract, a dried extract of young green barley leaves that is widely used in Japan and other countries as a nutritional supplement, contains a molecule(s) that enhances the release of growth hormone and/or prolactin from rat anterior pituitary cells in vitro. Using reverse-phase high performance liquid chromatography and fast atom bombardment-mass spectrometry (FAB-MS) we have now isolated and chemically characterized the molecule possessing this neuroendocrine activity. The molecule is α-tocopherol succinate, an analog of α-tocopherol or vitamin E. This is the first report documenting a role for this vitamin E analog on pituitary endocrine release. (J. Nutr. Biochem. 5:145–150, 1994.)

Keywords: green barley leaf extract; growth hormone; prolactin; vitamin E; α-tocopherol succinate

Introduction

Barley is widely used internationally as a food stuff and in beverages. The dried extract of the young green leaves barley (BLE) is also widely used in Japan and other countries as a nutritional supplement. In addition to being rich in vitamins and trace elements, extract of green leaves contains a complex mixture of largely undefined molecules with diverse putative biological activities.

Recent studies from our laboratory have established that heated and unheated BLE contain molecules that can enhance the release of growth hormone (GH) and prolactin beyond that achievable with GH releasing factor (GRF) or thyrotropin releasing hormone (TRH) alone.[1] GRF and TRH are the natural stimulators of growth hormone and prolactin, respectively. The objective of our present study was to identify the molecule(s) responsible for the stimulation of prolactin and growth hormone from rat anterior pituitary cells in vitro. Recently, we have developed a simple reverse-phase high performance liquid chromatography (RP-HPLC) procedure for the isolation of biologically active molecules from BLE in sufficient quantities for characterization. In the present study using our newly developed RP-HPLC, we report the isolation and chemical analysis of the biologically active molecule responsible for the enhancement prolactin and growth hormone.

This molecule is identified as α-tocopherol succinate, an analog of α-tocopherol or vitamin E. This is the first report concerning the prolactin/growth hormone stimulating activity of vitamin E and provides evidence for a novel biochemical action for vitamin E succinate at the level of the pituitary.

Methods and materials

Green barley leaf extract (BLE) was provided as a gift from Japan Pharmaceutical Development Co., LTD (Osaka, Japan). Delta Pak C_{18} Columns were obtained from Waters Chromatography Division of Millipore Corp. (Milford, MA USA). Water for HPLC was purified with a Milli-Q water system (Millipore Corp., Bedford, MA USA). All buffers and solvents were of HPLC grade (Fisher Scientific, Pittsburgh, PA USA). All HPLC solutions were filtered through a 0.45-μM membrane (Millipore) before use. TRH was obtained from Abbott Laboratories (North Chicago, IL USA), and human pancreatic GRF was a gift from Peptide Biological Laboratory, the Salk Institute (La Jolla, CA USA).

Barley leaves

Green barley leaves (110 g) were harvested 2 weeks after germination. The barley leaves were freeze dried for 3 days in a freeze dryer, Model 50-SRC-5 (Virtis Co., Gardiner, NY USA). The freeze-dried leaves were subsequently ground with a Wiley mill Standard Model 3 (Arthur H. Thomas Co., Philadelphia, PA USA) equipped with a

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mesh size 2-mm sieve to form a fine and uniform powder for extraction.

Extraction procedure

BLE powder (50 mg/mL) was suspended in water and stirred for 1 hr at room temperature. The mixture was then centrifuged at 3000*g* for 30 minutes using a bench-top centrifuge. The pellet was discarded and the supernatant was pre-filtered through a Millipore DEPTH filter. The filtrate was then filtered through 0.45 μM membrane and stored at −20° C for HPLC or biological assays.

HPLC

Preparative reverse phase chromatography of BLE (250 mg) was performed on a Model 600 HPLC system (Waters), equipped with a Model 441 absorbance detector (Waters) set at 214 nm, and a 300 × 19 mm DeltaPak, 300 Å, 15 μm C_{18} column. This was the initial step used for the fractionation of all the peptides/molecules in BLE. Eluent A was 0.1% trifluoroacetic acid (TFA) and Eluent B was acetonitrile containing 0.1% TFA. A 60-min linear gradient from 0 to 80% was run at a flow rate of 11.5 mL/min. One-min fractions were collected and combined into 10 different pools in order of increasing hydrophobicity as follows: P_1 (1-5), P_2 (6-8), P_3 (9-10), P_4 (11-15), P_5 (16-17), P_6 (18-20), P_7 (21-30), P_8 (31-40), P_9 (41-50), and P_{10} (51-60).

Analytical reverse-phase chromatography of BLE was performed on a 3.9 mm × 30 cm Delta-Pak 300 Å 5 μm C_{18} column. Eluent A was 0.1% trifluoroacetic acid in water, and eluent B was acetonitrile with 0.1% trifluroacetic acid. A 40-min linear gradient from 0 to 80% B, followed by a 5-min hold at 80% B was run at a flow rate of 1 mL/min. Detection was at 214 nm. Half-minute fractions were collected and assayed for hormone-releasing activity using normal anterior pituitary cells.

Anterior pituitary cell culture

Anterior pituitary cells from normal adult female or male Sprague-Dawley rats were dispersed as described by Spangelo et al.[2] Normal cells were seeded into 24-well tissue culture plates (Falcon, Oxnard, CA USA) at a density of 0.4 × 10^5 viable cells/well in 1.5 mL of RPMI-1640 medium supplemented with 2.5% fetal calf serum 7.5% horse serum, 7.5 μg/mL fungizone (Gibco, Grand Island, NY USA). The cells were allowed to attach to the wells in a humidified atmosphere of 5% CO_2 and 95% air at 37° C for a minimum period of 4 days before an experiment was performed. On the day of an experiment, the cells were rinsed twice (1 hour each) with serum-free RPMI-1640 medium containing antibiotics. Test substances were placed in the wells at varying concentrations and, following 30 min incubations, the media were quickly removed and saved for radioimmunoassay (RIA). At the termination of some experiments, 0.5 mL of 0.1 M HCl was added for a 24-hr incubation for extraction of intracellular cAMP.

Prolactin was determined by standard RIA techniques using materials and protocols supplied by the NIDDK Rat Pituitary Hormone Distribution Program (National Institute of Health, Bethesda, MD). Inter- and intra-assay variations for prolactin was less than 8%. All samples were assayed in duplicate, with results expressed in terms of NIDDK standards (rat prolactin RP-3). Intracellular cAMP concentrations were measured by Gammaflo Automated RIA.[3] The variability within a set of quadruplicate values was 5 to 10%.

Statistical analysis

The data are expressed as the mean ± SEM of groups consisting of at least four wells. Experiments were performed independently at least 2 to 3 times with representative results reported. Analysis of variance and the Bonferroni analysis for multiple comparisons were used for the statistical evaluation of these data. A P value of less than 0.05 was considered significant.

Mass spectrometric analysis

A VG ZAB-T Mass Spectrometer (VG Instruments, VG Analytical, LTD., Wythenshawe, Manchester, UK) was used for the analyses of the purified molecule. Three modes of analyses were conducted, namely, desorption chemical ionization (DCI), electron impact (EI), and fast atom bombardment (FAB). A high resolution mass spectrometry (HRMS) was also carried out at FAB mode to search for the elementary composition and hence molecular formula of the sample. DCI mode: 150° C a pressure of 0.3 Torr, a filament emission current of 200 uA; the primary electron energy was 70 eV. EI mode: Mass spectra were recorded using electron impact (70 eV) ionization at 150° C under 2-4 × 10-5 Torr. FAB mode: The technique employed was positive ion liquid secondary ion mass spectrometry (PI-LSIMS). The matrix used was "magic bullet" matrix, which was a mixture of dithiothreitol/dithioerythritol, 3:1, in methanol. The atom generating cesium (CS) gun anode was operated at a current of 2 uA and a voltage of 30 kV. Spectra were recorded with a scan time of 2 second/decade. The high resolution mass spectra were obtained at a resolution of 5000 after a mass calibration by matching the peaks with a polyethylene glycol (PEG) 2000 standard.

The sample was also analyzed by gas chromatography-mass spectrometry (GC-MS). No significant peaks were observed, apparently due to the involitality of the sample.

Results

Effects of BLE treatment on hormone release from pituitary cells in vitro

BLE stimulated prolactin and growth hormone release from rat anterior pituitary cells (*Figure 1*, A and B). Significant increases in the release of prolactin and growth hormone were observed with 500 and 50 μg/mL BLE, respectively; however, the release of these hormones was not accompanied by elevations in intracellular cAMP levels (*Figure 2*). Significant increase in the release of prolactin and growth hormone were also observed with boiled BLE samples (*Figure 3*, Panel A and B).

HPLC separation of BLE

BLE was separated into 10 pools (P_1 - P_{10}) using reverse phase HPLC (*Figure 4A*). Treatment of normal anterior pituitary cells with each of the 10 pools, P_1 - P_{10} caused significant increases ($P < 0.01$) in prolactin and growth hormone release in P_7 and P_8 - treated wells (*Figure 5*, A and B). However, P_8 (fractions 31-40) appeared more potent for stimulation of prolactin and growth hormone release than P_7.

Further analysis of individual fractions 31-40 in pool P_8 indicated that the peak hormone-releasing activity eluted in fraction 31 (F_{31}) and 32 (F_{32}) (*Figure 6*, A and B). Therefore, F_{32} were used for further purification.

Refractionation of F_{32} with the analytical HPLC procedure followed by hormone-releasing activity assays indicated that subfraction 27 showed the strongest activity (*Figure 4B*). Rechromatographing of subfraction 27 under the same analytical conditions resulted in a single peak designated as peak 1 (*Figure 4C*). Peak 1 was collected and subjected to amino acid, carbohydrate, and fast atom bombardment-mass spectometry (FAB-MS) analyses for chemi-

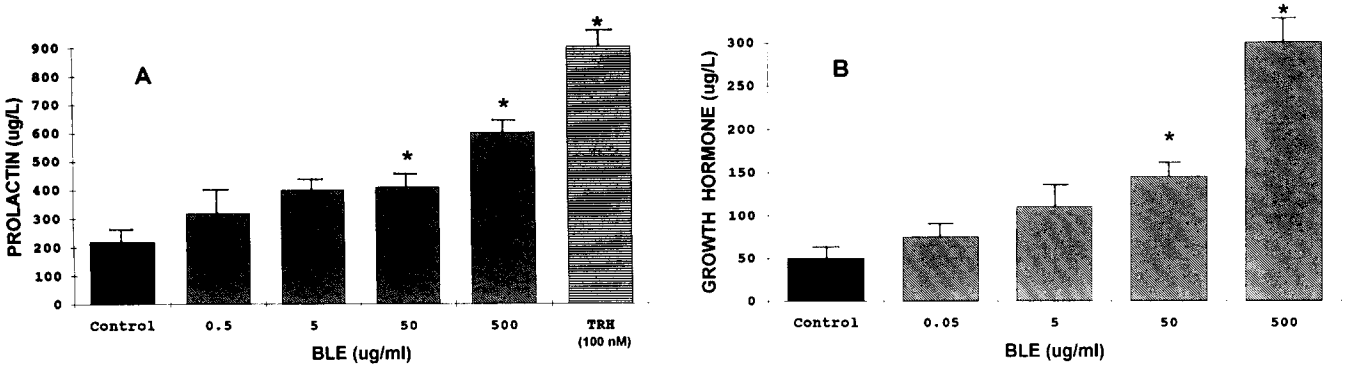

Figure 1 Concentration-dependent influence of green barley leaf extract (BLE) on (A):prolactin and (B):growth hormone release from female rat anterior pituitary cells. Cells were incubated in RPMI containing BLE (0.5 to 500 μg/mL) or 10 nM thyrotropin releasing hormone (TRH) for 30 min. BLE significantly stimulated the release of prolactin and growth hormone. The data are expressed as the mean ± SEM of groups consisting of four observations. *$P \leq 0.05$ compared with control.

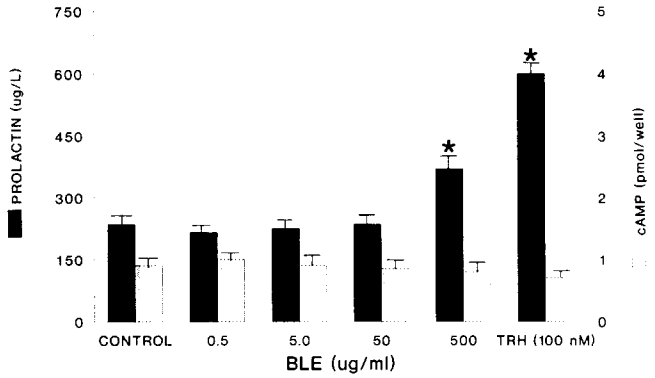

Figure 2 Effect of green barley leaf extract (BLE) on prolactin release from female rat anterior pituitary cells: comparison with cAMP accumulation. Incubation of different concentrations of BLE (0.5 to 500 μg/mL) and 100 nM Thyrotropin releasing hormone (TRH) with normal pituitary cells did not change cAMP levels. The data are expressed as the mean ± SEM of groups consisting of four observations. *$p \leq 0.05$ compared with control.

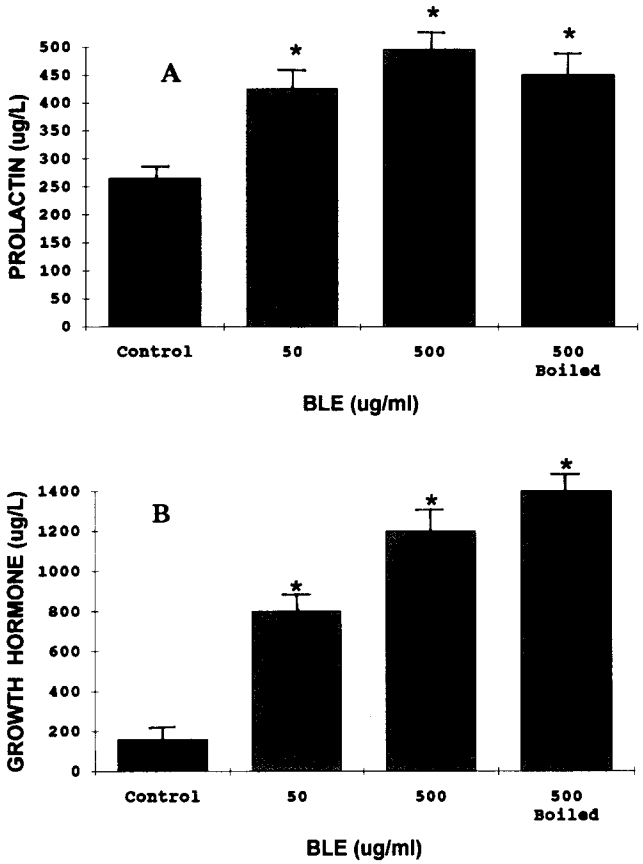

Figure 3 Effect of green barley leaf extract (BLE) and boiled BLE on (A) prolactin and (B) growth hormone release from female rat anterior pituitary cells. Final preparation of BLE was boiled for 5 min. and centrifuged at 3000*g* for 15 min. to remove pellet. The supernatant was used for prolactin and growth hormone assay. The data are expressed as the mean ± SEM of groups consisting of four observations. *$P \leq 0.05$ compared with control.

cal characterization. The yield of peak 1 from BLE is about 0.05%. *Figure 7* (A and B) shows the effect of peak 1 on prolactin and growth hormone release in vitro.

Characterization of peak 1

To investigate if peak 1 is a peptide, amino acid analysis of peak 1 was performed. Our result indicated that peak 1 only contains a low percentage of glycine (data not shown). The presence of small amounts of glycine in acid hydrolysates is attributed to minor contaminant in the preparation of the sample for amino acid analysis.

Carbohydrate analysis of peak 1 using the Dionex carbohydrate analysis system indicated that peak 1 does not contain any significant amount of monosaccharide (data not shown).

The FAB-MS of peak 1 gave an M + 1 ion peak at 531, suggesting that the molecular weight is 530 *(Figure 8)*. To find the elementary composition and possibly the molecular formula of peak 1, a high-resolution mass spectrometry was carried out, also in FAB mode. The measured exact molecular weight was 530.401561. Search for a match was conducted with a computer-aided library system in a range of ± 10 ppm of mass. α-tocopherol succinate, $C_{33}H_{54}O_5$, which has a molecular mass of 530.397125, was tentatively identified *(Figure 9)*. Confirmation of the identification was obtained by the coinciding retention times in a high perfor-

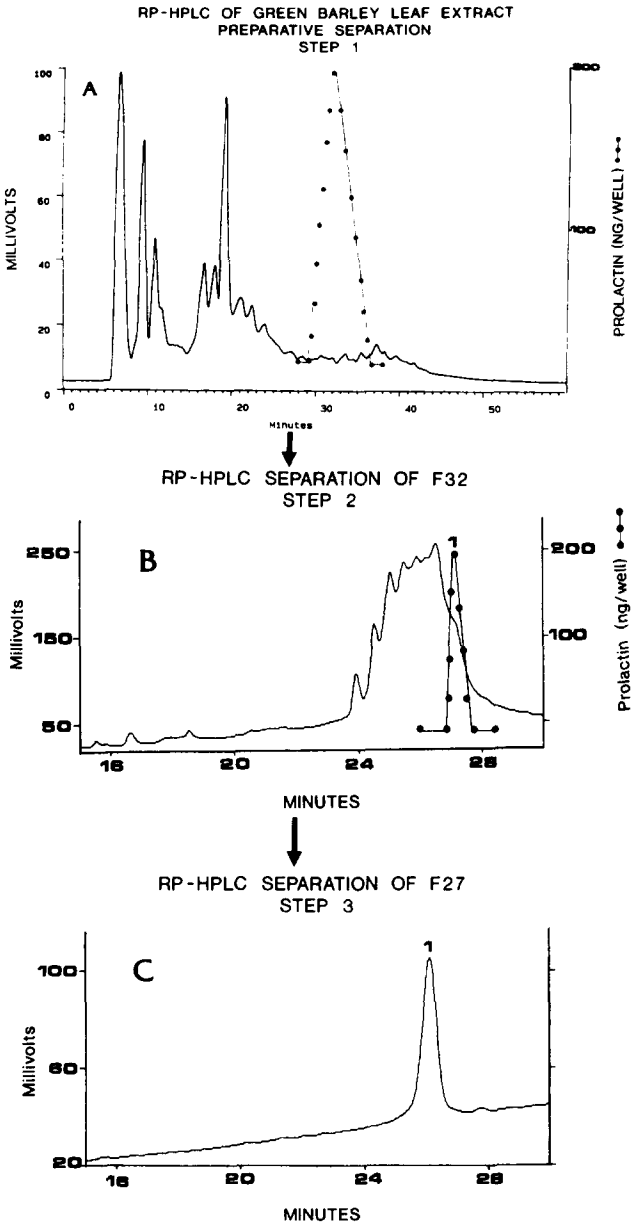

Figure 4 Flow diagram of the fractionation of endocrine active molecule (peak 1) from green barley leaf extract (BLE). A, RP-HPLC separation of 250 mg BLE (detection was at 214 nm). B, rechromatography of the active fraction from panel A, used for purifying endocrine active molecule (detection was at 214 nm). C, rechromatography of the active fraction (1) from panel B (detection was at 214 nm). The chromatographic condition is described in Methods and materials for panel A and in Results for panels B and C.

mance anion exchange chromatography-conducting detection (data not shown). Ultraviolet spectrophotometry (UV) on peak 1 revealed a UV maximum at 286 nm, further confirming the identification.

Treatment of normal anterior pituitary cells with different doses of peak 1 *(Figure 10A)* and commercial (±) α-tocopherol acid succinate *(Figure 10B)* results in significant increases in prolactin and growth hormone release (growth hormone results are not shown).

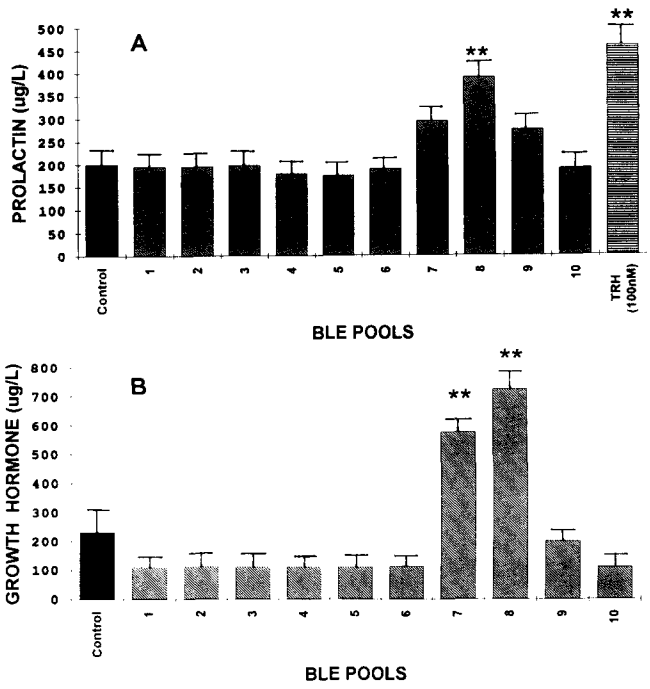

Figure 5 A, effect of pooled fractions P_1–P_{10} from the RP-HPLC of green barley leaf extract (BLE) *(Figure 4A)* on prolactin release from female rat anterior pituitary cells. Cells were incubated in RPMI containing P_1–P_{10} (100 μg/mL) for 30 min. B, effect of pooled fractions P_1–P_{10} from RP-HPLC of BLE *(Figure 4A)* on growth hormone release from normal female anterior pituitary cells. The data are expressed as the mean ± SEM of groups consisting of four observations. $^{**}P \leq 0.01$ compared with control.

Discussion

Young green barley leaves have been reported to contain biologically active substances such as antioxidants and anticarcinogens.[4,5] A variety of immunomodulatory properties of BLE have also been reported to date, such as in vitro anti-inflammatory and anti-leukemic activity and reduced healing time of ulcerous lesions in rats.[5,6]

In the present study we report the isolation and characterization of a molecule with hormone releasing activity that has been purified from BLE. This molecule has been identified as α-tocopherol succinate, an analog of vitamin E.

GRF stimulates growth hormone release in part through its ability to increase intracellular cAMP.[7] α-tocopherol succinate has no effect on normal cell cAMP accumulation *(Figure 2)*. The mechanism by which α-tocopherol succinate stimulates hormone release, therefore, does not appear to be dependent on changes in cAMP. Similarly, TRH stimulation of prolactin release is mediated through increased hydrolysis of polyphosphoinositide.[8] Coincubation of natural α-tocopherol succinate with an optimal amount of TRH causes an additive increase in prolactin release compared with the effect of natural α-tocopherol succinate and TRH alone (data are not shown). These results suggest that natural α-tocopherol succinate may act via a mechanism(s) different from that of TRH.

Although vitamin E has been found to have several distinct biochemical actions in vitro such as antioxidant function[9] intermediary in arachidonic acid and prostaglandin metabolism,[10–19] nucleic acid, protein, and lipid metabo-

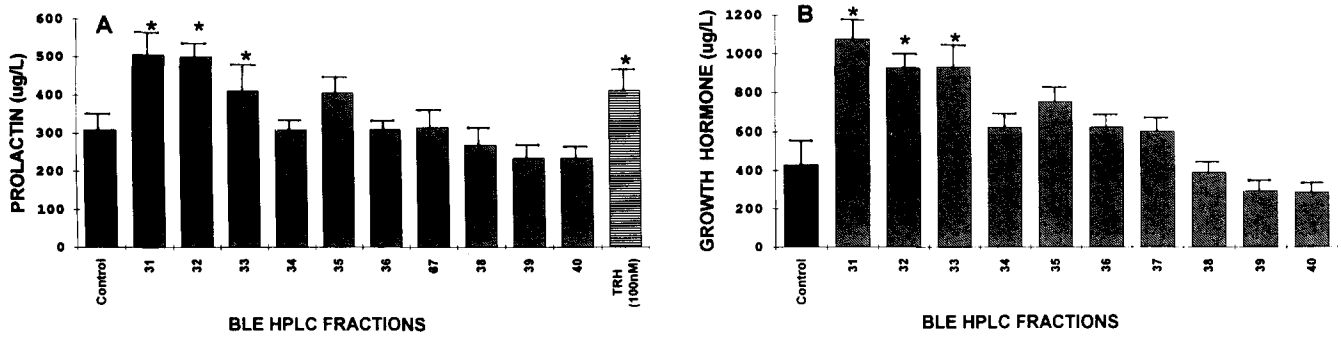

Figure 6 Effect of RP-HPLC fractions of green barley leaf extract (BLE) ($pool_8$, fractions: 31–40 from *Figure 4*) on: A, prolactin and B, growth hormone release from female rat anterior pituitary cells. Cells were incubated in RPMI containing HPLC fractions 31–40 (100 μg/mL) or 100 nM thyrotropin releasing hormone (TRH) for 30 min. Results are expressed as the mean ± SEM of groups consisting of four observations. $^*P \leq 0.05$ compared with control.

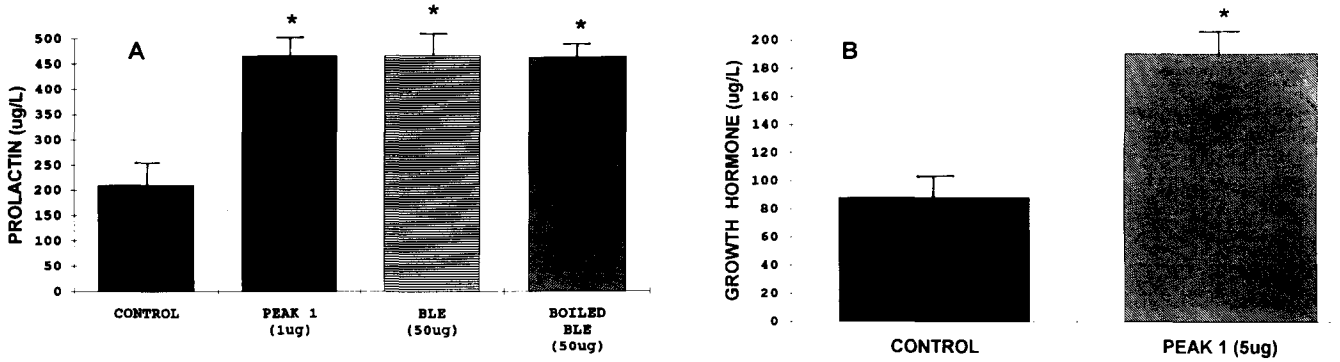

Figure 7 Effect of endocrine active molecule (peak 1) from the RP-HPLC of active fraction *(Figure 4C)* on: A, prolactin and B, growth hormone release from female rat anterior pituitary cells. The experimental conditions are described in *Figure 5A*. The concentrations are expressed as μg/mL. The data are expressed as the mean ± SEM of groups consisting of four observations. $^*P \leq 0.05$ compared with control.

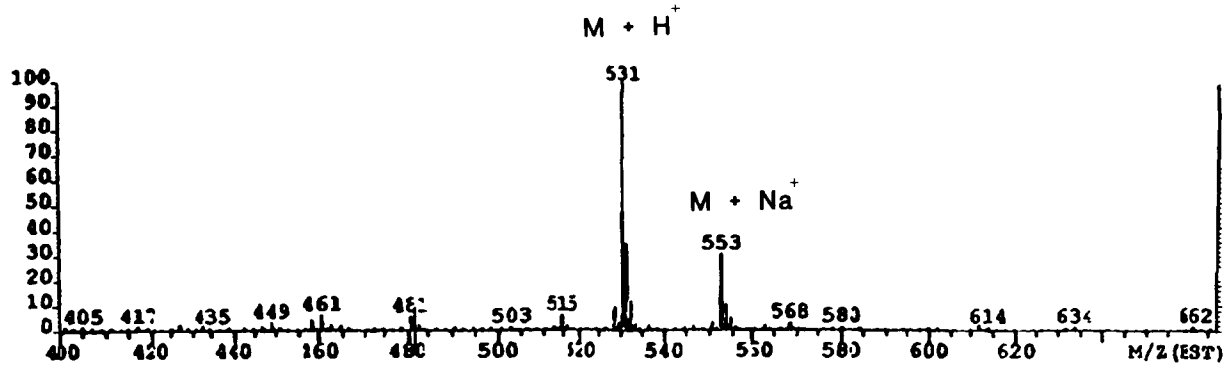

Figure 8 High resolution FAB-MS of endocrine active molecule (peak 1) isolated from green barley leaf extract. The experimental condition is described in Methods and materials.

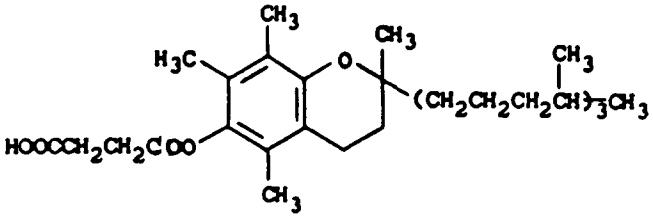

Figure 9 Chemical structure of α-tocopherol succinate.

lism,[20–27] mitochondrial function,[28] male and female sex hormone production,[29] until recently it was considered "a vitamin in search of disease," as vitamin E deficiency diseases have not been described.[30] Although this description is still accurate, we now know that the α-tocopherols play an important metabolic role in maintaining the integrity of membranes and most recently have been found to significantly reduce the risk of heart disease.[31,32] Our studies are the first to document a role for vitamin E in modulating prolactin

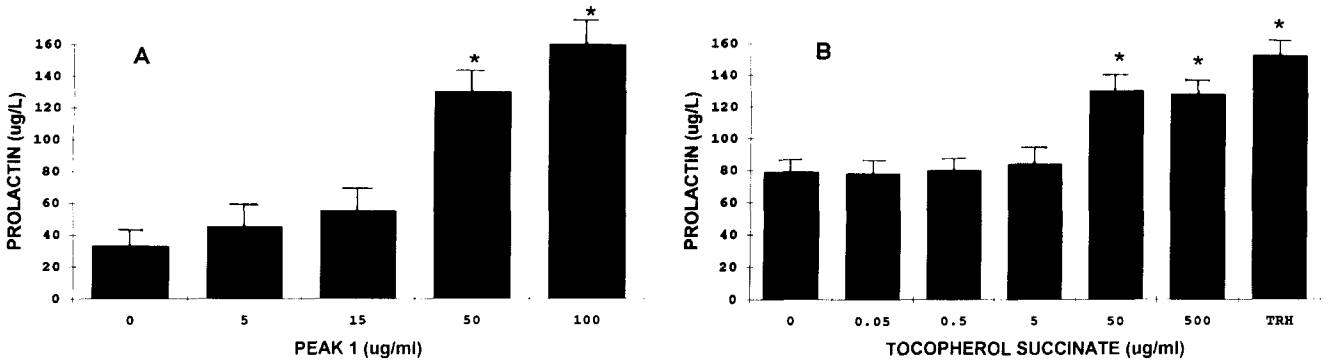

Figure 10 Concentration-dependent influence of: A, natural α-tocopherol succinate isolated from green barley leaf extract (BLE) and B, commercial α-tocopherol succinate on prolactin release from female rat anterior pituitary cells. The experimental conditions are described in *Figure 5A*. The data are expressed as the mean ± SEM of groups consisting of four observations. $^*P \leq 0.05$ compared with control.

and growth hormone release, suggesting that vitamin E may also have an important hitherto unknown role in regulating neuroendocrine responses.

Acknowledgments

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