Antigoitrogenic effect of combined supplementation with dl- α -tocopherol, ascorbic acid and β -carotene and of dl- α -tocopherol alone in the rat

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Abstract

The effects of the vitamins dl- α -tocopherol, ascorbic acid and β -carotene, free radical scavengers and lipid peroxidation inhibitors, were analyzed in male Wistar rats made goitrous by feeding a low iodine diet (<20 μ g iodine/kg) and perchlorate (1% in drinking water) for 4, 8, 16 and 32 days. Groups of control or goitrous rats received for at least 16 days before killing a diet containing 0.6% vitamin E (as dl- α -tocopherol acetate), 1.2% vitamin C (ascorbic acid) and 0.48% β -carotene, either simultaneously (vitamin cocktail) or separately. This treatment led to a 5-fold increase of vitamin E in the thyroid gland, a 24-fold increase in the liver and a 3-fold increase in the plasma. In control rats, vitamin cocktail administration increased slightly the thyroid weight with little changes in thyroid function parameters. During iodine deficiency, administration of the vitamin cocktail or vitamin E alone reduced

significantly the rate of increase in thyroid weight, and DNA and protein contents, as well as the proportion of [³H]thymidine labeled thyroid follicular cells, but not that of labeled endothelial cells. Plasma tri-iodothyronine, thyroxine, TSH levels, thyroid iodine content and concentration as well as relative volumes of glandular compartments were not modified. The proportion of necrotic cells rose from 0.5% in normal animals to about 2% after 16 days of goiter development. No significant protective effect of the vitamins was observed. These results suggest that these vitamins, particularly vitamin E, modulate one of the regulatory cascades involved in the control of thyroid follicular cell growth, without interfering with the proliferation of endothelial cells.

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Introduction

Hyperplastic goiter results from a lack of iodine, alone or in association with an ingestion of antithyroid drugs. Thyroid cell necrosis can be observed during iodine deficiency (Many *et al.* 1991) and is aggravated with an acute iodide refeeding (Wollman *et al.* 1968, 1990, Belshaw & Becker 1973, Many *et al.* 1992, Bagchi *et al.* 1995). When involution of hyperplastic thyroid glands is induced by thyroid hormones, apoptosis substitutes cell necrosis for cell loss (Mahmoud *et al.* 1986). The mechanisms by which iodide induces thyroid cell toxicity are still unknown. One of the proposed biochemical hypotheses is the generation of free radicals during the thyroid hormonogenesis which requires, besides peroxidase, iodide, thyroglobulin and hydrogen peroxide (Many *et al.* 1991, Denef *et al.* 1996).

The free radical hypothesis is difficult to test *in vivo*. Two approaches are possible: to reduce thyroid antioxidant defenses (Contempré *et al.* 1995), or to analyze the effects of free radical scavengers or of antioxidants known to stop lipid peroxidation. In order to assess the implication

of free radical generation in the induction of thyroid cell necrosis, we analyzed the effect of the vitamins known to be radical scavengers on thyroid iodine metabolism in rats during goitrogenesis.

Materials and Methods

Animals and treatments

For this study, three experiments were performed. Male Wistar rats (KUL, Leuven, Belgium) weighing approximately 140 g at the onset of the study were used. Rats were housed, two per cage, in a temperature- and light-controlled room. Goiter (hypothyroidism) was induced by feeding a low iodine diet (LID, <20 μ g iodine/kg, Animalabo, Brussels, Belgium), providing a daily iodine supply of about 0.1 μ g, with 1% (w/v) NaClO₄ in distilled water for 4, 8, 16 and 32 days in the first experiment, and for 16 days in the second and third experiments. Control rats received the same diet with 0.75 μ g iodine/ml as KI instead of perchlorate in the

drinking water, providing a daily iodine supply of about 12 µg.

For vitamin treatment, randomly selected control and goitrous rats (six/group, whatever the duration of the goitrogenic treatment) received for at least 16 days before killing, 0.6% vitamin E (as dl- α -tocopherol acetate), 1.2% vitamin C (ascorbic acid) and 0.48% β -carotene mixed in the food (Federa, Brussels, Belgium), either simultaneously (E β C cocktail), in the first experiment, or separately, in the second experiment. In the third experiment, vitamin-treated control and goitrous rats received only vitamin E for 16 days. Rats on different treatments were pair-fed. All animals were weighed before and after treatment and, in general, body weight gains were positive in all groups. Vitamin supplementation increased the body weight to the same extent in control and goitrous rats.

Rats were killed under nembutal anesthesia. Blood was collected by cardiac puncture, and plasma separated and stored at -20°C until use. A piece of liver was taken from each animal, fixed as described below, and 0.5 µm sections examined by light microscopy to examine the possible toxic effects of either vitamins or perchlorate on hepatocytes. No alterations were seen in any group.

For biochemical determinations, thyroid glands were directly dissected on ice, weighed, quick-frozen in liquid nitrogen and stored at -80°C . They were homogenized on ice in 20 mM phosphate buffer pH 7.4 for DNA, stable iodine and total protein content determinations.

Thyroid hormones assay

Concentrations of thyroid hormones were determined in duplicate by RIA using commercially available kits (Corning, Medfield, MA, USA) for tri-iodothyronine (T_3) and thyroxine (T_4). Plasma thyrotropin (TSH) concentrations were determined using a specific kit for rat TSH (Amersham International, Amersham, Bucks, UK).

Morphological and stereological analysis

In the first and second experiments, six rats of each group were injected with 30 µCi [methyl- ^3H]thymidine (Amersham International) 1 h before killing. They were perfused through the heart with saline for 1 min and with 2.5% glutaraldehyde (Taab, Reading, UK) in 0.1 M cacodylate buffer (Taab, pH 7.4, for 5 min as previously described (Mestdagh *et al.* 1990). Their thyroids were dissected carefully, weighed and further processed for light microscopy (Many *et al.* 1985) and autoradiography (see below). The thyroid was cut into fragments and immersed for 2 h in the fixative in cacodylate buffer. After fixation, thyroid fragments were rinsed in cacodylate buffer, post-fixed for 1 h in 1% osmium tetroxide, dehydrated in alcohol solutions of increasing strength and embedded

in LX112 resin (Ladd Research Industries, Burlington, USA). Thick sections (0.5 µm) were cut from the centre of each fragment and stained with toluidine blue.

Morphological changes induced by the different treatments were analyzed by light microscopy. The extent of cell necrosis was quantified by counting the number of necrotic cells (i.e. with pyknotic or karyolytic nuclei or with vacuolar cytoplasm) on semi-thin sections at a magnification of $\times 165$. Counting was performed on 1000 follicular cells per section and on two sections per gland. Relative volumes of glandular compartments (epithelium, follicular lumina, blood vessels and non-vascular interstitium) were determined by the point counting method as described previously (Weibel 1979). All measurements and analyses were made blind.

Autoradiography

Thick sections (1 µm) were collected on carefully cleaned glass slides and dipped in 50% diluted Ilford L4 (Ilford, Basildon, UK) emulsion. They were exposed for 2 months at 4°C , developed in Amidol and stained with a solution of 2% borax and 2% toluidine blue. Labeled nuclei of follicular epithelial cells were counted among a total of at least 1000 nuclei per section, on two sections per gland at a magnification of $\times 165$. The [^3H]thymidine incorporation index was expressed as the percentage of labeled nuclei. Endothelial labeled cells were counted in a total of 10 microscopic fields per section, on two sections per gland at the $\times 165$ magnification.

DNA, stable iodine and total protein contents determination

DNA and stable iodine contents were determined on one thyroid lobe from six rats as described previously (van den Hove *et al.* 1995).

Total protein content was measured with the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munchen, Germany) using BSA as standard (Sigma-Aldrich, Bornem, Belgium).

Plasma and tissues dl- α -tocopherol levels determination

In the third experiment, the level of dl- α -tocopherol was quantified by HPLC according to Ingold *et al.* (1987) for plasma, and to Burton *et al.* (1985) for liver and thyroid. Tocopherol acetate (Sigma-Aldrich) was used as internal standard. Plasma (100 µl) was mixed with 100 µl ethanol containing the internal standard. Then 200 µl *n*-hexane were added and vigorously mixed. The organic layer was carefully drawn off after centrifugation. Solvent was evaporated and the pellet redissolved in methanol and injected onto the HPLC column. In each group, one thyroid lobe or 100 mg of liver from six rats were homogenized in 100 mM SDS. The entire homogenate was then treated with ethanol and *n*-hexane in the same

Figure 1 Thyroid morphology of control and goitrous rats with or without vitamins. Panels A and B, 0.5 μm -thick toluidine blue stained sections from thyroids of control rats without any vitamin supplementation (panel A), or receiving (panel B) a vitamin cocktail for 16 days (0.6% vitamin E, 1.2% vitamin C and 0.48% β -carotene, w/w, mixed in the diet); the glands have a normal morphology. Panels C and D, [^3H]thymidine autoradiographs from thyroids of rats made goitrous by feeding an LID for 8 days and 1% NaClO_4 in drinking water without (panel C) or with (panel D) the vitamin cocktail given for 16 days; the glands have markedly enlarged blood vessels (*), hypertrophic epithelial cells and narrow lumina empty of colloid (empty arrows); some follicular (arrowhead) and endothelial nuclei (arrow) are labeled, no effect of vitamins is noticed on thyroid morphology, but the proportion of labeled follicular cells is reduced. Bar=25 μm .

way as plasma. Separation was performed on a $\mu\text{Bondapak C18}$ column 30×3.9 mm, equipped with a guard column (Waters Model 510, Waters Associates, Milford, MA, USA). Elution was performed with methanol–water (95:5, v/v) at a flow rate of 1.5 ml/min. The column effluent was monitored at 280 nm. Vitamin E content was determined from a standard curve of peak surfaces in which a constant amount of tocopherol acetate was combined with different concentrations of dl- α -tocopherol (Sigma-Aldrich). Peak surfaces corrected for the recovery of the internal standard were used to calculate the dl- α -tocopherol concentration in each sample.

Statistical analysis

Results are expressed as means $\pm$ s.d. ($n=6$). Comparisons between groups were made by one- or two-way analysis of variance (ANOVA) followed by an F-test. Significant differences between means were performed after ANOVA

by the least significant difference test. The accuracy of the point counting method was tested by repeating five times the measurements on the same section during a period of time. The overall coefficient of variation was about 6%. For morphometric analysis, differences were considered as significant only when they were larger than the coefficient of variation of the method and with $P < 0.05$. To analyze follicular necrotic cells and [^3H]thymidine labeling indexes, data were converted to a normal distribution by a logarithmic transformation and then analyzed by one- or two-way ANOVA followed by an F-test. Differences were considered significant at $P < 0.05$.

Results

Morphological changes

Thyroid morphological changes were analyzed in the first two experiments. After LID and perchlorate treatment, a

Figure 2 Effect of vitamin E (E), β -carotene (β) and vitamin C (C), as a cocktail (E β C) or separately, on the percentage of follicular necrotic cells observed in 0.5 μ m-thick sections from control or goitrous thyroids. Panel A, experiment 1: control rats or rats made goitrous for 4 to 32 days without (E β C -) or with (E β C+) the vitamin cocktail given for at least 16 days. Panel B, experiment 2: control or goitrous rats receiving the vitamin cocktail or each vitamin separately for 16 days. Each bar represents the mean $\pm$ S.D. of six rats; * P <0.01 vs control without vitamins.

typical hyperplastic goiter was obtained from day 8 on. Compared with the thyroid of control rats (Fig. 1A), blood vessels were markedly enlarged, epithelial cells were hypertrophic and follicular lumina were narrow, almost all of them empty of colloid (Fig. 1C). As illustrated in Fig. 1B in control rats and in Fig. 1D in goitrous rats at day 8, the treatment with the vitamin cocktail did not modify the thyroid morphology. Necrotic cells were observed in the epithelial wall, their number was significantly increased from day 8 of goiter but returned to the control value after 32 days (Fig. 2). Again, no significant effect of vitamins administered either as a cocktail (Fig. 2A) or separately (Fig. 2B) was noticed. Relative volumes of glandular compartments are given in Table 1. Goiter development was associated with a significant decrease in the relative volume of follicular lumina and with an increase in the relative volume of blood vessels. However, the relative volume of epithelium and of the non-vascular interstitium was unchanged. Vitamin treatments (as a cocktail or separately) did not modify these parameters.

Plasma hormone concentrations

In the first experiment (Table 2), T_3 levels remained unchanged up to day 8 of goiter and decreased significantly from day 16 to day 32, as compared with control values. In contrast, plasma T_4 levels showed a marked depression as early as day 4 of iodine deficiency, with a further decrease occurring at day 16. Administration of the E β C cocktail did not interfere with T_3 levels but induced a small but significant decrease in T_4 levels in control rats.

In the second experiment (Table 2), T_3 and T_4 levels, which were decreased significantly during goiter induction, were not affected by vitamin cocktail supplementation. When the effect of each vitamin was tested in control

rats, T_3 levels were decreased with the three vitamin treatments, whereas T_4 levels were reduced only after vitamin C and β -carotene supplementation. The plasma TSH levels (Table 2) were increased significantly at day 16 of goiter as compared with control value (55.5 ± 16.0 vs 9.5 ± 2.0 ng/ml). No change in TSH levels was observed after vitamin supplementation (as a cocktail or separately) in either control or goitrous rats.

Thyroid weight

After administration of the E β C cocktail or of vitamin E alone to control rats, a slight but significant increase of the thyroid weight was observed in all three experiments (Fig. 3). After LID-perchlorate treatment, a goiter developed in all groups from day 8 on. Supplementation with the E β C cocktail reduced significantly the goiter weight from day 8 in the first experiment (Fig. 3A). In the second experiment (Fig. 3B), this reduction was seen after administration of the cocktail and of the vitamin E alone whereas administration of vitamin C and β -carotene increased the goiter weight. In the third experiment, vitamin E supplementation reproduced similar effects (data not shown).

Thyroid cellularity

As shown in Fig. 4A and B, the follicular cell proliferation index was 0.4% in control rats, and it did not change after vitamin cocktail treatment. During hyperplasia in the first experiment (Fig. 4A), the [3 H]thymidine labeling index of follicular cells increased significantly until day 16 ($2.6 \pm 0.3\%$); it returned to control values at day 32. Supplementation with the vitamin cocktail reduced significantly the follicular cell proliferation index at day 8 and

Table 1 Relative volumes (% $\pm$ S.D., $n=6$) of thyroid compartments

	Vitamins	Epithelium	Colloid	Blood vessels	Interstitium
Experiment 1					
Control	—	40 $\pm$ 2	25 $\pm$ 5	18 $\pm$ 4	17 $\pm$ 1
	E β C	39 $\pm$ 4	21 $\pm$ 5	19 $\pm$ 3	21 $\pm$ 2
Goiter					
4 days	—	42 $\pm$ 4	10 $\pm$ 2°	28 $\pm$ 5°	20 $\pm$ 2
	E β C	41 $\pm$ 3	16 $\pm$ 3°	26 $\pm$ 4°	17 $\pm$ 2
8 days	—	43 $\pm$ 3	3.4 $\pm$ 1.0°	37 $\pm$ 4°	17 $\pm$ 3
	E β C	42 $\pm$ 3	3.1 $\pm$ 1.0°	39 $\pm$ 3°	16 $\pm$ 2
16 days	—	43 $\pm$ 3	0.5 $\pm$ 0.2°	40 $\pm$ 4°	17 $\pm$ 2
	E β C	42 $\pm$ 4	0.8 $\pm$ 0.1°	44 $\pm$ 4°	14 $\pm$ 1
32 days	—	44 $\pm$ 4	0.5 $\pm$ 0.1°	39 $\pm$ 2°	17 $\pm$ 3
	E β C	42 $\pm$ 3	0.6 $\pm$ 0.1°	42 $\pm$ 4°	15 $\pm$ 2
Experiment 2					
Control	—	41 $\pm$ 2	29 $\pm$ 8	9 $\pm$ 2	22 $\pm$ 1
	E β C	40 $\pm$ 3	28 $\pm$ 6	12 $\pm$ 2	20 $\pm$ 2
	E	40 $\pm$ 4	28 $\pm$ 6	12 $\pm$ 1	20 $\pm$ 3
	β	41 $\pm$ 3	26 $\pm$ 4	13 $\pm$ 2	20 $\pm$ 1
	C	39 $\pm$ 2	29 $\pm$ 4	11 $\pm$ 3	21 $\pm$ 1
Goiter					
16 days	—	43 $\pm$ 3	0.5 $\pm$ 0.2°	43 $\pm$ 3°	14 $\pm$ 2
	E β C	42 $\pm$ 4	0.5 $\pm$ 0.2°	43 $\pm$ 4°	15 $\pm$ 2
	E	39 $\pm$ 3	1.2 $\pm$ 0.8°	44 $\pm$ 4°	16 $\pm$ 1
	β	43 $\pm$ 4	0.5 $\pm$ 0.3°	41 $\pm$ 6°	15 $\pm$ 2
	C	41 $\pm$ 3	0.7 $\pm$ 0.2°	39 $\pm$ 4°	16 $\pm$ 3

Experiment 1: control rats or rats made goitrous for 4 to 32 days without (—) or with vitamin E (E), β -carotene (β) and vitamin C (C) given as a cocktail (E β C) for at least 16 days. Experiment 2: control or goitrous rats receiving the vitamin cocktail or each vitamin separately for 16 days.

° $P < 0.05$ and difference superior to coefficient of variation (see Materials and Methods) vs control without vitamins.

16. In the second experiment (Fig. 4B), the follicular cell proliferation index also increased significantly during iodine deficiency. When the effect of each vitamin was tested, vitamin E alone produced results similar to the vitamin cocktail, whereas no significant effect was observed with vitamin C or β -carotene. The endothelial cell proliferation index (Fig. 4C) increased significantly during iodine deficiency, as early as day 4. In contrast to the reduced proliferation index of follicular cells, no effect on endothelial cell proliferation index was noticed after vitamin cocktail supplementation.

In the first experiment, the goitrogenic treatment significantly increased the thyroid DNA content after 8 and 16 days (Fig. 5A). In vitamin-treated animals, a significant reduction in the thyroid DNA content was observed at day 16 (42.8 ± 11.0 vs 52.6 ± 11.0 $\mu\text{g}/\text{lobe}$). A similar reduction in DNA content was also observed in the third experiment when vitamin E was tested alone (Fig. 5B). Since DNA content varied in parallel with the thyroid weight, no difference in DNA concentration was noticed. In the first experiment, DNA concentration was 2.8 ± 0.9 $\mu\text{g}/\text{mg}$ tissue in control rats, 2.1 ± 0.8 in vitamin cocktail-treated normal rats, 3.7 ± 0.8 in iodine-deficient rats at day 16 and 3.9 ± 0.8 in iodine-deficient vitamin cocktail-treated rats at day 16. Similar concentrations were observed in the third experiment (data not shown).

Protein content and concentration

Protein contents and concentrations showed the same evolution as DNA. The protein content increased significantly after the goitrogenic treatment at day 16 only (806 ± 180 vs 389 ± 81 $\mu\text{g}/\text{lobe}$). It decreased markedly after vitamin cocktail supplementation (569 ± 105). However, the protein concentrations remained unchanged whatever the treatment (66 ± 12 , 67 ± 14 , 56 ± 6 and 53 ± 5 $\mu\text{g}/\text{mg}$ tissue in control rats, control vitamin cocktail-treated rats, iodine-deficient rats at day 16 and in iodine-deficient vitamin cocktail-treated rats at day 16 respectively).

Thyroid stable iodine

During iodine deficiency, the decrease in stable iodine content of the glands (Fig. 6) confirmed the well-known perchlorate effect on thyroid iodine metabolism (Ortiz-Caro *et al.* 1983). After 16 days of the goitrogenic treatment, the thyroid was almost empty of iodine. Thyroid iodine concentration decreased with the goitrogenic treatment duration, from 730 ± 250 $\mu\text{g}/\text{g}$ tissue in control rats to 8 ± 3 $\mu\text{g}/\text{g}$ tissue in goitrous rats at day 16. Vitamin cocktail treatment did not modify the glandular iodine content in either control or goitrous rats.

Table 2 Plasma T₃, T₄ and TSH (mean ± s.d., n=6)

	Vitamins	T ₃ (ng/dl)	T ₄ (µg/dl)	TSH (ng/ml)
Experiment 1				
Control	—	74 ± 9	3.2 ± 0.6	nd
	EβC	74 ± 13	2.3 ± 0.4°	nd
Goiter				
4 days	—	65 ± 23	1.7 ± 0.5°	nd
	EβC	64 ± 14	1.5 ± 0.4°	nd
8 days	—	80 ± 24	1.8 ± 0.4°	nd
	EβC	81 ± 26	1.8 ± 0.5°	nd
16 days	—	47 ± 25°	<1.25°	nd
	EβC	30 ± 12°	<1.25°	nd
32 days	—	13 ± 12°	<1.25°	nd
	EβC	24 ± 17°	<1.25°	nd
Experiment 2				
Control	—	85 ± 10	2.7 ± 0.7	9.5 ± 2
	EβC	68 ± 9°	2.4 ± 0.4	11.8 ± 3
	E	63 ± 10°	2.5 ± 0.7	11.1 ± 2
	β	61 ± 17°	2.2 ± 0.7°	9.2 ± 2
	C	72 ± 12°	1.9 ± 0.4°	8.5 ± 3
Goiter				
16 days	—	20 ± 5°	nd	55.5 ± 16°
	EβC	26 ± 13°	nd	57.0 ± 08°
	E	28 ± 15°	nd	62.7 ± 29°
	β	23 ± 5°	nd	52.0 ± 09°
	C	19 ± 5°	nd	47.4 ± 14°

Experiment 1: control rats or rats made goitrous for 4 to 32 days without (—) or with vitamin E (E), β-carotene (β) and vitamin C (C) given as a cocktail (EβC) for at least 16 days. Experiment 2: control or goitrous rats receiving the vitamin cocktail or each vitamin separately for 16 days. °P<0.05 vs control without vitamins; nd=not done.

Figure 3 Effect of vitamin E (E), β-carotene (β) and vitamin C (C), as a cocktail (EβC) or separately, on the thyroid weight in control and goitrous rats. Panel A, experiment 1: control rats or rats made goitrous for 4 to 32 days without (EβC —) or with (EβC+) the vitamin cocktail given for at least 16 days. Panel B, experiment 2: control or goitrous rats receiving the vitamin cocktail or each vitamin separately for 16 days. Other experimental conditions were as for Fig. 2. °P<0.01 vs control without vitamins, *P<0.01 vs goiter without vitamins.

Plasma, liver and thyroid dl-α-tocopherol concentrations

To assess whether supplementation with vitamins affected thyroid vitamin content, the plasma, liver and thyroid vitamin E concentrations were determined in the third experiment (Fig. 7). During goiter development, in the

absence of vitamin E supplementation, vitamin concentrations were significantly increased in plasma and thyroid (11.2 ± 1.5 vs 7.8 ± 0.5 and 33 ± 6 vs 14 ± 4 µg/g tissue respectively) whereas they were significantly decreased in the liver (10 ± 1 vs 17 ± 6 µg/g tissue)

compared with controls. After 16 days of dl- α -tocopherol acetate supplementation, the vitamin E concentrations were increased in the liver (approximately 24-fold), in the thyroid (5-fold) and in the plasma (3-fold), in both control and goitrous rats. Vitamin E concentration in the liver was lower in vitamin-treated goitrous rats than in vitamin-treated control rats (260 ± 77 vs 375 ± 110 $\mu\text{g/g}$ tissue). In contrast, the vitamin E concentrations in the plasma and in the thyroid were higher in vitamin-treated goitrous rats than in vitamin-treated control rats (39.5 ± 6 vs 29.2 ± 8 and 169 ± 30 vs 72 ± 14 $\mu\text{g/g}$ tissue respectively).

Discussion

The present data clearly show that the concentration of vitamin E in the thyroid gland is similar to that found in the liver. Administration of vitamin E has an antigoitrogenic effect during iodine deficiency; it reduces follicular cell proliferation, but does not interfere with either endothelial cell proliferation or iodine metabolism. Neither β -carotene nor vitamin C, two other free radical scavengers, has a similar effect. Furthermore, we did not observe any additive effects of these two antioxidant molecules when given in association with vitamin E.

To the best of our knowledge, no data concerning thyroidal concentrations of vitamin E have been published so far, either in normal or in iodine-deficient animals. Vitamin E concentration in the thyroid of normal rats was almost as high as in liver, and higher than in plasma. It decreased during iodine deficiency in the liver while it increased in thyroid and plasma. It is well known that total lipid content is raised in serum during hypothyroidism and that a high correlation exists between serum total lipids and tocopherol levels (Machlin 1984). The increased concentration of vitamin E in the thyroid is larger than that which could be expected by multiplying the plasma concentration observed in our hypothyroid animals by the increased relative volume of the vascular bed measured in goiters. This strongly suggests that the increased vitamin E concentration during goitrogenesis is tissue specific. Mano *et al.* (1995) reported that hypothyroidism has no effect on vitamin E levels in rat heart muscle and we observed a significant reduction of the concentration in the liver, both with or without vitamin E administration. This suggests

Figure 4 Effect of vitamin E (E), β -carotene (β) and vitamin C (C), as a cocktail (EBC) or separately, on the [^3H]thymidine labeling indexes in follicular (panels A and B) and endothelial (panel C) cells of control and goitrous rats. Panels A and C, experiment 1: control rats or rats made goitrous for 4 to 32 days without (EBC-) or with (EBC+) the vitamin cocktail given for at least 16 days. Panel B, experiment 2: control or goitrous rats receiving the vitamin cocktail or each vitamin separately for 16 days. Other experimental conditions were as for Fig. 2. * $P < 0.01$ vs control without vitamins, * $P < 0.01$ vs goiter without vitamins.

Figure 5 Effect of vitamin E (E), β -carotene (β) and vitamin C (C), as a cocktail (E β C) or vitamin E alone, on the DNA content in control or goitrous thyroids. Panel A, experiment 1: control rats or rats made goitrous for 4 to 32 days without (E β C-) or with (E β C+) the vitamin cocktail given for at least 16 days. Panel B, experiment 3: control or goitrous rats receiving vitamin E alone for 16 days. Other experimental conditions were as for Fig. 2. * $P < 0.01$ vs control without vitamins, * $P < 0.01$ vs goiter without vitamins.

that liver stores are diverted in favor of the goiter and raises the question as to whether vitamin E may play a role during goitrogenesis or not. Mobilization of hepatic stores of vitamin E during response to an oxidative stress *in vivo* has been reported (Warren & Reed 1991). The liver α -tocopherol content decreased in rats in which high doses of butylated hydroxytoluene induced oxidation in hepatic tissue (Siman & Eriksson 1996). In the thyroid, a molecular mechanism has been proposed in which the oxidative H_2O_2 -peroxidase pathway could generate oxygen-derived free radicals during iodine deficiency (Denef *et al.* 1996). Whether activation of this H_2O_2 -peroxidase

Figure 6 Effect of vitamin E (E), β -carotene (β) and vitamin C (C), as a cocktail (E β C), on the stable thyroid iodine content in rats during goiter development. Control rats or rats made goitrous for 4 to 32 days without (E β C-) or with (E β C+) the vitamin cocktail given for at least 16 days (experiment 1). Other experimental conditions were as for Fig. 2. * $P < 0.05$ vs control without vitamins.

pathway during goiter development might account for the raised vitamin E concentration in the thyroid tissue remains to be elucidated. After vitamin E administration to control rats, the increase in concentration in liver is much larger than the increase observed in thyroid and plasma. This is in line with previous reports showing that the increase in α -tocopherol content in liver exceeds that in other tissues (Machlin & Gabriel 1982, Konneh *et al.* 1995).

In rats treated with the vitamin cocktail the rate of goiter development was slowed down. This antigoitrogenic effect of the vitamin complex was illustrated by a reduced increase in thyroid weight, in the [3H]thymidine labeling index of follicular cells and in DNA and protein glandular contents. In contrast, the [3H]thymidine labeling index of endothelial cells was not altered by the vitamin complex administration. This clearly suggests a specific action of the vitamin cocktail on the replication of follicular cells. As already mentioned (Denef *et al.* 1981, Many *et al.* 1984), an early peak of [3H]thymidine labeling index is observed in endothelial cells, before the rise of [3H]thymidine incorporation in follicular cells. Of interest, the administration of the vitamin cocktail did not alter the peripheral levels of thyroid hormones and TSH, or the relative volumes of glandular constituents, or the iodine metabolism during goitrogenesis. Surprisingly, in control rats, administration of vitamin cocktail or vitamin E alone increased the thyroid weight in all three experiments with variable and inconstant effects on the thyroid functional parameters. We have indeed been puzzled by the

Figure 7 Vitamin E concentration in plasma, thyroid and liver of control and goitrous rats. Results are means $\pm$ S.D. of six rats. Data are expressed as $\mu\text{g/g}$ plasma or tissue. $^{\circ}P<0.01$ vs control without vitamin E, $^{*}P<0.01$ vs control with vitamin E, $^{*}P<0.001$ vs goiter without vitamin E.

observation of a slight decrease of T_4 plasma level and the increase of the thyroid weight in control animals treated either by the vitamin cocktail or by vitamin E alone. These changes, although not very large, suggest that vitamin E acts differently depending upon whether the gland is stimulated or not. Further experiments are necessary to elucidate this point.

Vitamin E was the only component of the vitamin cocktail that reproduced its antigoitrogenic effect, while vitamin C or β -carotene increased the goiter weight. This increase was not correlated with an increase in the [^3H]thymidine labeling index or with changes in the relative volumes of glandular constituents. A small interstitial edema could explain this effect.

Although a vitamin E reducing effect on goiter development has never been reported, several papers have mentioned an inhibitory effect of vitamin E on cell proliferation in various tissues, e.g. smooth muscle cells (Mahoney & Azzi 1988, Boscoboinik *et al.* 1991a), epithelial cells (Sakamoto *et al.* 1996), fibroblasts (Haas *et al.* 1996), and blood leukocytes (Turley *et al.* 1995). In some of these reports, vitamin E was proposed to inhibit the protein kinase (PK) C (PKC) activity (Boscoboinik *et al.* 1991b, Chatelain *et al.* 1993, Ozer *et al.* 1993, Stauble *et al.* 1994, Azzi *et al.* 1995). This inhibition of the PKC activity could be due to the antioxidative properties of vitamin E (Kunisaki *et al.* 1994) or to the binding of vitamin E to a specific protein reducing the phosphorylation by PKC of the activator protein-1 transcription factor (Stauble *et al.* 1994, Azzi *et al.* 1995, 1996). PKC is one of the key steps in the signaling pathways regulating thyroid cell proliferation (Omri *et al.* 1986, Hagiwara *et al.* 1990, Shimizu *et al.* 1991, Feliars & Pavlovic-Hournac 1994) and its stimulation is observed during

goitrogenesis (Omri & Pavlovic-Hournac 1985). Thus, vitamin E could be antigoitrogenic through its PKC-inhibition mechanism. Whether the effect of vitamin E is related to an oxidative metabolic pathway cannot be excluded from our data. Goiter development involves other signaling pathways, e.g. the cAMP-dependent cascade (Dumont *et al.* 1992, Ledent *et al.* 1992), in which vitamin E could also have some inhibitory effect. In addition, interaction between the PKC-inositides cascade and the cAMP-dependent cascade have been described in the regulation of thyroid cell proliferation (Fujimoto & Brenner-Gati 1992, Kraiem *et al.* 1995, Cowin *et al.* 1996, Ledent *et al.* 1997). The absence of effect of vitamin E administration on endothelial cells, whose proliferation is generally regulated through the tyrosine kinases signaling pathway (Guo *et al.* 1995) could be an indirect argument in favor of the PKC or PKA pathways as targets of the vitamin E action. In addition, the absence of any antigoitrogenic effect of β -carotene and vitamin C, two potent antioxidant molecules, when given separately suggests a specific rather than an antioxidative action of vitamin E.

Follicular cell necrosis has been reported to increase during goiter development in the rat (Contempre *et al.* 1995). In our study, cell necrosis increased as early as day 8 after goiter induction and returned to control values at day 32. This increase was not reduced significantly after vitamin treatment, and thus present data do not support the free radical hypothesis proposed to explain cell necrosis during goitrogenesis (Denef *et al.* 1996). However, we think that the low incidence of cell necrosis occurring during goiter formation in the rat, when compared with that observed in the mouse (Many *et al.* 1991), could explain the lack of significance of the reduction observed in our rat model.

In conclusion, vitamin E alone or in association with β -carotene and vitamin C reduces goiter development in rats without any change in thyroid hormone metabolism and in relative volumes of glandular constituents. This effect is at the level of thyroid follicular cell proliferation and does not depend on TSH variation. It is probably due to a direct action of vitamin E on intracellular signaling pathways controlling thyroid growth. However, further experiments will be necessary to confirm the exact site of vitamin E action. Our results show that vitamin E reduces goiter development and might thus be administered to goitrous patients without any change in their thyroid metabolism.

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