HNUC #1328605, VOL 0, ISS 0

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NUTRITION AND CANCER
2017, VOL. 0, NO. 0, 1–11
https://doi.org/10.1080/01635581.2017.1328605

Denatonium and Naringenin Promote SCA-9 Tumor Growth and Angiogenesis: Participation of Arginase

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ABSTRACT
Submandibular gland (SMG) is one of the major salivary glands, and is formed by acinar cells that are conveyed to the oral cavity by a duct system. We had previously reported that T2R receptors that were originally identified in gustatory tissues were also present in murine SMG. The addition of bitter compounds to the gland reduced nitric oxide production and downregulated amylase secretion. In this work, we investigated the effect of two different bitter compounds namely denatonium and naringenin on tumor progression as well as the presence of T2R in SCA-9 cells derived from a murine tumor induced in SMG. Both compounds increased tumor cell proliferation in bi- and three-dimensional cultures. These effects were mediated by the activation of arginase and the inhibition of nitric oxide synthase. Denatonium and naringenin also increased vascular endothelial growth factor-A expression via arginase and tumor neovascularization in vivo. T2R6 and T2R4 were identified in SCA-9 cells by immunostaining. Also, Gi and Ggust proteins, which usually couple to T2R receptors, are expressed in these cells. Finally, we demonstrated for the first time that bitter compounds can exert pro-tumor actions that should be taken into account as side effects when they are used as nutraceuticals.

ARTICLE HISTORY
Received 28 July 2016
Accepted 5 April 2017

Introduction

Submandibular gland (SMG) is one of the major salivary glands, and is formed by acinar cells that are conveyed to the oral cavity by a cell-lined duct system, where the proteins and electrolyte components are subjected to secondary modifications (1). Salivary protein secretion is evoked when neurotransmitters bind to β adrenergic and muscarinic acetylcholine receptors. These types of receptors belong to the family of G-protein-coupled receptors (GPCRs), and both are located in the basolateral membrane of secretory cells (2). Regarding the latter, we reported that amylase secretion is regulated by the muscarinic agonist carbachol in murine SMG (3). In addition, we documented that amylase secretion is also modulated by the activation of other GPCRs known as T2Rs (4). These receptors were originally identified in gustatory tissues and linked to bitter taste perception (5). Their activation by synthetic or natural bitter compounds leads to the dissociation of a heterotrimeric G protein called Ggustducin (Ggust) that belongs to the Gi/o class (6,7). The dissociation of Ggust protein into α subunit and $\beta\gamma$ dimer may activate on the one hand phosphodiesterase reducing the synthesis of cAMP, and on the other hand phospholipase Cβ2 (PLCβ2), which liberates inositol tris phosphate (IP3), a Ca^{2+} ionophore (6). Subsequently, Ca^{2+} binds to calmodulin, activating several enzymes including the nitric oxide synthase (NOS), producing nitric oxide (NO), which is an important mediator of several cellular processes. To briefly synthesize the NO pathway, NOS enzyme goes through two main steps. In the first one, NOS transforms L-arginine to N^{ω}-hydroxy-L-arginine (L-NOHA). In the second step, NOS oxidizes L-NOHA to L-citrulline and NO. All isoforms of NOS bind calmodulin. In neuronal NOS or NOS1 and in endothelial NOS or NOS3, calmodulin binding is brought about by an increment in intracellular Ca^{2+} (half-maximal activity between 200 and 400 nM). On the other hand, in inducible NOS or NOS2, calmodulin already binds at extremely low intracellular Ca^{2+} concentrations (below 40 nM) (8). In many tumors, NOS2 expression is high, but the role of this enzyme during tumor development is complex and is not completely understood. NOS shares the substrate, L-arginine, with the enzyme

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arginase, which produces polyamines necessary for cell division (9).

T2Rs that belong to GPCRs family have been identified in different regions of the gastrointestinal tract and organs unrelated to the taste perception regulating physiological functions such as hormone secretion and immune response (10–13).

T2R receptors have been recently identified in mammary tumor cells (14). In this work, we investigated the effects of two bitter agonists namely denatonium and naringenin, during proliferation and angiogenesis of SCA-9 tumor cells by considering arginase participation. Moreover, we characterized the expression of T2R eceptors and the G proteins that couple to these receptors, particularly in these cells that are derived from a tumor induced in murine SMG.

Methods and Materials

Cell Culture and Proliferation Assays

SCA-9 clone 15 (ATCC® CRL-1734™) derived from an undifferentiated chemically induced carcinoma of murine SMG and CT26. WT cells (ATCC® CRL-2638™) from a murine colon carcinoma were used. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 2 mM glutamine, and RPMI-1640 from Gibco (MA, USA), respectively, supplemented with 80 μg/ml gentamycin, 50 U/ml penicillin, and 10% heat-inactivated fetal bovine serum (FBS) from Internegocios (Buenos Aires, Argentina) at 37°C in 5% CO_2 air atmosphere. Cells were detached using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in Ca^{2+} and Mg^{2+} free phosphate buffered solution (PBS). The medium was replaced three times a week. The absence of mycoplasma was confirmed by Hoechst staining (15).

Proliferation in bi-dimensional cultures was evaluated using the salt 3-(4,5-dimethyl-2-thiazolyl-2)-2,5-diphenyl-tetrazolium bromide (MTT) from MP Biomedicals (Buenos Aires, Argentina) as a colorimetric metabolic activity indicator. MTT is converted to water-insoluble formazan by mitochondrial dehydrogenases of living cells. SCA-9 and CT29 cells were seeded in 96-well plates at a density of 7×10^3 cells per well in DMEM and RPMI-1640, respectively, supplemented with 5% FBS and were left to adhere. Then, cells were deprived of FBS for 18 h to induce quiescence, and were treated with different concentrations of denatonium or naringenin during 20 min in the absence or presence of 10^{-4} MNOHA, aminoguanidine, or N^G-methyl-L-arginine (L-NMMA) added 30 min before bitter compounds. After treatment, the culture medium was replaced by a fresh medium with 2% of FBS, and cells were cultured during 96 h. Then, the medium was replaced by 100 μl of DMEM: F12 (1:1) without phenol red, containing 10 μl of MTT (5 mg/ml) in PBS. After 4 h at 37°C, the supernatant was discarded and intracellular formazan was solubilized with 50 μl of dimethyl sulfoxide (DMSO) from Research AG (Buenos Aires, Argentina). The absorbance was measured with an ELISA reader Quant (Bioteck Instruments Inc., VT, USA) at 540 nm. Values are mean ± SEM of 3 independent experiments performed in quadruplicate. The results were expressed as percentage of stimulation in relation to control, cells without treatment considered as 100%.

Three-dimensional culture of SCA-9 cells was analyzed inducing spheroids formation. Tumor cells (2×10^4 per drop of 20 μl in complete medium) were seeded on the inner side of a lid of 100-mm Petri dish. Then, the lid was inverted letting the drops hang over the plate that contained PBS to avoid evaporation, and the plate was incubated during 48 h at 37°C. Then, spheroids were placed in 96-well plates covered with 1% agarose, containing 100 μl/well of DMEM supplemented with 5% FBS to prevent spheroid attaching, and cultured during 4 days at 37°C with 5% CO_2, until they reached a diameter of 400 μm. The culture medium was replaced 3 times a week by a fresh medium. To measure spheroids growth, they were treated with denatonium or naringenin in the absence or presence of 10^{-4} M NOHA or aminoguanidine. Photographic records were obtained with an inverted microscope equipped with a digital camera (Arcano, Beijing, China) and analyzed with Image-Pro Plus software. Volume was calculated as $4/3\,\pi r^3$ in which $r = đ/2$ (đ is the media of two perpendicular diameters measured). The values are mean ± SEM of at least 3 independent experiments performed with ten replicates.

Expression of Arginase by Western Blot

SCA-9 cells (1.5×10^6) were grown in 35-mm culture dishes, washed twice with PBS and lysed in 500 μl:50 mM Tris–HCl, 150 mM NaCl, 1 mM NaF, EDTA and PMSF, 1% Triton X-100, 4 μg/ml trypsin inhibitor, 5 μg/ml aprotinin and 5 μg/ml leupeptin, pH 7.4. After 1 h in ice bath, lysates were centrifuged at 10,000 rpm for 10 min at 4°C and supernatants were stored at −80°C until use. Protein concentration was determined by the method of Bradford (16).

Using a Mini-PROTEAN electrophoresis system (Bio-Rad, CA, USA), samples were run in 8–12% SDS-polyacrilamide gels loading 80 μg of protein per lane, followed by liquid electrotransference into nitrocellulose membranes. Blotted proteins were then stained with Ponceau 2R to corroborate transfer efficiency, washed in distilled water and incubated for 1 h at room

temperature in 20 mM Tris–HCl buffer, 150 mM NaCl, and 0.05% Tween 20 (TBS-T) with 5% skim milk. Then, membranes were incubated overnight with the following antibodies: monoclonal mouse antihuman arginase I antibody from BD Transduction Laboratories (CA, USA) or polyclonal rabbit antiarginase II antibody (kindly gifted by Dr. Masataka Mori), both diluted 1:500 in TBS-T. Then, membranes were blocked in TBS-T with 5% skim milk for 1 h at room temperature and subsequently incubated overnight at 4°C. After several washes in TBS–T, membranes were incubated during 1 h at room temperature with antimouse (1:10,000) or antirabbit (1:20,000) IgG secondary antibodies linked to horseradish peroxidase (Sigma-Aldrich, Buenos Aires, Argentina). After 3 washes in TBS-T, bands were visualized by ECL using 250 mM luminol, 90 mM p-coumaric acid, 1 M Tris–HCl (pH 8.5) in distilled water and H_2O_2. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control.

Arginase Activity Assay

SCA-9 cells (2×10^4 per well) were seeded in 24-well plates and left to adhere in DMEM with 10% of FBS. Then, the culture medium was replaced by a fresh medium without FBS to achieve quiescence before treatment. Then, cells were treated with denatonium or naringenin during 20 min in the absence or presence of 10^{-4} M NOHA or aminoguanidine added 30 min before bitter compounds, and left with a fresh medium with 2% of FBS for 96 h. After washing with PBS, cells or spheroids were lysed in distilled water with 0.1% of Triton X-100.

To activate arginase enzyme, buffer containing 25 mM Tris–HCl, pH 7.4, and 5 mM $MnCl_2$ was added to samples in equal volume and heated during 10 min at 56°C. Arginine hydrolysis was performed by incubating 25 μl of the activated lysate with 25 μl of 0.5 M arginine, pH 9.7, at 37°C for 1 h. The reaction was stopped in an acidic medium. Urea concentration was measured at 540 nm in an ELISA reader. The amount of urea produced in the unknown samples was determined by extrapolation using a urea standard curve. Results were expressed as micromoles of urea per mg of protein and per hour (μmol/mg prot.h) (17).

Nitric Oxide Production

NO production was determined by measuring nitrite (NO_2^-) accumulation in culture supernatants. SCA-9 cells (7×10^3 per well) were seeded in 96-well plates in DMEM supplemented with 10% FBS and left to adhere overnight. Then, the medium was replaced by DMEM without SFB during 18 h and treated with denatonium or naringenin during 20 min in the absence or presence of 10^{-5} M H-89 or 10^{-4} M aminoguanidine added 30 min before bitter compounds. After treatment, the medium was replaced by DMEM:F12 without phenol red and supplemented with 2% FBS during 96 h. Q2

Nitrite accumulation was measured after 96 h in culture supernatants by Griess reagent [1% sulfanylamine in 30% acetic acid with 0.1% N-(1-naphtyl)-ethylenediamine in 60% acetic acid)] (18). Absorbance was measured at 540 nm with an ELISA reader. The amount of nitrite produced in the unknown samples was determined by extrapolation using a $NaNO_2$ standard curve and expressed as micromolar (μM) concentration of nitrite. Values are mean ± SEM of 3 independent experiments performed in quadruplicate.

Expression of Vascular Endothelial Growth Factor-A by Western Blot

Cell lysates were prepared as mentioned above. For protein extraction, spheroids were sonicated for 10 s in cold-ice lysis buffer. After 1 h in ice bath, lysates were centrifuged at 10,000 rpm for 10 min at 4°C, and supernatants were stored at −80°C. Protein concentration in samples was measured by the method of Bradford (16). Then, samples were seeded in 10–12% SDS-polyacrilamide gels and transferred to nitrocellulose membranes as mentioned above. Then, the membranes were blocked in TBS-T with 5% skim milk for 1 h at room temperature, and incubated overnight with a polyclonal goat antimouse vascular endothelial growth factor-A (VEGF-A) antibody diluted 1:100 (Santa Cruz Biotechnology, CA, USA) at 4°C. After washing with TBS-T, the membranes were exposed to a secondary antigoat antibody diluted 1:15,000 during 1 h at room temperature. Bands were visualized by ECL. Bands were quantified by densitometric analysis with ImageJ software. Results were expressed as relative optical density (rOD) with respect to the expression of GAPDH that was used as loading control.

Tumor-Induced Angiogenesis

To analyze the induction of angiogenesis by tumor cells, we employed an in vivo assay previously described (19,20). SCA-9 cell concentration was adjusted to 2×10^6/ml in DMEM, and cells were treated with denatonium or naringenin for 20 min. Then, cells were washed and resuspended in DMEM medium and 2×10^5 cells/0.1 ml were inoculated intradermically (i.d.) in both flanks of three-month Swiss male mice purchased by the Animal Division of the Pharmacy and Biochemistry School, University of Buenos Aires. Mice

were handled according to the Guide for the Care and Use of Experimental Animals (NIH, 1986). On day 5, animals were sacrificed, and the internal layer of skin was dissected from the underlying tissues with a microscope Konus, Crystal 45 (IL, USA) under 7.5 × magnification and the vascular response was photograph with an incorporated digital camera (Canon Power Shot A45, Buenos Aires, Argentina). Images were projected on a reticular screen to count the number of vessels per mm^2 of skin. Angiogenesis was quantified as vessel density (δ), calculated as the total number of vessels divided by the total number of squares.

$$\delta = \frac{\Sigma \text{ Number of vessels per square}}{\text{Number of total squares}}$$

Expression of T2R by Immunocytochemistry

SCA-9 cells were seeded over cover glasses and left to attach overnight in DMEM with 10% of FBS. After washing with PBS, cells were fixed with ice-cold methanol during 5 min. After several washes with PBS, endogenous peroxidase was blocked with 6% H_2O_2 in methanol for 30 min. Then glasses were incubated with blocking solution and exposed to goat antimouse T2R6 or T2R4 polyclonal antibodies diluted 1:300 or 1:100, respectively (Santa Cruz Biotechnology, CA, USA) overnight at 4°C in a humidified chamber. After several washes, glasses were exposed to a secondary antigoat biotinylated antibody during 1 h at room temperature and immune reaction was detected using a Vectastin Elite ABC kit from Vector Laboratories (CA, USA). Control glasses were prepared by omitting primary or secondary antibody. Inverted microscope Nikon Eclipse E200 equipped with a digital camera DS-Fi1, Nikon (NY, USA) was used to observe and photograph cell preparations.

Expression of T2R and G Proteins by Western Blot

SCA-9 cells (1.5 × 10^6) were grown and lysed as mentioned above. Lysates were centrifuged at 10,000 rpm for 10 min at 4°C and supernatants were stored at −80°C until use. Protein concentration was determined by the method of Bradford (16) and subjected to electrophoresis as mentioned previously. After liquid electrotransference into nitrocellulose membranes, blotted proteins were washed in distilled water and incubated for 1 h at room temperature in 20 mM Tris–HCl buffer, 150 mM NaCl, and 0.05% Tween 20 (TBS-T) with 5% skim milk. Then, membranes were incubated overnight with the polyclonal antibodies such as goat antimouse T2R6 or T2R4 (both at 1:350), goat antihuman Gαi or rabbit antirat Gαgust (both at 1:250) from Santa Cruz Biotechnology (CA, USA) at 4°C, under agitation. After several washes with TBS-T, the membranes were incubated with secondary antigoat or antirabbit antibodies (1:10,000) linked to horseradish peroxidase. After 3 washes with TBS–T, bands were revealed by ECL. Bands were quantified by densitometric analysis with ImageJ software (NIH, USA). Circumvallate papillae homogenates were used as positive controls following previous procedures (4). The expression of GAPDH was used as loading control.

Chemicals

All drugs were purchased from Sigma-Aldrich (Buenos Aires, Argentina) unless otherwise stated. Solutions were prepared fresh daily.

Statistics

The statistical significance of differences between groups was analyzed by one-way analysis of variance complemented with Tukey's test using GraphPad Prism 4. $P < 0.05$ was considered to be statistically significant.

Results

Effect of Bitter Compounds on SCA-9 Cell Proliferation

In our first experiment, we tested the ability of bitter compounds to modulate proliferation on two different tumor cell lines: SCA-9 and CT26 were derived from murine SMG and colon carcinomas, respectively. The addition of different concentrations of denatonium or naringenin significantly increased cell proliferation at almost all concentrations tested (Table 1). Focusing our study on SCA-9 cells, in bi-dimensional cultures, both 10^{-8} M denatonium and 10^{-7} M naringenin were equally effective in triggering cell duplication ($P < 0.001$ vs. control, cells without treatment considered as 100%) (Fig. 1A). The action of both compounds was reduced in the presence of the arginase inhibitor NOHA (10^{-4} M) indicating that this enzyme is involved in the proliferative action triggered by bitter compounds (Fig. 1B). The preincubation of cells with L-NMMA or aminoguanidine potentiated the action of denatonium but did not modify that of naringenin on cell proliferation (Fig. 1B). Denatonium and naringenin were also able to promote cell proliferation in three-dimensional cultures since both compounds added at the same concentrations that promoted cell duplication in bi-dimensional cultures increased volume in SCA-9 spheroids by 32.9 ± 2.9 and 36.5 ± 4.1%, respectively ($P < 0.001$ vs. control considered as 100%). This effect was totally reduced in the presence of NOHA (Fig. 1C and D)

Table 1. Effect of denatonium and naringenin on tumor cells.

	Proliferation (% Control)					
	Denatonium			Naringenin		
	10^{-8} M	10^{-7} M	10^{-6} M	10^{-8} M	10^{-7} M	10^{-6} M
SCA-9	$148 \pm 12^{**}$	$129 \pm 11^{**}$	$125 \pm 10^{**}$	$124 \pm 18^{*}$	$147 \pm 13^{**}$	$116 \pm 10^{*}$
CT26	$133 \pm 12^{\#}$	113 ± 18	$137 \pm 15^{*}$	$157 \pm 20^{**}$	$196 \pm 25^{***}$	$171 \pm 10^{**}$

Tumor cells were seeded at 7×10^3 cells/well and treated during 20 min with denatonium or naringenin and then cultured during 96 h as it was indicated for bi-dimensional cultures in Methods and Materials.
Values are mean $\pm$ SEM of three independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$; $^{\#}P < 0.05$ vs. control (cells without treatment) considered as 100%.

confirming that arginase is involved in the initial steps of SCA-9 tumor progression mimicked with this technique. The addition of aminoguanidine only potentiated the action of denatonium on spheroids growth (Fig. 1C and D).

Arginase Expression and Activity in SCA-9 Cells

Considering the fact that arginase is involved in SCA-9 cell proliferation, we analyzed its expression by Western blot, confirming the presence of both isoforms (I and II) of the enzyme in these cells (Fig. 2A). Moreover, when we analyzed the effect of denatonium and naringenin, we observed that both bitter compounds increased urea production in relation to control, cells without treatment (control: 97.7 ± 10.9 μmol/mg prot.h; denatonium: 144.6 ± 11.8 μmol/mg prot.h; naringenin: 151.4 ± 19.9 μmol/mg prot.h; $P < 0.05$ vs. control). This effect was reduced to control value in the presence of 10^{-4} M NOHA for both bitter compounds (Fig. 2B).

Similar to that observed in bi-dimensional cultures, bitter compounds upregulated arginase activity in SCA-9

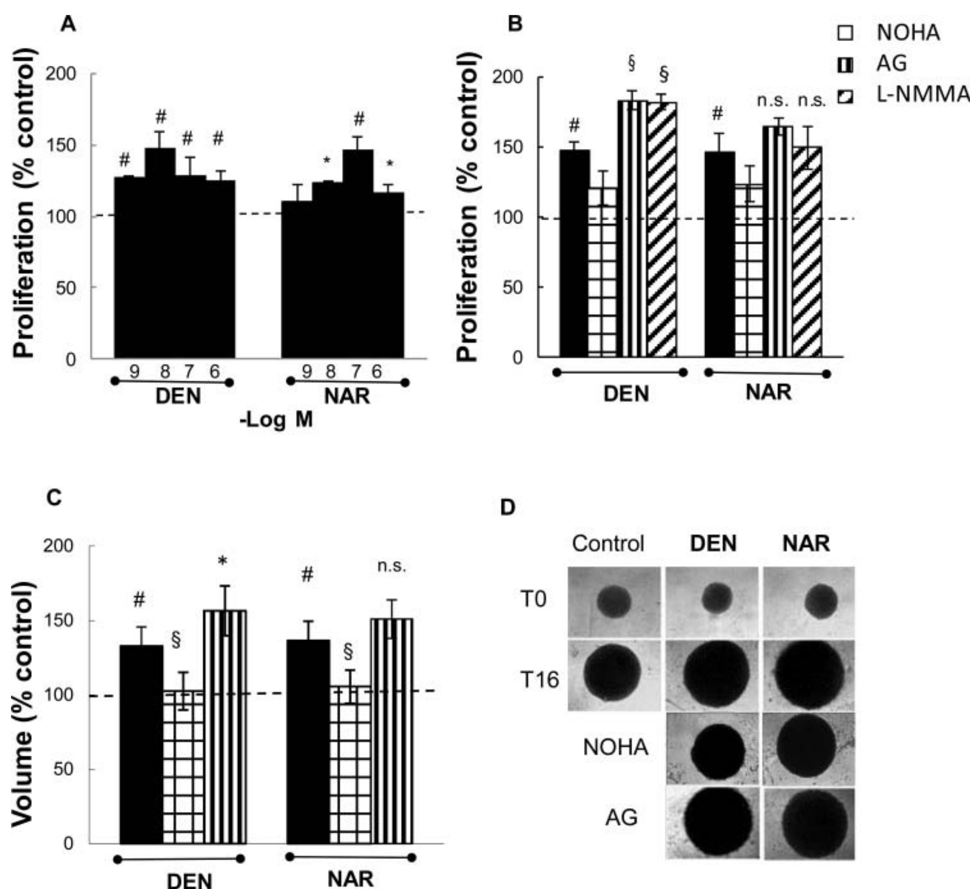

Figure 1. Effect of bitter compounds on SCA-9 cell proliferation. A: Concentration-response effect of denatonium (DEN) or naringenin (NAR) on bi-dimensional cultures, B: Effect of 10^{-4} M N^{w}-hydroxy-L-arginine (NOHA), aminoguanidine (AG) or N^{G}-methyl-L-arginine (L-NMMA) on the action of DEN (10^{-8} M) or NAR (10^{-7} M) in bi-dimensional or C: three-dimensional cultures, and D: representative photographs of spheroids for each treatment. Magnification: 7.5 ×. Values are mean $\pm$ SEM of at least 3 experiments performed in triplicate. $^{*}P < 0.01$ vs. control; $^{\#}P < 0.001$ vs. control; $^{\S}P < 0.001$ vs. DEN or NAR; n.s. not significant.

spheroids increasing urea production by more than 60% ($P < 0.001$ vs. control). This effect was also reduced in the presence of NOHA (Fig. 2C).

Role of Bitter Compounds on Urea/NO Balance on SCA-9 Cells

Considering the fact that arginase shares its substrate, L-arginine with NOS, and that aminoguanidine potentiates the action of denatonium on SCA-9 cell proliferation, we analyzed the effect of this NOS2 inhibitor on urea production. Aminoguanidine only potentiated denatonium-triggered urea production in SCA-9 cells revealing a cross-talk between arginase and NOS2 in this mechanism (Fig. 3A). It is important to note that both bitter compounds inhibited nitrite formation, but the inhibition of protein kinase A (PKA) with H-89 turned to stimulate the effect of denatonium on NO formation in SCA-9 cells (Fig. 3B).

Bitter Compounds Action on VEGF-A and Tumor-Induced Angiogenesis

Considering the fact that denatonium and naringenin increased tumor cell proliferation via arginase pathway, we analyzed the effect of both compounds on angiogenesis, another important step of tumor progression. The addition of bitter compounds increased the expression of VEGF-A in tumor cells, with this effect being more potent for naringenin than for denatonium. NOHA (10^{-4} M) completely reduced the effect of both bitter agonists in bi-dimensional and in three-dimensional SCA-9 cell cultures (Fig. 4A–D).

By *in vivo* experiments, we confirmed the effect of denatonium and naringenin in tumor-induced angiogenesis. SCA-9 cells promoted the formation of new blood vessels in the skin in relation to control skin (SCA-9 cells: 2.31 ± 0.26; (control: 1.43 ± 0.14; $P < 0.001$) (Fig. 4E). The addition of denatonium or naringenin to SCA-9 cells significantly increased the neovascular response in the skin in comparison to the inoculation of untreated tumor cells ($P < 0.001$ vs. SCA-9 cells).

Expression of T2R Receptors and G Proteins in SCA-9 Cells

We have previously demonstrated by immunohistochemistry that T2R6 receptors are expressed in murine SMG. Our results obtained by immunocytochemistry demonstrate that SCA-9 cells express not only T2R6 but also T2R4 receptors (Fig. 5A and B). In addition, we confirmed the expression of both subtypes of receptors by Western blot, which is similar to that observed in circumvallate papillae (Fig. 5E). SCA-9 cells also express the subunit α of both Gi and Ggust proteins, with the latter being in lower amounts than the first one in comparison to circumvallate papillae (Fig. 5F).

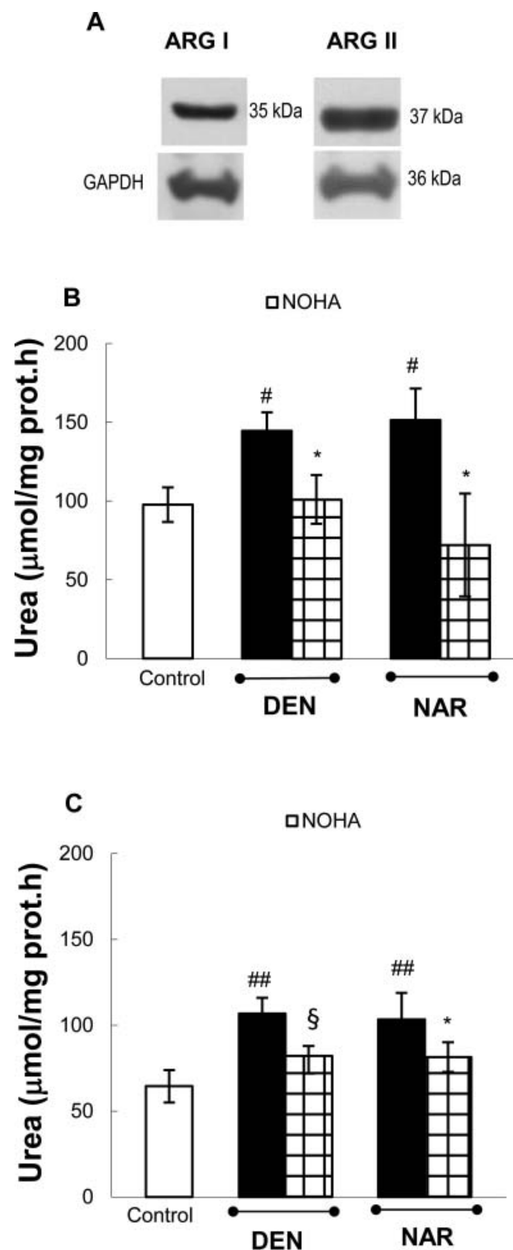

Figure 2. Effect of bitter compounds on arginase expression and activity in SCA-9 cells. Western blot assay of (A) arginase (ARG). Molecular weights are indicated on the right. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. One representative experiment of 3 is shown. Cells in (B) bi-dimensional or (C) three-dimensional culture were treated with denatonium (DEN; 10^{-8} M) or naringenin (NAR; 10^{-7} M) in the absence or presence of 10^{-4} M N-hydroxy-L-arginine (NOHA) to evaluate urea production Values are mean ± SEM of at least 3 experiments performed in duplicate. #$P < 0.05$; ##$P < 0.001$ vs. control (cells without treatment); § or $^{*}P < 0.01$ vs. DEN or NAR. Values are mean ± SEM of at least 3 experiments performed in triplicate.

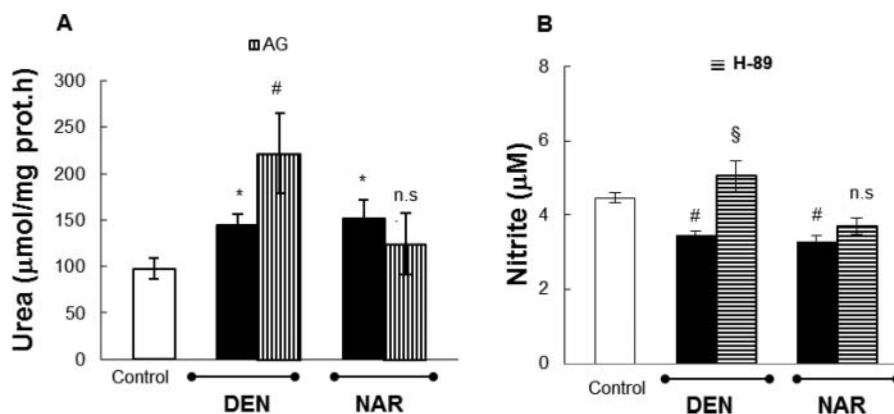

Figure 3. Participation of nitric oxide in the effect of bitter compounds on SCA-9 cells. Cells were treated with denatonium (DEN; 10^{-8} M) or naringenin (NAR; 10^{-7} M) in the absence or presence of 10^{-4} M aminoguanidine (AG) to evaluate (A) urea production in bi-dimensional cultures. Cells were treated with denatonium (DEN; 10^{-8} M) or naringenin (NAR; 10^{-7} M) in the absence or presence of 10^{-5} M H-89 to evaluate (B) nitrite production in culture supernatants. Values are mean ± SEM of at least 3 experiments performed in triplicate. $^{\#}P < 0.001$ or $^{*}P < 0.05$ vs. control (untreated cells) § or $^{\#}P < 0.001$ vs. DEN or NAR; n.s. not significant.

Discussion

Salivary glands are necessary for maintaining oral cavity and upper gastrointestinal tract health because of their exocrine and endocrine secretion (21,22). Malignant transformation in these glands has been reported to generate nearly 3% of head and neck tumors in adults. Moreover, salivary gland tumors are the most frequent pediatric malignancies with head and neck localization (23). The heterogeneity of histological types and complexity of salivary gland tumors represents a challenge for diagnosis, treatment, and outcome of these malignancies (24). The SMG-derived tumor cell line SCA-9 is considered a useful tool to study malignant proliferation, signaling pathways, and their regulation, which could be specifically activated by different stimuli or agonists. In our laboratory, we extensively studied the role of different agonists that activate GPCRs in tumor progression (25,26). Particularly, we analyzed the activation of muscarinic acetylcholine receptors that belong to the GPCRs family, with carbachol. This synthetic agonist triggers phospholipase C/A_2 pathways in SCA-9 cells increasing proliferation (27).

Recent studies on T2Rs, another subfamily of GPCRs, showed that these receptors are expressed in several extra-oral tissues, including the respiratory system, brain, reproductive tissues, and airways mediating physiological functions (28,29). However, the expression and possible functions of T2Rs in cancer cells were not elucidated yet. Singh et al. (14) documented that T2Rs are present in human breast cancer cells. Genetic variants in T2R genes have been hypothesized to impact negatively in health outcomes and/or influence dietary intake. Using a case-control study, Schembre et al. (30) postulated associations among colorectal adenoma risk, dietary intake, and genetic variation in 3 encoding genes TAS2R38, TAS2R16, and TAS2R50. In the same way, our results show that two different bitter compounds, denatonium and naringenin, can trigger cell growth in CT26 cells from murine colon carcinoma.

In the present study, we described for the first time that SCA-9 tumor cells express T2R6 and T2R4 receptors. Then, we analyzed the action of two different bitter compounds: denatonium and naringenin on tumor progression. The first one belongs to quaternary amine group and can activate at least eight different subtypes of T2Rs, among which T2R4 is included; while the latter one is a natural flavonone glycoside that can be found in citric fruits. It has been recently identified as a ligand of Tas2R14 in humans, one of the most broadly tuned bitter taste receptors (31,32). Both bitter compounds increased SCA-9 tumor growth either in bi- or in three-dimensional cell cultures. Other authors have reported beneficial effects of naringenin in tumor models, like the inhibition of proliferation and/or the increment in apoptosis (33,34). But these actions were observed at very high concentrations (5–500 mM), added during the whole time of culturing, and/or targeting other receptors than T2Rs. The range of concentrations of bitter compounds used in our experiments (10^{-9} M–10^{-6} M) is the one that can trigger in a specific manner the activation of GPCRs like T2R receptors (25,27).

Our results demonstrate that both isoforms of arginase are expressed in SCA-9 tumor cells. Arginase is involved in the production of L-ornithine for polyamine and proline synthesis necessary for cell proliferation and development (35). But, the effect exerted by bitter compounds on SCA-9 cell proliferation was due not only to an upregulation in arginase activity but also to a downregulation in NO production. We

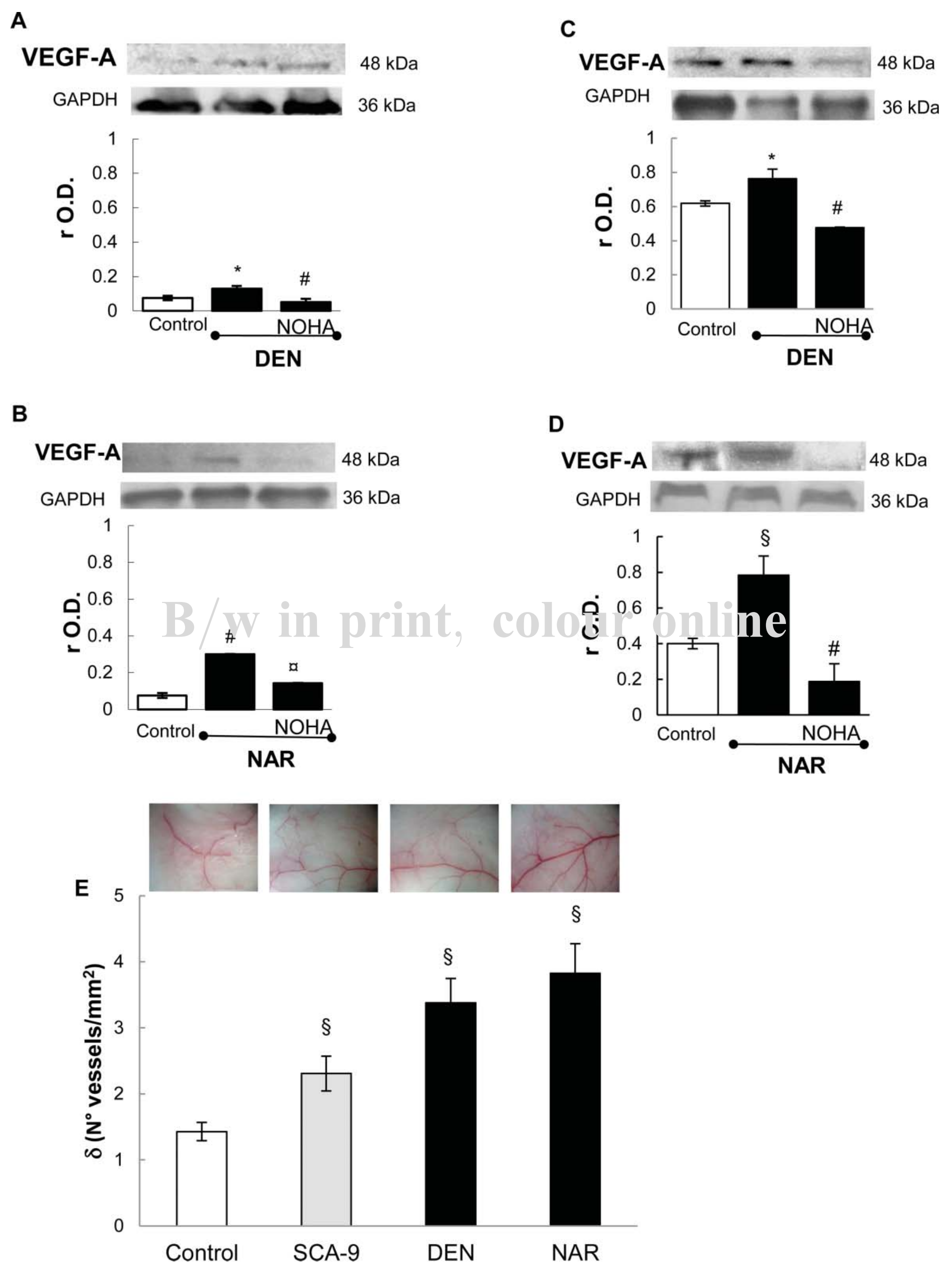

Figure 4. Effect of bitter compounds on SCA-9 induced angiogenesis. Expression of vascular endothelial growth factor-A (VEGF-A) in tumor cells by Western blot. Cells were treated with (A) and (C) denatonium (DEN; 10^{-8} M) or (B) and (D) naringenin (NAR; 10^{-7} M) in the absence or presence of 10^{-4} M N-hydroxy-L-arginine (NOHA) and grown in bi-dimensional (A, B) or tri-dimensional (C, D) cultures. Values are mean ± SEM of at least 3 experiments performed in duplicate. (E) Neovascular response (d) induced by tumor cells in vivo. Cells were adjusted to 2×10^6 cells/ml and treated during 20 min with denatonium (DEN; 10^{-8} M) or naringenin (NAR; 10^{-7} M). After washing, cell suspensions (0.1 ml) were inoculated (i.d.) in both flanks of Swiss mice. Values are means ± SE of three experiments performed in triplicate. Photographs of the angiogenic sites from each experimental group. Magnification: 6.4 ×. $^{*}P < 0.05$; $^{\#}P < 0.01$; $^{\S}P < 0.001$ vs. control or SCA-9 cells; $^{\#}$ or $^{¤}P < 0.01$ vs. DEN or NAR.

have previously reported that NOS is expressed in SCA-9 cells (26), and it is well known that it shares its substrate, L-arginine with arginase. In spite of this, the addition of aminoguanidine, a selective NOS2 inhibitor, only potentiated cell growth and urea production induced by dentonium but not by naringenin. This could reveal differences in the activation of each subtype of T2R and/or in the mechanism of action for each agonist. Regarding the latter one, the addition of H-89 turned to stimulate the inhibitory action triggered by denatonium on NO production, involving PKA in a negative regulation of NOS activity. Similar results were obtained by us in SMG, since the addition of the PKA inhibitor, H-89 prevented the downregulation in phospholipase C/NOS pathway produced by T2R agonists (4).

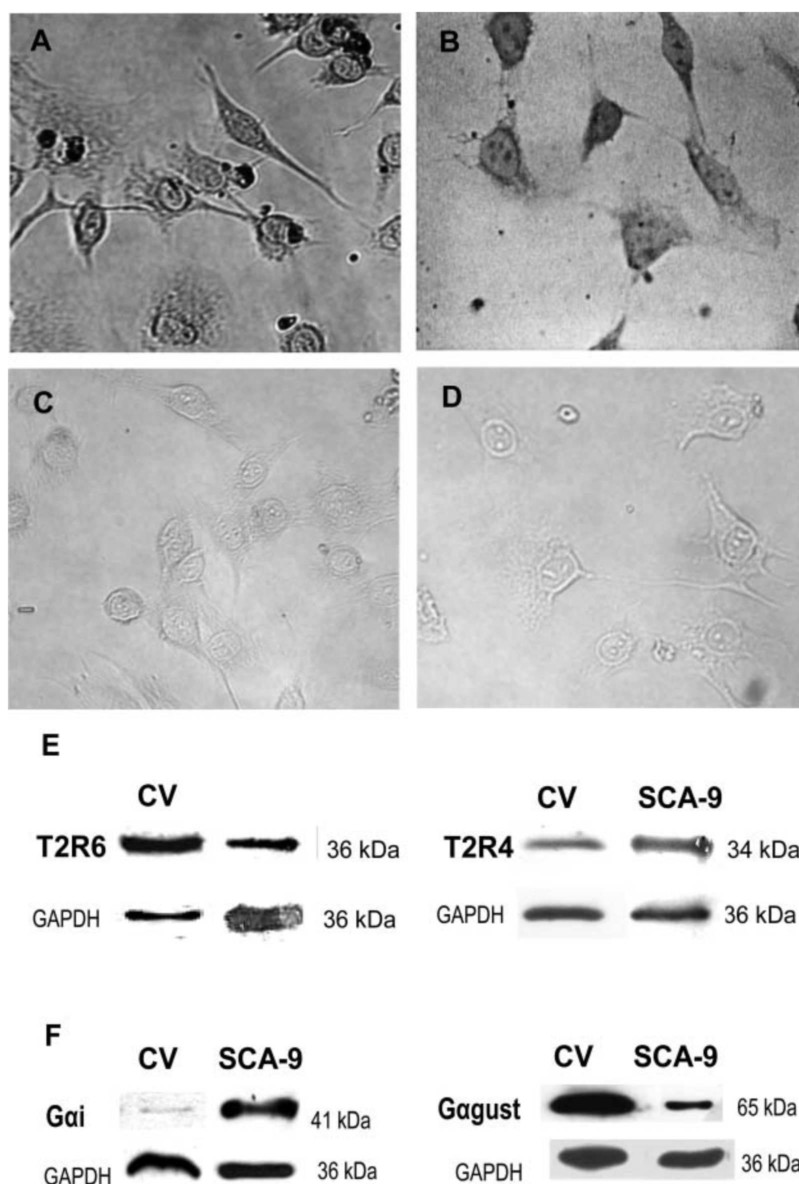

Figure 5. T2Rs and G protein expression in SCA-9 cells. Immunocytochemistry assay to detect (A) T2R6 and (B) T2R4 with specific antibodies in tumor cells. Negative control omitting (C) the primary or D) the secondary antibodies were performed. Magnification: 640 ×. Western blot assay to detect (E) T2R6 and T2R4 in SCA-9 cells [positive control was performed in murine circumvallate papillae (CV) of the tongue) or F] Gai and Gagust in SCA-9 cells. Molecular weights of proteins are indicated on the right. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. One representative experiment of 3 is shown.

Furthermore, bitter compounds activate neovascular response induced by SCA-9 cells by increasing VEGF-A expression in tumor cell lysates and also in vessel count in the skin of SCA-9 tumor-induced angiogenesis *in vivo*. The effect of denatonium and naringenin on VEGF-A levels was demonstrated to be arginase-dependent, since NOHA decreased the increment in VEGF-A levels in tumor cells. Concordantly, Prati et al. (36) described the curative effect of nor-NOHA, a potent arginase inhibitor in the endothelial dysfunction associated with rheumatoid arthritis induced in rats. Their results showed that the beneficial effect is mediated by a decrement in plasma levels of IL-6 and VEGF in arthritic rats.

It has been reported that many different bitter compounds contained in phytonutrients exert antitumor actions. Li et al. (37) documented that dietary flavones and flavonones can be the most potent aromatase-inhibitory flavonoids. Their effects can be exerted on the transcription regulation of CYP19 in MCF-7 cells. However, naringenin was unable to reduce CYP 19 mRNA expression in these breast tumor cells. But recent evidence not only identified T2Rs in tumor cells but also related its activation with tumor progression. Gaida et al.

(38) confirmed the expression of T2R38 in tumor cells in patients with pancreatic cancer and in tumor-derived cell lines. The activation of these receptors with phenylthiourea, and N-acetyl-dodecanoyl homoserine from the bonafide flora activated phosphorylation of the MAP kinases p38 and ERK1/2, and upregulated of NFAT and multidrug resistance protein 1, indicating that T2R38 could link microbiota and cancer (38).

Conclusion

Our work demonstrates for the first time the expression of two different subtypes of T2Rs in murine SCA-9 tumor cells derived from SMG. The treatment of these cells with bitter compounds promotes two essential steps of tumor progression such as proliferation and angiogenesis activating arginase metabolic pathway. These results point out the undesirable effects of bitter compounds that are frequently used as nutraceuticals, revealing the need to prevent their indiscriminate use in cancer patients.

Acknowledgments

The authors want to thank Mr. Francisco Sánchez for his excellent technical assistance and for the correction of the manuscript. They also want to thank Mrs. Patricia Fernández for her excellent management of financial support. This work was supported by grants from the National Research Council from Argentina (CONICET) PIP 2012–2014 N° 11220110100108 and from the University of Buenos Aires (UBA) UBACYT 2014–2017 N° 20020130100168BA.

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