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Effects of Dietary Linoleic Acid on Beta-Adrenergic Responsiveness of the Guinea Pig Respiratory System

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Abstract — Respiratory autonomic β-adrenergic receptor function was investigated in isolated tracheal spirals of guinea pigs receiving different diets. Comparison was made between control and *Haemophilus influenzae* treated animals; this latter group serving as animal model for atopy. The different semi-synthetic diets (35 energy%) varying in their linoleic acid contents (5.85, 11.25 and 22.05 en%), exerted profound effects on membrane fatty acid composition. No influence of these diets on either food intake or growth could be detected. Isoprenaline induced relaxation of guinea pig tracheal spirals was maximal in the dietary group receiving moderate linoleic acid (11.25 en%). Both the addition and the withdrawal of linoleic acid to this diet resulted in a significant impairment of β-adrenergic receptor function, to the same extent as can be induced by *Haemophilus influenzae*.

The results are discussed in view of current concepts for atopy.

Introduction

Asthma is a disease of the respiratory system, characterized by a hyperreactivity of the respiratory tract for a variety of stimuli. An imbalance between the bronchoconstrictive α-adrenergic and cholinergic receptor system and the bronchodilatory β-adrenergic receptor system may contribute to the bronchial hyperreactivity (1). We previously showed that administration of the Gramnegative bacterium *Haemophilus influenzae* to guinea pigs gave rise to responses comparable to those found in atopic patients (2–4). The predominant feature being a bronchial hyperreactivity to histamine *in vivo* (5, 6), and a decrease in number of β-adrenergic receptor binding sites in guinea pig lung. The function of the β-adrenergic receptor-adenylate cyclase system is also impaired, causing inadequate physiological responses (2–4). Recently it was postulated that the cellular defect in asthmatic patients was not located in the adrenergic or other receptors themselves but somewhere in the subsequent biochemical steps that link the activation of the receptor to the final cellular events under consideration, i.e. a defect in the stimulus-response coupling (7).

Membrane polyunsaturated fatty acids (PUFA's) are known to modulate adenylate cyclase activity, (for reviews see: 8, 9) probably by modulating membrane lipid composition. A possible role for fatty acids in atopy was already suggested in 1937 by Hansen (10), who postulated an essential fatty acid (EFA) deficiency in atopy. More recently it was suggested that not a deficiency of EFA but rather a disturbed EFA-metabolism accounted for the observed findings (11).

Membrane polyunsaturated fatty acids (PUFA's) also play an important role in asthma via their metabolites, the prostanoids and leukotrienes (12–16).

Considering that membrane fluidity, which is known to influence the β-adrenergic adenylate cyclase coupling, can be modulated by PUFA's (for reviews see: 9) and also that altered PUFA-metabolism might result in the formation of different mediators important in bronchial hyperreactivity, this study using an animal model for atopy was undertaken. It was investigated whether dietary linoleic acid is able to affect bronchial reactivity (*in vitro*) and the changes in bronchial reactivity induced by *Haemophilus influenzae* in guinea pigs. Deliberately, a moderate variance in dietary regimen under very strictly controlled experimental circumstances was chosen.

Materials and methods

Chemicals

Nembutal (pentobarbital sodium) was purchased from Abbott Laboratories, N. Chicago, Ill. USA. Carbamylcholine (carbachol), isoprenaline sulphate and atropine were from the Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. 1-[Propyl-2,3-^{3}H]-dihydro-al-prenolol (spec.act. 60 Ci/mmole) was from the Radiochemical Centre, Amersham, UK. L-Propranolol hydrochloride was a generous gift from The Imperial Chemical Industries, Macclesfield, Ches., U.K. All other chemicals used were of reagent grade.

Animals and diet

Sixty male guinea pigs weighing between 195 and 210 g [CPB-TNO, Zeist, The Netherlands] were randomly divided into 3 groups immediately after weaning. Each group was fed a semi-synthetic diet (35% of digestible energy = 35 en%) that differed in the amount of linoleic acid 18:2 (n-6). The basic dietary composition is described elsewhere (17). Composition of experimental fat is given in Table 1. The animals were put on a diet during a six week period after which they were used in the experiments. Dietary group I received low, group II moderate and group III high linoleic acid 18:2 (n-6). Fatty acids were added to the diet as vegetable oils. Sunflower seed oil being rich in linoleic acid (63%), palm oil being rich in palmitic and oleic acid. (Table 1). The semi-synthetic diets were prepared weekly for this experiment by Unilever Research Laboratory, Vlaarding-en, The Netherlands. Food was stored at 4°C, food was refreshed every other day. Water and food were given *ad libitum*.

Table 1 Vegetable Oils Added to the Basic Semi-Synthetic Diets (35 en%) to Give The Stated Amounts of Linoleic Acid

Group	*Sunflowerseed oil* %	*Palm oil* %	*18:2 en% fat*
I	5	30	5.85
II	15	20	11.25
III	35	—	22.05

Haemophilus influenzae administration

Four days before the start of the experiment 10 of the 20 animals per dietary group, their body weight (mean ± SD) being 528 ± 32 g, were injected intraperitoneally with 10^8 heat killed (30 min. 80°C, washed twice and resuspended in saline) *H. influenzae* particles per 100 g body weight; the other animals (matched pairs on weight base) received an equivalent volume of a saline (0.9% NaCl) solution. Four days later, as is described below, the *in vitro* tests were performed.

Tracheal preparation

The method used is a modification of that of Schreurs et al. (4). The animals were injected with a lethal dose of 0.1 ml/100 g b.w. of pentobarbial sodium (Nembutal) given intraperitoneally. Subsequently tracheae were removed and placed in ice cold oxygenated Krebs bicarbonate solution of the following composition: 25 mM sodium bicarbonate, 118 mM sodium chloride, 4.6 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM magnesium sulphate, 1.2 mM potassium hydrogenphosphate and 5 mM glucose.

The trachea was dissected free of connective tissue and blood vessels and subsequently cut in

a spiral fashion (7 rings per trachea) (18). The tracheal spirals were mounted into 7.5 ml organ baths and connected to isotonic transducers (Harvard Bioscience) under 0.8 g tension. Changes in lengths were recorded (Servogor SE 120, linear scale recorder, Brown Boveri, Austria), and paper length was calibrated to tracheal relaxation. After equilibration (30 min.), tracheal spirals were precontracted with 10^{-7} M carbachol (standardization of tissue tone) and relaxation upon cumulative doses of isoprenaline were measured. All drugs were added in distilled water.

Lung membrane preparation

The method used is a modification of that described earlier (2, 3). Immediately after removal of the tracheae, lungs were flushed twice through the pulmonary artery with 30 ml Krebs bicarbonate and dissected free of major bronchi. One half of the lung was homogenized in 30 ml of an ice cold 50 mM Tris-HCl buffer (pH 7.8) using an Ystral homogenizer (Ystral GmbH, Dottingen, GDR.) at setting 8 for 10 seconds. This homogenate was filtered through a single layer of cheese cloth and subsequently centrifuged at 60.000 *g* for 20 min. at 4°C. The final pellet was resuspended in ice cold Tris buffer at a concentration of about 1.6 mg/ml and stored at −80°C. Protein was assayed according to Lowry (19).

Receptor binding assay

The method used to assay β-adrenergic binding sites was described earlier (4), minor modifications were made. Deep frozen (−80°C) lung homogenates were thawed at 37°C and resuspended using the Ystral homogeniser setting 8 for 5 sec. in ice. Lung membranes were incubated in a final assay volume of 1 ml, that contained approximately 500 μg protein with increasing concentrations of the tritiated β-adrenergic receptor antagonist dihydro-alprenolol (0.2–5.0 nM). Aspecific binding was determined in presence of 1 μM L-propanolol. Incubations were carried out at 37°C for 20 min. and were terminated by rapid dilution with 5 ml ice cold Tris-HCl buffer (50 mM, pH 7.8), followed by rapid filtration through Whatman GF/B glass fiber filters. The filters were washed with another 5 ml ice cold Tris-HCl buffer. The radioactivity bound to the filters was counted in 3 ml scintillation fluid (Picofluor TM3, Packhard Indstr. Co. Ill., USA) using a liquid scintillation counter, (LKB, Sweden) at an efficiency of 33%. Specific binding was defined as total reactivity bound minus aspecific binding (i.e. in presence of propanolol), and it represented 95–98% of the total binding. The affinity of binding (Kd) and the maximal number of binding sites (Bmax) were determined according to Scatchard (20), using least square regression analysis.

Fatty acid determination

After the flushing and the dissection procedure, the other half of the lung was immediately frozen in liquid nitrogen and stored at −80°C, until fatty acid composition could be determined.

Tissue lipids were extracted with chloroform-methanol (1:2, v/v). Tissue lipid extracts were then separated by two dimensional thin layer chromatography (toluene/heptane, 50/50; ether/heptane, 80/20) into cholesteryl ester, triglyceride and total phospholipids (TPL) (21). Phospholipid fractions were methylated (2 hr, 4% methylchloride in methanol, 70°C) and the composition of fatty acid methylesters were analysed by gas-liquid chromatography using a HP 5700 (DEGCS column, 100–190°C).

Statistical analysis

Significance of concentration-response curves were tested using a two way Anova. Other statistical analyses were performed using Students' t-test. Results were expressed as mean ± standard error of the mean.

Results

Weight gain (Fig. 1) and food intake (results not shown) of all animals (n = 60) was identical during the experiment.

Fatty acid composition of the lung membranes (Table 2) varied considerably between the dietary groups. Significant differences were present in all fatty acids with exception of 16:0 and 22:4 (n-6). Marked differences between dietary group I and the other dietary groups are found in 20:4 (n-6), 20:3 (n-6) and in two fatty acids that are not derived from the essential fatty acids namely 20:1 and 22:3 (n-9). Dietary group III differed from all other dietary groups in the 16:1; 18:0, 18:3 and in the 20:5 (n-3) content. All dietary groups differed significantly from each other in the 18:1, 18:2 (n-6) as well as in the 22:5 content.

Saturated/unsaturated fatty acid ratios (S/U

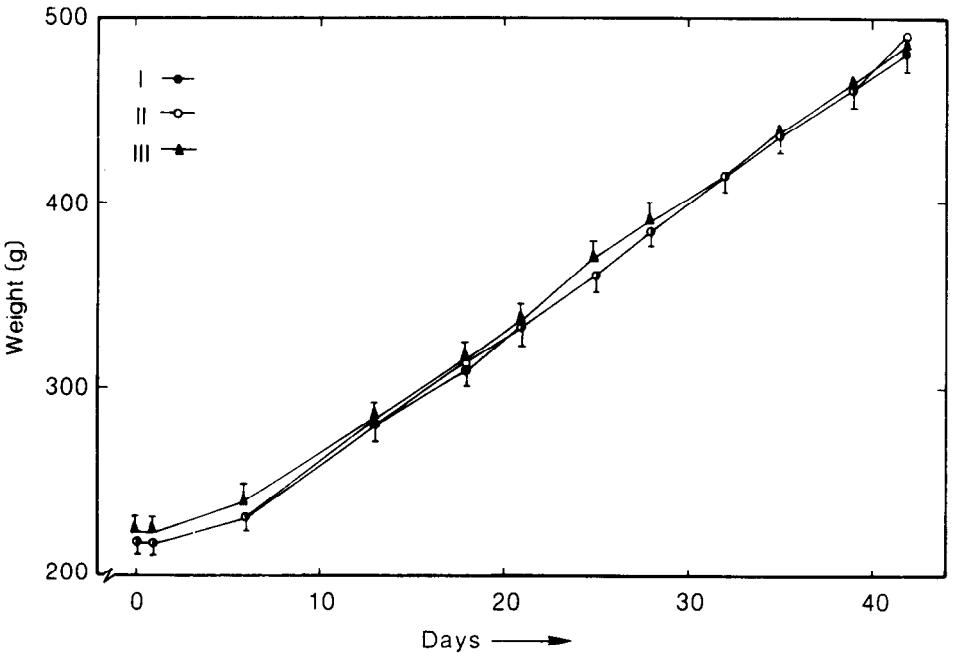

Fig 1 Weight Gain of the Guinea Pigs in the Three Dietary Groups. Each point is the mean weight (in gram per day) of 20 animals ± standard error of the mean. Dietary group I (•), group II (○), and group III (▲).

Table 2 Fatty Acid Composition of Lung Membranes of Guinea Pigs Fed Diets that Differed in the Amount of Linoleic Acid. (meanvalues in % of total phospholipid ± SE, n=10)

Fatty acid	*Diet I*	*Diet II*	*Diet III*	*Significance*
14:0	3.0 ± .1	3.3 ± .2	2.6 ± .1	b*,c*
16:0	42.9 ± 1.0	45.1 ± .8	43.0 ± .8	
16:1	3.6 ± .1	3.5 ± .1	2.9 ± .1	b*,c*
18:0	6.4 ± .2	6.1 ± .2	8.1 ± .2	b*,c*
18:1	15.6 ± .4	13.5 ± .3	10.7 ± .1	a*,b*,c*
18:2 (n–6)	7.6 ± .3	9.8 ± .3	13.3 ± .6	a*,b*,c*
18:3 (n–6)	.10 ± .01	.10 ± .01	.18 ± .01	b*,c*
18:3 (n–3)	.26 ± .02	.24 ± .02	.32 ± .01	b,c
20:1	.47 ± .03	.33 ± .02	.31 ± .02	a*,b*
20:3 (n–9)	.68 ± .05	.84 ± .05	1.30 ± .05	b*,c*
20:3 (n–6)	.47 ± .03	.41 ± .03	.42 ± .01	a ,b*
20:4 (n–6)	5.6 ± .2	4.9 ± .1	4.6 ± .1	a*,b*
22:1	.30 ± .01	.23 ± .02	.20 ± .00	a ,b*
20:5 (n–3)	.20 ± .01	.23 ± .02	.37 ±.02	b*,c*
22:3	.16 ± .02	.11 ± .01	.09 ± .02	a*,b*
22:4 (n–6)	2.7 ± .1	2.6 ± .1	2.6 ± .1	
22:5 (n–6)	3.5 ± .1	2.7 ± .1	2.2 ± .1	a*,b*,c*
22:5 (n–3)	.76 ± .04	1.00 ± .05	1.98 ± .05	a*,b*,c*

a : dietary group I vs. dietary group II $P< 0.05$ a* $P< 0.01$
b : dietary group I vs. dietary group III $P< 0.05$ b* $P< 0.01$
c : dietary group II vs. dietary group III $P< 0.05$ c* $P< 0.01$

Table 3 Saturated/Unsaturated and Polyunsaturated/Monounsaturated Fatty Acid Ratios of the Membranes of Guinea Pigs Fed Diets The Differed in Linoleic Acid Composition.

Dietary group†	S/U ratio	P/M ratio
I	1.25	1.06
II	1.34	1.35
III	1.29	1.93

† Composition of the dietary fats is given in Table 1.

ratio) and polyunsaturated/monounsaturated fatty acid ratios (P/M ratio) are given in Table 3.

After an equilibration period of 30 min. tracheal spirals were precontracted with 10^{-7} M carbachol, subsequently tracheal relaxations upon cumulative doses of isoprenaline were measured. Tracheal spirals of saline injected animals showed a maximal isoprenaline relaxation in group II. This concentration response curve (diet II) is located significantly below the curves of dietary group I ($P<0.05$) and dietary group III ($P<0.01$). Tracheal spirals of *H. influenzae* treated animals did not behave differently between the dietary groups. In dietary group II, administration of *H. influenzae* resulted in a significantly ($P<0.01$) decreased responsiveness to isoprenaline (Fig. 2), this responsiveness was identical to that observed in the other dietary groups after *H. influenzae* administration. Experiments were repeated twice with 60 different guinea pigs. Results (not shown) were similar: The largest relaxation upon isoprenaline administration and a significant decrease after administration of *H. influenzae* was always found in the dietary group receiving moderate (11.25 en%) linoleic acid.

The specific binding of ^{3}[H]-dihydro-alprenolol to guinea pig lung membranes was saturable and reached a plateau between 3 and 5 nM. Scatchard analysis resulted in a mean equilibrium constant of 1.1 and did not differ between the different dietary groups. Administration of *H. influenzae* had no effect on the equilibrium constant (Table 4). Maximal binding capacity (Bmax) of saline injected animals did not differ significantly between the dietary groups. Administration of *H. influenzae* resulted in a significant ($P<0.05$) decrease in the total number of binding sites in dietary group II.

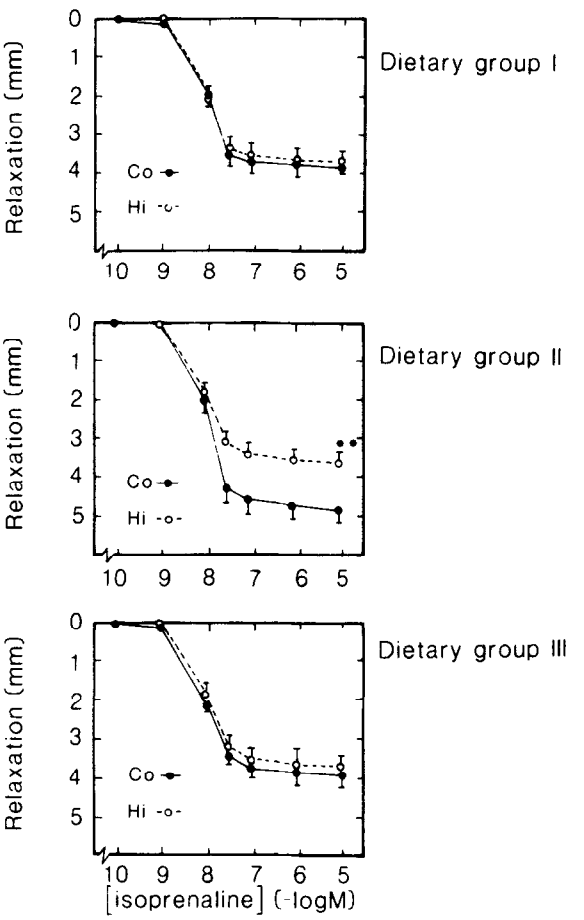

Fig 2 Cumulative Dose Response Curves to Isophrenaline in Isolated Tracheal Spirals of Guinea Pigs Receiving Different Diets in Normal [solid lines, closed symbols] and Stimulated (H. influenzae treated) Conditions [dotted lines, open symbols]. Each point is expressed as the mean ± standard error of the mean of 10 animals.
** $P< 0.01$ *H. influenzae* vs. control of the same diet.

Table 4 Maximal Number of Beta-Adrenergic Binding Sites (Bmax) of Guinea Pig Lung Membranes and the Affinity of the Receptors (Kd).

Dietary group†		β-adrenergic receptors Bmax	Kd	
I	saline	601 ± 32	.96± 23	(9)
	H.i.	634 ± 23	1.17 ± .20	(10)
II	saline	656 ± 39	1.21 ± .40	(9)
	H.i.	554 ± 29*	1.03 ± .30	(9)
III	saline	653 ± 25	1.14 ± .21	(9)
	H.i.	642 ± 38	1.12 ± .30	(9)

† see Table 2 for composition of dietary fatty acids.
* $P<0.05$ *H. influenzae* vs. control within the same dietary group.

Discussion

Since Szentivanyi (1) proposed his *Bordetella pertussis* concept for atopy, animals injected with bacterial cells are widely used to provide a model for this syndrome. In our laboratory the Gramnegative bacterium *H. influenzae* is used instead. Administration of *H. influenzae* seemed a better, clinically more relevant model for human atopy because *H. influenzae* is one of the most common bacteria found in the lower respiratory tract of patients with chronic asthmatic bronchitis (22–25). Administration of the bacteria to guinea pigs resulted in effects commonly observed in atopic patients. The predominant feature being a hyperreactivity to histamine *in vivo* (5) and a diminished tracheal β-adrenergic receptor responsiveness (2–4). Recently it was suggested that the small decrease in β-adrenergic receptor function, observed in guinea pigs after *H. influenzae* administration, was responsible for the observed hyperreactivity for histamine *in vivo* (6, 26).

The present study shows that dietary linoleic acid affects the normal (saline injected animals) airway responsiveness of guinea pig tracheae to the β-agonist isoprenaline. *H. influenzae* administration to animals receiving moderate linoleic acid (dietary group II) showed an effect on β-adrenergic receptor responsiveness comparable to that described (2, 3) for animals receiving ordinary laboratory chow (Hope Farm, Woerden, The Netherlands); a significant ($P<0.01$) decreased isoprenaline responsiveness of guinea pig tracheae in *H. influenzae* injected animals as compared to control animals and a concommittant fall in the total number of β-adrenergic receptor binding sites in peripheral lung ($P<0.05$).

However, not only administration of *H. influenzae* to the animals could affect the β-adrenergic responsiveness of guinea pig tracheal spirals, addition or withdrawal of linoleic acid in the diet also resulted in a significant ($P<0.05$) decrease of the β-adrenergic receptor responsiveness in saline-injected animals. The decrease induced by linoleic acid was comparable to but not synergistically with the decrease induced by *H. influenzae* treatment. Down regulation of β-adrenergic receptors might be limited and the maximal effect determined, as was suggested previously (4).

The effects were not due to differences in weight between the dietary groups since both weight gain and food intake (results not shown) did not differ between the dietary groups (Figure 1). It was noted in 1937 (10) that patients with atopic eczema had reduced levels of plasma EFA; an EFA deficiency was postulated in atopy. More recently it was shown that both plasma levels and red blood cells from those patients had elevated concentrations of linoleic acid and low levels of its metabolites, suggesting that atopy is associated with altered EFA metabolism (11). This hypothesis is substanciated by recent findings showing differences in fatty acid spectra in polymorphonuclear and mononuclear cells from patients with allergic rhinitis and asthma (27).

As in most tissues in animals in a non-pathological state the only members of the (n-6) family to accumulate in relatively large quantities are 18:2 (n-6) and 20:4 (n-6), much lower levels of the intermediates 18:3 and 20:3 (n-6) are detected (Table 3). The polyunsaturated acyl chains 22:4 (n-6) and 22:5 (n-6) are also present in significant amounts. Unlike Lokesh et al. (28), who could only detect 22:5 in lung microsomes of guinea pigs fed a Menhaden oil rich diet and not in control animals, we found considerable amounts of 22:5 (n-3) especially in dietary group III.

Surprisingly the arachidonic acid content of dietary group I, the dietary group receiving low linoleic acid, was significantly increased as compared to the other groups. The tendency of a decreased arachidonic acid content in dietary groups receiving increasing concentrations of linoleic acid content was also observed by Croft et al. (29). This indicates that at some point a balance is reached between increased availability of linoleic acid for conversion to arachidonic acid, increased inhibition of the delta-6-desaturation and chain elongation system and competition for incorporation into phospholipids.

No differences could be detected in the amount of 16:0 being present. Palmitic acid (16:0) is abundant in lung membranes, dipalmitoylphosphatidylcholine being the major phospholipid component, and the phospholipid largely responsible for surface (surfactant) activity (30). Similar results with respect to 16:0 were found by Alam and Alam (31) in rat lung membranes.

Moderate variations in guinea pig dietary PUFA's not only exert profound and complex effects on lung metabolism, but modifications in dietary linoleic acid can also modulate the β-

adrenergic receptor responsiveness of guinea pig tracheal spirals to isoprenaline. A bell shaped correlation between these differences in tracheal responsiveness and linoleic acid was observed in the fatty acid profiles of the membranes of animals on different dietary regimens. The β-adrenergic receptor responsiveness was maximal at moderate dietary linoleic acid contents. This result was found to be reproducible in two other (independent) experiments (results not shown). Bell shaped dose-response curves for linoleic acid are commonly observed, for instance in immune responses (for review see: 32). Further investigations of the role of linoleic acid metabolites in atopy seem useful. Especially since it was demonstrated that considerable amounts of 9-hydroxylinoleic acid are formed in guinea pig tissues even under non-stimulated conditions (33).

Results of an enhanced β-adrenergic functionality are often explained as an increased fluidity of the membranes. The polyunsaturated/monounsaturated fatty acid ratio (P/M ratio) however, was intermediate in dietary group II. The saturated/unsaturated fatty acid ratio (S/U ratio) was even increased in the dietary group showing maximal β-adrenegic responsiveness (dietary group II). A discrepancy in ratios was reported previously and suggests that there is no direct correlation between these parameters and membrane fluidity (34). Although encounter between the β-adrenergic receptor and other components of the adenylate cyclase system requires mobility of the receptors relative to the other components in order to produce activation, the receptor need not necessarily be the mobile partner, or if it is need not move over large (μm) distances. Nevertheless, data concerning the influence of lipids as a whole seems to suggest that the β-adrenergic receptor whose function depends solely upon the interactions with other membrane proteins (guanyl nucleotide regulatory protein and the catalytic moiety of adenylate cyclase; for reviews see 35, 36) can exercise optimal function only in an ordered membrane (37). Structural and spatial order seem to be more important than fluidity for the function of membraneous multicomponent systems, although viscosity changes may result from structural changes and vise versa (for review see; 38). This study, using well controlled dietary conditions, clearly showed that modulation of the linoleic acid composition of the diet can lead to alterations in the airway responsiveness of guinea pigs. The impairment of β-adrenergic receptor function due to less optimal linoleic acid conditions is comparable to that resulting from *H. influenzae* treatment, an animal model for atopy.

Therefore results substantiate evidence that alterations in linoleic acid metabolism might be associated with atopy. Dietary correction of the EFA-abnormality in eczema was associated with clinical improvement (39). This study indicates that it might be possible to devise nutritional strategies helpful in forms of atopy other than eczema.

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