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BRIEF COMMUNICATION

Ultrastructural analysis of pancreatic acinar cells from mice fed on genetically modified soybean

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Abstract

No direct evidence that genetically modified (GM) food may represent a possible danger for health has been reported so far; however, the scientific literature in this field is quite poor. Therefore, we investigated the possible effects of a diet containing GM soybean on mouse exocrine pancreas by means of ultrastructural, morphometrical and immunocytochemical analyses. Our observations demonstrate that, although no structural modification occurs in pancreatic acinar cells of mice fed on GM soybean, quantitative changes of some cellular constituents take place in comparison to control animals. In particular, a diet containing significant amount of GM food seems to influence the zymogen synthesis and processing.

Key words amylase; electron microscopy; exocrine pancreas; genetically modified organisms.

Introduction

Genetically modified (GM) crops, in which new genes have been inserted into the original genome, are nowadays distributed all over the world. No direct evidence that GM food may represent a possible danger for health has been reported so far; however, the scientific literature in this field is quite poor and heterogeneous. Nutritional evaluations of GM crops have been carried out (Edwards et al. 2000), and the potential problems related to the introduction of GM food in animal and human diets, such as antibiotic resistance, allergenity and food safety, have been discussed (see review in Halford & Shewry, 2000). Some authors have investigated the fate of ingested foreign DNA (Schubbert et al. 1994, 1997, 1998), demonstrating that it can enter the bloodstream and various organs; others have studied the optimal conditions to ensure sufficient DNA fragmentation to a size where it would be unlikely to be stably transferred to host cells (Chiter et al. 2000). Finally, in a controversial report, Ewen & Pustzai (1999) described changes in the gastrointestinal tract of rats fed on GM potatoes. In the light of this, we decided to analyse some of the possible effects of a diet containing GM plants.

Exocrine pancreatic function is required for the processing of food by the body. Pancreatic acinar cells have a particular role in the synthesis, storage and regulated secretion of the different digestive enzymes present in pancreatic juice. Extensive information exists on the effects of diet and pancreatic secretagogues on acinar cell components (e.g. Ermak & Rothman, 1981; Bendayan et al. 1985; Aughsteen & Cope, 1987; Grégoire & Bendayan, 1987; Malatesta et al. 1998). However, no data are available about the possible effects of GM food on pancreatic activity.

In the present study, we investigated the possible effects of a diet containing GM soybean on mouse exocrine pancreas. We have examined the pancreatic acinar cells of control and GM soybean-fed mice at the electron microscopic level and further evaluated these cells by means of morphometrical and immunocytochemical analyses.

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Materials and methods

Twelve female Swiss mice were fed *ad libitum* on a standard laboratory chow (Mulino & Frantoio del Trasimeno, Castiglione del Lago, PG, Italy) containing 14% GM soybean (Padgette et al. 1995); in parallel, 12 (control) mice were fed on the same diet with wild soybean. Both animal groups started their respective diets at weaning. The animals were weighed and then killed by cervical dislocation at 1, 2, 5 or 8 months of age. Samples of pancreas were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen phosphate buffer at 4 °C for 3 h, washed, post-fixed with 1% OsO_4 and 1.5% potassium ferrocyanide at 4 °C for 45 min, dehydrated with acetone and embedded in Epon. Other fragments were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M Sörensen phosphate buffer, pH 7.4 at 4 °C for 2 h, dehydrated with ethanol and embedded in LR White resin.

Semithin sections from Epon-embedded samples were stained with 1% toluidine blue and observed with an Olympus BX51 light microscope. Ultrathin sections from the same samples were stained with lead citrate and observed in a Zeiss EM 902 electron microscope operating at 80 kV. Morphometric analysis was carried out on Epon-embedded samples. Thirty pancreatic acinar cells were chosen from each animal, taking care that each cell contained the nucleus and zymogen granules. In order to consider homogeneous samples, the acinar cells were chosen from tele-insular regions only, since peri-insular acinar cells differ from tele-insular cells in size and zymogen content (Gingras & Bendayan, 1992). The cellular, nuclear and total zymogen granule areas were measured on toluidine blue-stained sections at a fixed magnification (×100) by means of an Olympus BX51 light microscope connected to a computerized image analysis system (Olympus DP-Soft ver. 3.0 for Windows 98). The cytoplasmic area, the nucleus/cytoplasm (N/C) ratio and the percentage of zymogen area per cell were then calculated.

In order to investigate the fine cellular distribution of α-amylase, immunocytochemical analyses were carried out on samples embedded in LR White resin. Ultrathin sections were floated for 3 min at room temperature in normal goat serum (NGS) diluted 1 : 100 in phosphate-buffered saline (PBS) and then incubated for 17 h at 4 °C with the rabbit anti-α-amylase antibody (Sigma, Buchs, Switzerland; product No. A-8273) diluted 1 : 1500 in a solution containing 0.1% bovine serum albumin (Fluka, Buchs, Switzerland) and 0.005% Tween 20 in PBS. After rinsing in PBS, sections were floated on NGS diluted 1 : 100 in PBS and then reacted for 20 min at room temperature with the goat antirabbit IgG secondary 12 nm gold-conjugated antibody (Jackson ImmunoResearch, Cardiff, UK; code 111-205-144) diluted 1 : 10 in PBS. Sections were then rinsed with PBS and bidistilled water, air-dried and finally stained with lead citrate. As controls, some grids were treated with the incubation mixture without the primary antibody and then processed as described above.

In order to assess the presence of α-amylase quantitatively, the labelling density over some acinar cell compartments was evaluated. The surface area of each compartment was measured on 16 randomly selected electron micrographs (×36 000) from each animal group by using a computerized image analysis system (Image Pro-Plus for Windows 95). The following cellular compartments were investigated: (1) rough endoplasmic reticulum (RER); (2) Golgi area, comprising the smooth vesicles, tubules and cisternae and the condensing vacuoles; (3) zymogen granules; (4) mitochondria (as negative control). The gold grains present over the compartments were counted and the labelling density was expressed as the number of gold grains per square micrometre. For each variable a two-way ANOVA test (with age and food factors) was performed. The ANOVA models included an interaction term between the factors. A correction term for multilevel design was introduced to take into account that cellular data from different animals were pooled. When necessary, data were transformed to achieve either normalization or variance stabilization, as appropriate. Significance level was fixed at $P \leq 0.05$. Moreover, the comparison between each GM-fed group and its control was calculated by Least Significant Difference (LSD) test. The Jonckheere test against ordered alternative was applied to verify the existence of a gradient of α-amylase in RER – Golgi complex – zymogen granule compartmental sequence.

At the time the mice were killed some samples of pancreas were quickly removed into ice-cold homogenization buffer (in mM): 280 mannitol, 10 KCl, 1 $MgCl_2$, 0.2 Pefabloc SC, 10 Hepes, pH 7.0 adjusted with Tris (Thevenod et al. 1999). The tissue was minced into a fine paste and homogenized manually. The crude homogenate was centrifuged at 10 000 r.p.m. for 10 min in a Microfuge 18 Centrifuge (Beckman Coulter, Inc.), total protein content was determined according to Bradford (1976) and α-amylase activity was evaluated enzymatically by

means of an Ektachem 250 Analyser (Johnson & Johnson Clinical Diagnostic).

Blood samples, also collected at the time of death, were analysed both for α-amylase as described above and for insulin by radioimmunoassay, using a kit with a sensitivity of 1 μIU mL^{-1} (BioChem ImmunoSystem Italia s.p.a., Casalecchio di Reno, Bo, Italy). Statistical comparisons were performed by the non-parametric Mann–Whitney *U*-test and the significance level was set at $P \leq 0.05$.

In order to investigate the presence in the two diets of the soybean trypsin inhibitor, a protein known to stimulate pancreatic α-amylase secretion (reviews in Gallaher & Schneeman, 1984; Birt, 2001), its presence was assessed spectrophotometrically (UV-VIS DU 640 spectrophotometer, Beckman Coulter, Inc.) by measuring at 405 nm the absorbance variation of the chromogen substrate BApNA (Kassel, 1981).

Results

Body weight of mice at the time of death ranged from 26 to 38 g, without significant differences between control and GM soybean-fed animals; moreover, no macroscopic modifications of pancreatic gland was observed.

In all mice the pancreatic acinar cells showed the typical aspect of secretory polarized cells (Fig. 1). They had the general shape of the frustum of a cone, with a base resting on a basal lamina and a narrower apical part in contact with the acinar lumen. The nuclei were roundish and lay in the basal region of the cell. The RER was abundant and arranged in parallel cisternae. The Golgi apparata were numerous and well developed. Many secretory zymogen granules were present in the apical part of the cell, approaching the acinar lumen, which was generally open. Mitochondria were generally located in the basal region of the cell and exhibited elongated shapes and well-developed cristae. Few lysosomes were present in these cells and the smooth endoplasmic reticulum appeared poorly developed.

The mean (± SE) values of the variables analysed in control and GM soybean-fed mice are shown in Table 1. Briefly, all variables analysed showed statistical significance for the complete model; however, cell and cytoplasm areas were smaller in young than in mature mice thus indicating that the differences were related to the age factor only, whereas nuclear area, N/C ratio, total zymogen area and zymogen percentage displayed differences related to the age as well as to the age–food interaction term. Finally, zymogen granule area was always smaller in GM soybean-fed mice with respect to controls and the statistical significance was related to age, food and interaction term age–food.

In pancreatic acinar cells of all mice, labelling with anti-α-amylase antibody (Fig. 2) was distributed over the RER, Golgi area and zymogen granules. Mitochondria and nuclei appeared devoid of gold grains.

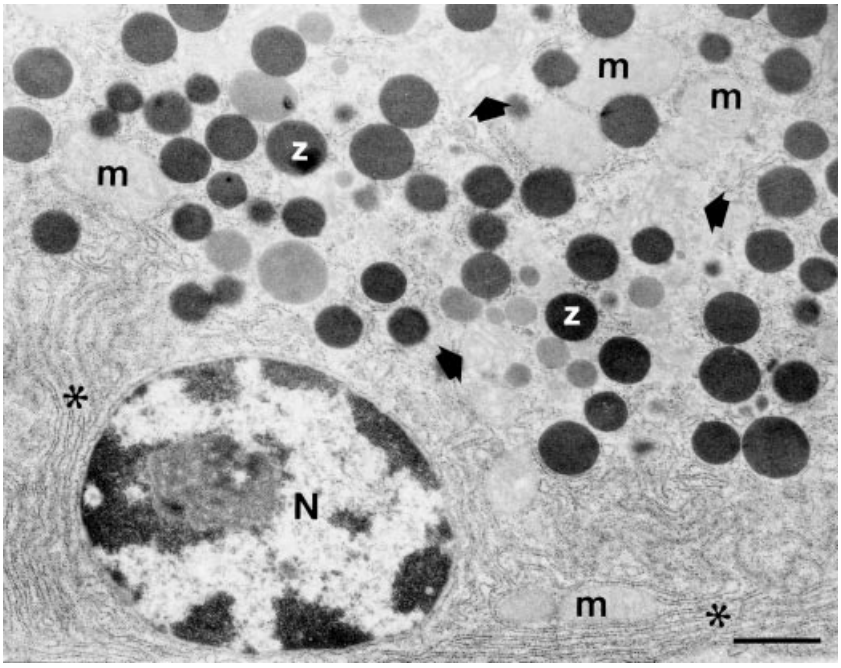

Fig. 1 Pancreatic acinar cell from a 2-month-old mouse fed on GM soybean. The cell nucleus (N) shows a roundish shape. In the cytoplasm, RER cisternae (asterisks), Golgi complexes (arrows), zymogen granules (z) and mitochondria (m) are abundant. Scale bar = 1 μm.

Table 1 Means ± SE values of variables considered in pancreatic acinar cells. Areas are expressed as μm^2. Different symbols indicate statistical significance for complete model (*), age (†), food (‡) and interaction term age–food (§). C, control animals. *P* values refer to comparison between each GM-fed group and its control, performed by Least Significant Difference test

Age	Cell area (*†)	Nucleus area (*†§)	Cytoplasm area (*†)	N/C ratio (*†§)	Total zymogen area (*†§)	Zymogen percentage (*†§)	Zymogen granule area (*†‡§)
1 month (C)	106.63 ± 3.39	18.20 ± 0.71	86.45 ± 3.12	0.25 ± 0.01	40.93 ± 2.57	46.21 ± 1.91	0.32 ± 0.02
1 month	103.48 ± 2.74	16.85 ± 0.52	86.24 ± 2.48	0.21 ± 0.01	24.47 ± 1.09	28.29 ± 0.93	0.24 ± 0.02
	P = 0.485	*P* = 0.109	*P* = 0.936	*P* = 0.018	*P* < 0.0001	*P* < 0.0001	*P* = 0.001
2 months (C)	112.97 ± 3.76	16.77 ± 0.62	95.93 ± 3.55	0.19 ± 0.01	38.03 ± 2.53	38.82 ± 1.64	0.37 ± 0.02
2 months	120.20 ± 3.56	19.19 ± 0.61	100.58 ± 3.30	0.21 ± 0.01	44.72 ± 2.12	43.94 ± 1.33	0.32 ± 0.01
	P = 0.176	*P* = 0.008	*P* = 0.344	*P* = 0.114	*P* = 0.044	*P* = 0.016	*P* = 0.069
5 months (C)	139.61 ± 5.36	21.70 ± 0.81	116.84 ± 4.94	0.21 ± 0.01	43.30 ± 2.47	37.13 ± 1.28	0.36 ± 0.01
5 months	129.48 ± 4.24	21.79 ± 0.85	106.71 ± 3.82	0.23 ± 0.01	48.53 ± 2.66	44.60 ± 1.43	0.22 ± 0.01
	P = 0.138	*P* = 0.936	*P* = 0.105	*P* = 0.182	*P* = 0.176	*P* < 0.0005	*P* < 0.0001
8 months (C)	120.61 ± 4.10	20.01 ± 1.09	99.67 ± 3.76	0.23 ± 0.01	39.30 ± 1.84	39.61 ± 1.18	0.34 ± 0.01
8 months	110.74 ± 2.41	18.21 ± 0.68	90.28 ± 2.16	0.24 ± 0.01	34.41 ± 1.29	38.39 ± 1.14	0.25 ± 0.01
	P = 0.029	*P* = 0.146	*P* = 0.022	*P* = 0.618	*P* = 0.025	*P* = 0.479	*P* < 0.0001

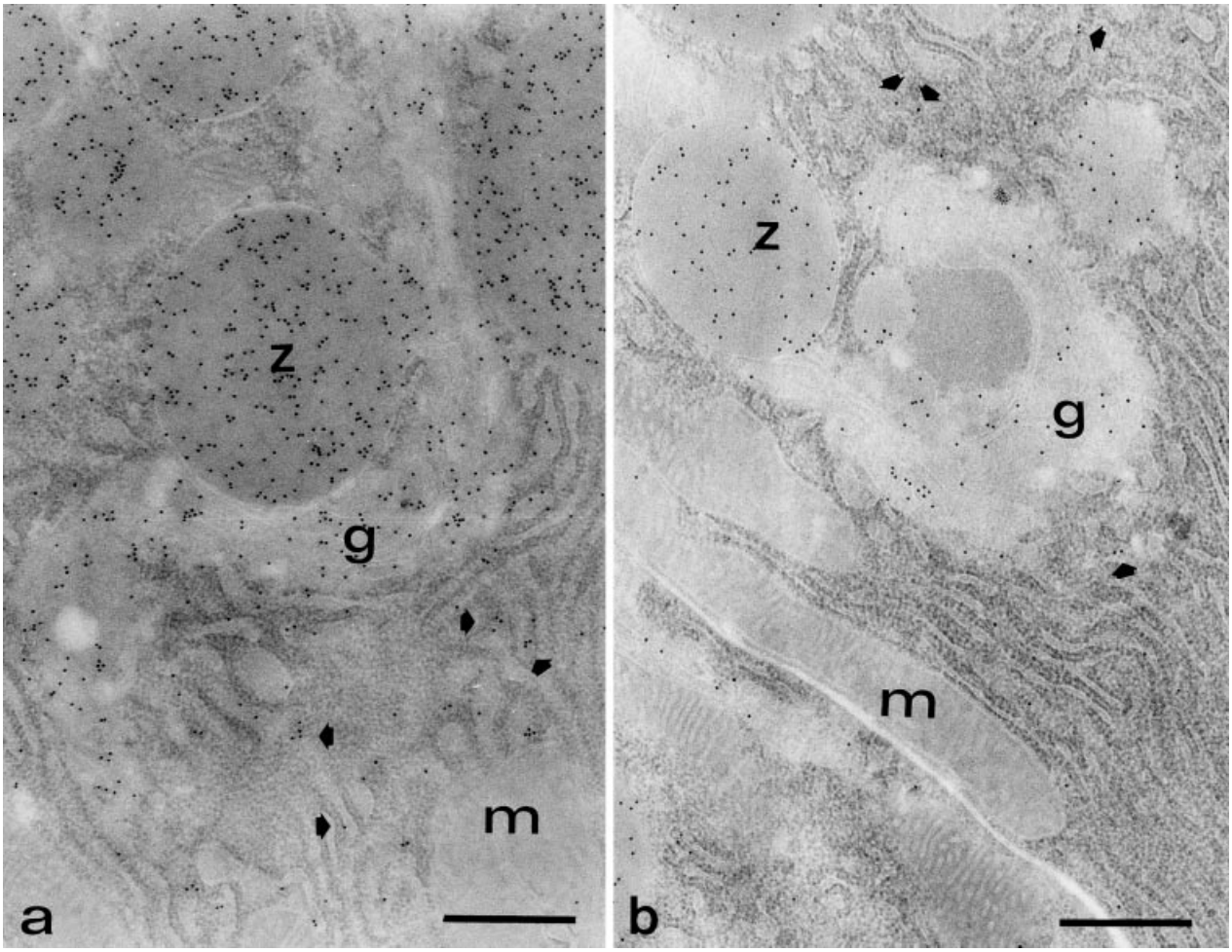

Fig. 2 Distribution of α-amylase in pancreatic acinar cells from 5-month-old control (a) and GM soybean-fed (b) mice. RER (arrows), Golgi complexes (g) and zymogen granules (z) are specifically labelled, whereas mitochondria (m) are devoid of gold grains. The labelling over all cellular compartments appears stronger in (a) than in (b). Scale bars = 0.5 μm.

Table 2 Density of labelling obtained with the anti-α-amylase antibody over various cellular compartments of pancreatic acinar cells from the eight groups of animals (gold particles μm^{-2}, mean values ± SE). Different symbols indicate statistical significance for complete model (*), age (†), food (‡) and interaction term age–food (§). C, control animals. *P* values refer to comparison between each GM-fed group and its control, performed by Least Significant Difference test

Age	RER (*†‡§)	Golgi complex (*†‡§)	Zymogen (*†‡§)	Mitochondria
1 month (C)	4.31 ± 0.50	19.23 ± 2.14	62.85 ± 0.19	0.66 ± 0.24
1 month	3.51 ± 0.34	20.87 ± 2.07	41.25 ± 2.06	0.70 ± 0.34
	P = 0.184	*P* = 0.592	*P* < 0.0001	*P* = 0.952
2 months (C)	13.22 ± 0.96	61.04 ± 4.89	116.38 ± 4.23	0.67 ± 0.21
2 months	4.03 ± 0.36	54.95 ± 4.93	94.61 ± 2.15	0.73 ± 0.33
	P < 0.0001	*P* = 0.401	*P* < 0.0001	*P* = 0.905
5 months (C)	16.93 ± 0.91	96.04 ± 5.04	124.06 ± 2.20	0.76 ± 0.26
5 months	9.05 ± 0.80	35.45 ± 4.86	108.27 ± 2.26	0.53 ± 0.23
	P < 0.0001	*P* < 0.0001	*P* < 0.0001	*P* = 0.511
8 months (C)	12.08 ± 1.12	63.86 ± 4.97	121.96 ± 2.53	0.48 ± 0.22
8 months	10.30 ± 0.57	37.94 ± 3.46	69.42 ± 3.04	0.64 ± 0.26
	P = 0.183	*P* < 0.0001	*P* < 0.0001	*P* = 0.676

Table 3 Alpha-amylase levels in pancreatic tissue and in serum of the eight groups of animals (mean values ± SE). Values identified by common symbols are not significantly different from each other. C, control animals

Age	Pancreas α-amylase (U/mg proteins)	Serum α-amylase ($U\ mL^{-1}$)
1 month (C)	41.9 ± 4.0*	2.8 ± 0.1†
1 month	39.0 ± 3.8*	3.0 ± 0.1†
2 months (C)	79.5 ± 1.7	3.0 ± 0.1†
2 months	18.6 ± 4.2	3.0 ± 0.2†
5 months (C)	81.8 ± 4.3	2.9 ± 0.1†
5 months	20.5 ± 2.1	2.9 ± 0.1†
8 months (C)	79.0 ± 2.4	3.0 ± 0.2†
8 months	31.4 ± 3.3	3.0 ± 0.1†

The results of the labelling density evaluation are given in Table 2. In summary, RER, Golgi complex and zymogen granules generally showed lower labelling densities in GM soybean-fed mice than in control animals, and the statistical significance was due to both age and food factors. However, in 1-month-old animals such differences were less marked. Because the labelling over the mitochondria was constant and significantly lower than that found in the other cellular compartments measured, it was considered as background.

The Jonckheere test demonstrated a significant increase in labelling density from the RER to the Golgi complex to the zymogen granules in all groups considered ($P < 0.05$).

The biochemical evaluation of α-amylase in pancreatic tissue (Table 3) confirmed the lower enzyme content in GM soybean-fed mice than in controls, with the exception of 1-month-old animals. On the other hand, serum levels were similar in all animals (Table 3).

Blood insulin levels (mean values ± SD) were 20.67 ± 3.45 $\mu IU\ mL^{-1}$ for control mice vs. 21.08 ± 3.99 $\mu IU\ mL^{-1}$ for GM soybean-fed mice, without significant differences between the two animal groups.

Finally, soybean trypsin inhibitor activity was similar in the two diets.

Discussion

Our observations demonstrate that, although no structural modification occurs in pancreatic acinar cells of mice fed on GM soybean, quantitative changes of some cellular constituents take place in comparison with control animals. Cellular, nuclear and cytoplasmic areas as well as N/C ratio change in relation to the age only, probably because of the postnatal development of pancreatic acinar cells (Taga et al. 1998).

On the other hand, zymogen seems to be influenced by food also. The zymogen content – both total zymogen area and the percentage of cytoplasmic area occupied by zymogen – varies in relation to the age and to the age–food interaction. In particular, in 1-month-old mice the zymogen is less abundant in GM soybean-fed than in control animals, while, subsequently, the differences become negligible, probably because of the functional maturation of pancreatic acinar cells (review in Githens, 1990). Zymogen granule size changes in relation to age and food: granules are always smaller in GM soybean-fed than in control mice. Moreover, quantitative immunocytochemical and biochemical evaluations of α-amylase, a major pancreatic protein, demonstrated that this

enzyme is generally less abundant in pancreatic tissue of GM soybean-fed than of control animals. However, serum α-amylase levels are similar in all animals.

It has been reported that the zymogen granule size differs according to the secretory activity of the cell, the stimulation of secretion inducing a decrease in size of the newly formed granules (Ermak & Rothman, 1981; Bendayan et al. 1985; Aughsteen & Cope, 1987). It could therefore be hypothesized that GM soybean-fed mice are characterized by a higher synthetic rate of zymogen granules containing lower amounts of α-amylase in comparison to control animals. The lower α-amylase content found in 1-month-old mice in comparison to older animals is probably due to the postnatal functional maturation (Githens, 1990); but our data indicate that the reduction in α-amylase synthesis and secretion is related to GM food at all ages considered.

A similar decrease in α-amylase over RER, Golgi complex and zymogen granules has been observed under starvation conditions (Bendayan et al. 1985); however, our animals always had free access to food and water. Alternatively, a significant reduction in α-amylase in pancreatic acinar cells has been reported, together with an altered gradient along the secretory pathway, under diabetic conditions (Grégoire & Bendayan, 1987). It is in fact known that insulin plays a key role in the regulation of α-amylase synthesis (Korc et al. 1981). However, in our animals an increased gradient of labelling intensity from the RER through the Golgi complex and to the zymogen granules was always found, thus indicating that no alteration of the protein concentration process along the secretory pathway takes place (Palade, 1975). Moreover, insulin levels do not differ significantly between control and GM soybean-fed mice.

A further hypothesis could be based on the dietary regulation of pancreatic activity. It is in fact known that dietary substrates alter the synthesis of their respective digestive enzymes through not well understood mechanisms (e.g. Brannon, 1990), which probably involve also the intestinal microflora (Lhoste et al. 1996). However, the levels of soybean trypsin inhibitor, a well-known protein able to increase pancreatic α-amylase secretion (reviews in Gallaher & Schneeman, 1984; Birt, 2001) and therefore the major candidate to explain the differences found in our animals, are similar in control and GM-containing food.

In conclusion, a diet containing significant amounts of GM food seems to influence the zymogen synthesis and processing in mouse pancreatic acinar cells. The reasons leading to these modifications remain unknown and further investigations are needed to understand this phenomenon.

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