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Modulation of Binding Characteristics of Peripheral Benzodiazepine Receptors in Vitamin A-Deficient Guinea Pig Lung

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ABSTRACT. Both vitamin A and peripheral benzodiazepine receptors (PBRs) are involved in the control of cell proliferation and differentiation. The objective of this study was to determine whether vitamin A deficiency causes any modulation in the binding characteristics of the PBRs. Forty-five weanling guinea pigs were divided into three groups (control, pair-fed control, and vitamin A-deficient). Vitamin A-deficiency status was achieved after 90 days of feeding. It caused atelectasis, hyperplasia and metaplasia of alveolar epithelium, acute inflammation of the tracheobronchial tree, and a significant increase in the number of alveolar type II cells. In comparison to the control and pair-fed control groups, the lung of vitamin A-deficient animals had 40.6 and 42.8% less PBR density, respectively. There was no significant difference in the equilibrium dissociation constant (K_D) of PBRs between the control and the pair-fed control groups, whereas the K_D value was 77.7 and 60% higher in the vitamin A-deficient group than in the control and the pair-fed control groups, respectively. Furthermore, vitamin A deficiency caused a decrease in the binding capacity of PBRs in both nuclear and mitochondrial fractions. These results may suggest a close functional relationship between vitamin A and PBRs. BIOCHEM PHARMACOL 51;9:1203–1209, 1996.

KEY WORDS. guinea pig; lung; vitamin A; peripheral benzodiazepine receptors; diet; ligand binding

Vitamin A contributes to the maintenance of the structural integrity of the respiratory mucosa by controlling the proliferation and differentiation of epithelial cells [1], and it plays a key role in vertebrate development [2]. Retinoids, in general, are important regulatory signaling molecules for cell growth and differentiation during fetal and adult life [3]. Retinol deficiencies or excesses induce embryonic malformations in several mammalian species, including humans [4, 5]. Moreover, retinoids can inhibit or reverse the process of malignant transformation in some cell types [6]. They are also important regulatory factors of humoral and cell-mediated immune function [7]. An association between the risk of impaired lung function [8, 9] and an increase of epithelial cancer and vitamin A deficiency has been well established [10, 11].

We have reported recently the presence of high density PBRs† in lung [12] and alveolar type II cells [13], which are involved in surfactant synthesis and secretion [14]. Recent reports also suggest that drugs selective for PBRs cause a pleiotropic spectrum of pharmacological actions, including stimulation or inhibition of cell proliferation and differentiation [15, 16], chemotaxis [17], alteration of immune function and protooncogene expression [15, 18, 19], inhibition of mitogenesis and growth of thymoma cells [20], malignancy [16, 20, 21], ion transport and mitochondrial respiration [22]. We reported earlier that there is a substantial increase in the concentration of PBRs in the nucleus of malignant submandibular glands of rats, suggesting a possible role of PBRs in nuclear events [21]. Recent evidence indicates that there is a close relationship between thyroid receptors and PBRs [23, 24]. It is also evident that vitamin A treatment is beneficial to the hyperthyroidic state due to down-heteroregulation of thyroid receptors by RA [25]. It is not known whether there is any functional relationship between retinol and PBRs.

Therefore, the present study was undertaken to determine whether vitamin A deficiency causes any modulation of the binding characteristics of PBRs in lungs, information that will be useful in understanding the molecular mechanism of action of vitamin A. Guinea pigs were selected as the animal model since in the developmental aspect their lungs appear more similar to human lungs than do the lungs of other species [14, 26].

MATERIALS AND METHODS

Materials

All biochemicals were purchased from the Sigma Chemical Co., St. Louis, MO. [^{3}H]Ro 5-4864 (86.4 Ci/mmol) was

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† *Abbreviations:* PBRs, peripheral benzodiazepine receptors; RA, retinoic acid; and RARs, retinoic acid receptors.

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purchased from New England Nuclear Research Products, Boston, MA. Non-radioactive Ro 5-4864 was donated by Dr. Peter F. Sorter, Hoffmann-La Roche, Nutley, NJ.

Animals

Weanling male guinea pigs (Hartley strain), weighing 200 ± 20 g, were obtained from Camm Research Laboratories, Wayne NJ. Forty-five animals were divided into three groups (control, pair-fed control, and vitamin A-deficient) after an acclimatization period of 3 days. The animals were housed in individual stainless steel cages in a temperature-controlled (26°) 12:12 hr light:dark cycle room. They were fed a diet prepared by Teklad, Madison, WI (control diet TD 93145, vitamin A-deficient diet TD 93187). The vitamin A-deficient diet was similar to the control diet except that vitamin A palmitate was omitted (Table 1). All animals had free access to drinking water. Feed consumption was recorded daily, and the animals were weighed every other day. Control animals and vitamin A-deficient animals were fed *ad lib*. Animals of the pair-fed control group received control diet in the same amount as was consumed by the vitamin A-deficient animals on the previous day. After reaching the growth plateau indicating the beginning of deficiency, all animals were killed at day 90 of experimentation. Lung lavage was performed as described earlier [27], and plasma, liver, lung, and lung lavage were collected for retinol analysis.

Morphological Examination

A portion of the lungs from each guinea pig in a set was inflation-fixed *in situ* with neutral buffered 10% formalin, pH 7.4, at an infusion pressure of 25 cm of water pressure. After overnight fixation at room temperature, 4-mm mid-sagittal sections were processed for embedding in paraffin. Five-micron sections were stained with hematoxylin and eosin, and the stained sections were examined from 10 to 15 fields under a light microscope. A linear calibrating scale was photographed and printed along with the photographs for accurate calculations of the final magnification in the photographic prints.

TABLE 1. Diet composition for guinea pigs

Ingredients	Amount (g/kg)
Soy assay protein	212.0
L-Cysteine	2.0
L-Methionine	2.0
Sucrose	300.2
Corn starch	240.0
Corn oil	70.0
Vitamin mix, Teklad (40060)	10.0
Ascorbic acid, coated (97%)	2.4
Choline dihydrogen citrate	0.7
Vitamin A palmitate (500,000 U/g)	0.01
Folic acid	0.01
Mineral mix	60.9

The diet was prepared by Teklad, Madison, WI. The control diet (TD 93145) and the vitamin A-deficient diet (TD 93187) were similar in all respects, except that vitamin A palmitate was omitted in the deficient diet.

Isolation of Alveolar Type II Cells

Type II cells were isolated by digestion of lung with elastase and purified by centrifugal elutriation as previously described [14]. In brief, animals were anesthetized by intraperitoneal injection of pentobarbital containing heparin. The chest cavity was opened and lungs were perfused by gravity with 2.6 mM phosphate-buffered saline containing 5.6 mM glucose. Following perfusion, the intact lungs were removed from the thorax, and lavaged five times with the perfusion solution. Lungs were instilled with a solution of elastase via a cannula inserted into the trachea, and the perfused lungs were immersed in a solution of 145 mM NaCl for 20 min at 37°. Then lungs were minced with scissors, mixed with fetal bovine serum, and filtered sequentially through cotton gauze, 100 and 20 μm nylon mesh. Next, the cell suspension was centrifuged at 200 *g* for 10 min. The cells were washed and suspended in Hanks' balanced salt solution, pH 7.4, containing 1 mg/mL bovine serum albumin. With the rotor spinning at 2000 rpm (385 *g*) the cell suspension was injected into the mixing chamber and then pumped into the separation chamber of the rotor at a flow rate of 10 mL/min. After all the samples were in the separation chamber, they were eluted by a stepwise increase in flow rate. The fraction collected between 22 and 26 mL/min represented homogeneous type II cells. For cellular identification, the sample was stained with modified Papanicolaou stain, and the percentages of type II cells were estimated with a fluorescent compound, phosphine 3R, in a Carl Zeiss Standard 16 microscope [14].

Subcellular Fractionation

Lungs were excised, washed in ice-cold saline (0.9% NaCl), blotted dry, and weighed. Then lungs were minced with scissors, homogenized in 10 vol. of 0.25 M sucrose, 1 mM EDTA (pH 7.4), in a Potter-Elvehjem homogenizer and then passed through a cheesecloth. The homogenate was centrifuged at 200 *g* for 10 min in a refrigerated Beckman TJ-6 centrifuge to remove any tissue debris. The supernatant fraction was centrifuged at 600 *g* for 10 min to sediment the nuclear fraction. The postnuclear supernatant was centrifuged at 10,000 *g* for 10 min in a refrigerated Sorvall RC-5 centrifuge using an SS-34 rotor to obtain the mitochondrial fraction. The supernatant fraction was centrifuged in a Beckman L8-M ultracentrifuge at 105,000 *g* for 60 min using a 70 Ti rotor to obtain the microsomal fraction. Nuclear, mitochondrial, and microsomal fractions were washed twice successively with 1.15% KCl and 0.1 M Tris–HCl, 1 mM EDTA (pH 7.4), and then suspended in a known volume of the same buffer. All fractions were stored at −70° until analyzed.

Receptor Binding Assay

Lungs were placed in ice-cold 50 mM Tris–HCl buffer (pH 7.4) and homogenized by using a Brinkmann Polytron (setting 6–7, 15 sec). The homogenate was centrifuged at 200 *g* for 10 min in a refrigerated Beckman TJ-6 centrifuge to remove any tissue debris. The supernatant fraction was then centrifuged at 20,000 *g* for 20 min (4°) using an SS-34 rotor, and the pellet was washed three times and resuspended in 50 mM Tris buffer, pH 7.4 (~1 mg membrane protein/mL). The binding of [^{3}H]Ro 5-4864 to the crude cell membrane fraction, as well as to each subcellular fraction, was determined as described earlier [12]. The assay was done in a volume of 1 mL containing 0.1 mL radioligand (0.5 to 32 nM), 0.1 mL Ro 5-4864 (5 μM) (or buffer), 0.7 mL assay buffer (Tris–HCl, 50 mM, pH 7.4), and 0.1 mL membrane or subcellular fraction. The reaction was initiated by the addition of either whole lung membrane fraction or individual subcellular fraction and incubated at 0–4° for 60 min. Then the reaction was terminated by rapid filtration over Whatman GF/B strips using a Brandel M-24 R filtering manifold (Brandel Instruments, Gaithersburg, MD), with two 5-mL washes of ice-cold buffer. The specific binding of [^{3}H]Ro 5-4864 was defined as the difference in binding obtained in the presence and absence of Ro 5-4864 (5 μM). The radioactivity retained by the filters was measured in a Beckman LS 355 liquid scintillation spectrometer, using 8 mL Universal Cocktail (ICN Radiochemicals, Irvine, CA) as a scintillant. Scatchard analysis and determination of binding constants were performed according to Bennet [28].

Biochemical Assay

In plasma and lavage samples, retinol was determined by the spectrofluorometric method of Thompson *et al.* [29]. Total retinol in the liver and lungs was assessed by the same method after alkaline hydrolysis [29]. Thus, the values represent a sum of free retinol and retinol present in the form of retinyl esters in these tissues. Protein concentrations were determined according to Peterson [30]. DNA content of lung was measured by the method of Burton [31].

Statistical Analysis

Data were analyzed statistically using Student's *t*-test [32]. The variation of data is presented as means ± SEM. Differences at $P < 0.05$ were considered significant.

RESULTS

There were no significant differences in either food intake or growth and development among the three groups up to day 62 of feeding (data not shown). From day 63 onward, acute vitamin A-deficiency symptoms started to develop in the experimental group of animals; these symptoms included loss of body weight associated with loss of appetite (feed efficiency was 0.25, 0.25, and 0.13 in the control, pair-fed control, and vitamin A-deficient group, respectively), prominence of blood vessels in the external ear, puffy appearance in the facial region with coarse body fur, eye infections, and eventually blindness. No such changes were observed in other groups. Vitamin A-deficiency status was confirmed by depletion of retinol in plasma, lung, and lung lavage, as well as liver, in the experimental group (Table 2).

Histological studies of lung indicated that in control diet fed animals the vascular endothelial and smooth muscle cells were normal, and the alveoli contained mostly alveolar macrophages and an occasional polymorphonuclear leukocyte (Fig. 1). The vitamin A deficiency caused morphological changes and enlargement of airspaces with destruction of alveoli (arrow, Fig. 1, center panel), showing evidence of lung injury (emphysema). Furthermore, there was atelectasis (small arrows, Fig. 1, bottom panel) and metaplasia (large arrow, Fig. 1, bottom panel) of alveolar epithelium accompanied by acute inflammation of the tracheobronchial tree.

The weight of the lung was increased markedly in the vitamin A-deficient group (0.41, 0.54, and 0.70 g/100 g of body weight for control, pair-fed control, and vitamin A-deficient animals, respectively), whereas there was no remarkable difference in liver weights between the groups (data not shown). We also observed an increase in the number of type II cells in lung of vitamin A-deficient animals ($20.2 \pm 4.2 \times 10^6$, $8.2 \pm 2.2 \times 10^6$, and $5.8 \pm 3.0 \times 10^6$

TABLE 2. Effects of vitamin A deficiency on retinol levels in plasma, lung lavage, liver, and lung of guinea pigs

	Retinol levels in:			
Group	Plasma (μg/100 mL)	Lung lavage (ng/mg protein)	Liver (μg/g)	Lung (μg/g)
Control	70.0 ± 1.6	154.9 ± 10.1	163.2 ± 4.5	0.5 ± 0.01
Pair-fed control	48.5 ± 0.9	93.6 ± 7.1	178.7 ± 2.4	0.4 ± 0.01
Vitamin A-deficient	4.7 ± 0.8	55.5 ± 4.2	1.0 ± 0.1	0.1 ± 0.01

Values are means ± SEM of 15 animals. Control and vitamin A-deficient groups were fed *ad lib*. The pair-fed group received control diet in the same amount as was consumed by the vitamin A-deficient group on the previous day. All animals were killed at day 90 of experimentation.

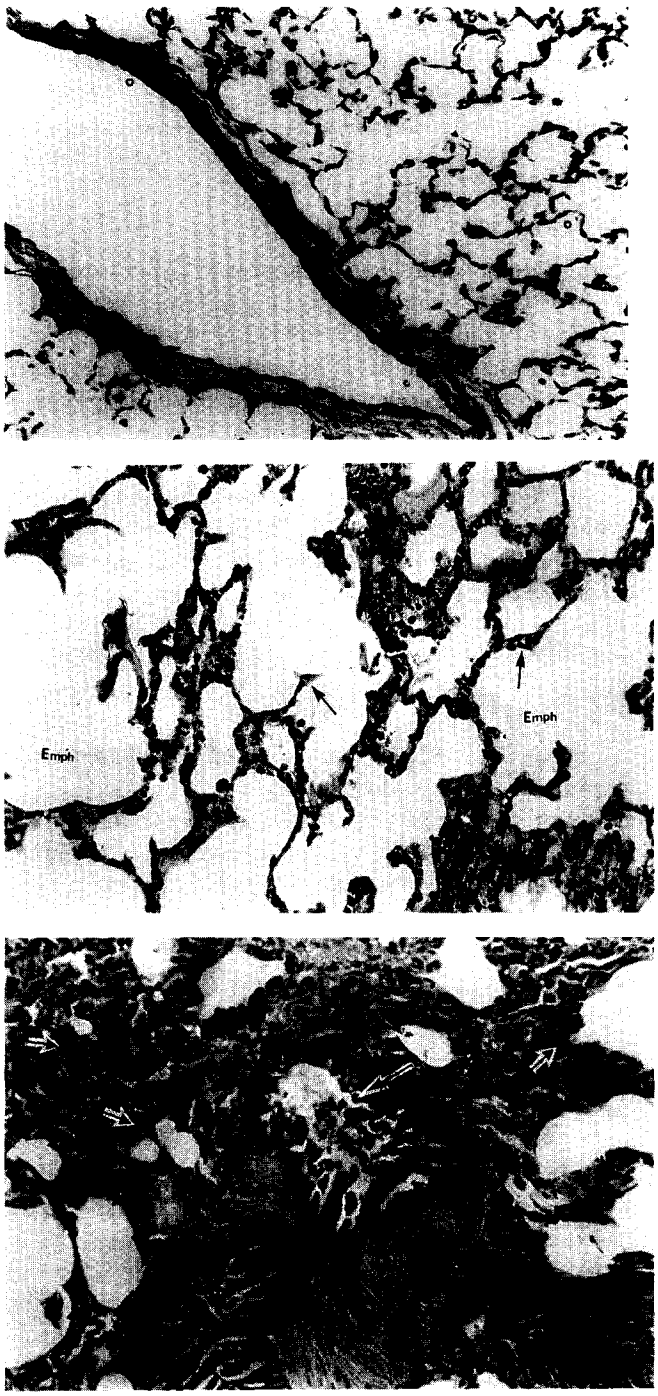

FIG. 1. Top panel: section from lungs of a guinea pig fed control diet. The vascular endothelial and smooth muscle cells were normal as were the mucosal and muscular layers of the bronchial tree. Center and bottom panels: light microscopic structure of vitamin A-deficient guinea pig lung (400×, hematoxylin and eosin). Center panel: section shows morphological changes and enlargement of air-spaces (emphysema) with destruction of alveoli (arrow). Bottom panel: section shows atelectasis (small arrows) and metaplasia (large arrow) of alveolar epithelium accompanied by acute inflammation of the tracheobronchial tree.

cells/g lung in vitamin A-deficient, control, and pair-fed control animals, respectively). In conjunction with an increase in the number of type II cells, there was an increase in the DNA content of lung due to vitamin A deficiency. For example, the control, pair-fed control, and vitamin A-deficient animals contained 11.9, 13.7, and 19.1 mg DNA/g lung, respectively.

To evaluate the effects of vitamin A deficiency on the binding characteristics of PBRs, the binding of [^{3}H]Ro 5-4864 to lung membrane fraction was determined. Scatchard plots for [^{3}H]Ro 5-4864 binding to control, pair-fed control, and vitamin A-deficient animals are shown in Fig. 2. The data on the binding characteristics (Table 3) indicate that there was no difference in the equilibrium dissociation constant values (K_D) between the control and pair-fed control groups; however, the vitamin A-deficient group had 77.7 and 60% higher K_D values than the control and the pair-fed control group, respectively. Furthermore, in comparison to the control and pair-fed control animals, the vitamin A-deficient animals had a 40.6 and 42.8% decrease in the maximal number of binding sites (B_{max}), respectively, when the results were expressed on a per milligram protein basis. However, if we normalize the data on the basis of DNA content, the B_{max} value for the deficient animals was decreased by 63 and 39%, respectively, in comparison to the control and pair-fed control group.

The binding capacity of [^{3}H]Ro 5-4864 to whole homogenate and different subcellular fractions of lung of control, pair-fed control, and vitamin A-deficient animals is illustrated in Table 4. Mitochondria had the highest ligand binding capacity of all the groups.

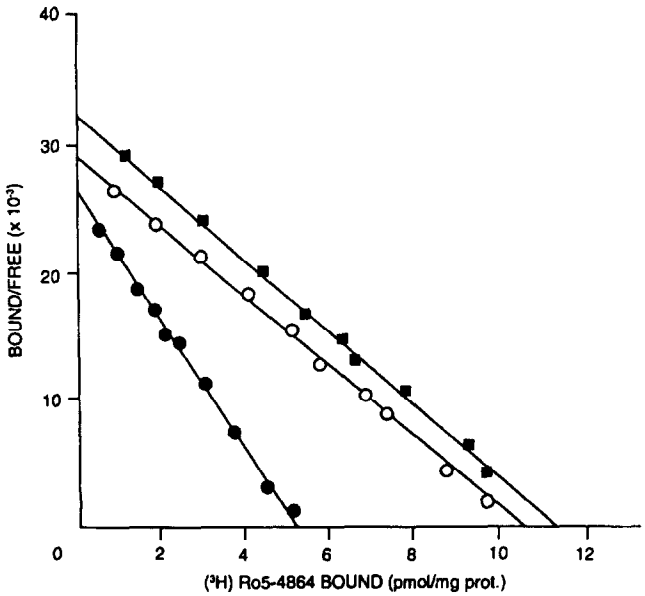

FIG. 2. Scatchard analysis of [^{3}H]Ro 5-4864 binding to guinea pig lung membranes. Data shown are representative of five independent experiments. Bound = pmol of specifically bound radioligand per mg protein. Free = total concentration of radioligand added in the incubation medium—specifically bound radioligand per mg protein. Key: (■—■) control group (C), (○—○) pair-fed group (PF), and (●—●) vitamin A-deficient group (E). The regression line (γ = 0.97 for both C and PF; and γ = 0.98 for E) indicates K_D values of 1.6, 0.94, and 1.03 nM and B_{max} values of 11.4, 10.6, and 5.2 pmol/mg for C, PF, and E groups, respectively.

TABLE 3. Effect of vitamin A deficiency on [^{3}H]Ro 5-4864 binding to guinea pig lung membrane

Group	K_D (nM)	B_{max} (pmol/g lung)	B_{max} (pmol/mg DNA)
Control	0.9 ± 0.1	1215 ± 32	102.1 ± 2.6
Pair-fed control	1.0 ± 0.1	1261 ± 12	92.1 ± 0.8
Vitamin A-deficient	1.6 ± 0.2	721 ± 28	37.8 ± 1.5

Values are means ± SEM of 15 experiments. There was no difference in the binding affinity (K_D) between the two control groups; however the K_D for the deficient group was significantly higher than the values in either the control or the pair-fed control group ($P < 0.05$). The binding capacity (B_{max}), measured in pmol/g lung, was reduced significantly in vitamin A-deficient animals ($P < 0.001$).

DISCUSSION

The observed weight loss due to vitamin A deficiency appeared to be mostly due to anorexia. The cessation of body weight gain is known to be a reliable indicator of vitamin A-deficiency status [33, 34]. Vitamin A deficiency caused morphological changes in the lung without any sign of malignancy. It is known that human neonates who have low serum retinol levels develop chronic respiratory distress, chronic lung disease, and bronchopulmonary dysplasia [35]. Furthermore, it has been reported by others for rat [36] and guinea pig [37] that in marginal vitamin A deficiency, there is a uniform basal cell hyperplasia with definite widening of the intracellular spaces and an increase in intracellular cytofilaments, which are typical signs of beginning metaplasia [38]. It should be noted that these lesions in the respiratory system precede well known consequences of retinol deficiency in visual processes [39].

Vitamin A deficiency caused a significant increase in the number of alveolar type II cells. Alveolar type II cells play an important role in lung injury and repair because they serve as the stem cells for both type II cell proliferation and differentiation into type I cells; the population of these cells changes dramatically with alveolar injury [40]. In fact, Zachman *et al.* [41] also observed slightly increased type II cells in marginal vitamin A-deficient rats. It is known that type II cells from adult rat lung have the ability to synthesize both retinyl palmitate and RA, which are important for stem cell proliferation and differentiation into type I cells [41].

Vitamin A deficiency caused a significant decrease in the binding capacity of PBRs in lung. The relationship between an increase in epithelial cancer and vitamin A deficiency has been well established [10, 11]. Recent reports suggest that malignant tissues contain higher levels of these receptor sites compared with nonmalignant tissues [42]. Katz *et al.* [43] observed a significant increase in the receptor density in the neoplasm compared with benign ovarian tumors and normal tissues, without a concomitant change in affinity values. They also observed that the PBR density was lower in the benign ovarian tumors than in normal ovaries. Thus, our present finding in vitamin A-deficient lung correlates with that of benign ovarian tumors. It remains to be seen whether the PBR density is increased in the vitamin A-deficient animals developing cancer by exposure to carcinogens.

The decrease in PBR density in the mitochondria due to vitamin A deficiency may result from a decrease in the number of mitochondria as well as down-regulation of receptor density. It was reported earlier that mitochondrial density decreased significantly in goblet cells of vitamin A-deficient lung [37]. The nuclei of the vitamin A-deficient lung had 50% lower PBR density than that of the control lung (Table 4). However, we previously reported that the nuclei of malignant submandibular gland have a higher density of PBRs than nuclei of normal submandibular gland [21].

Several benzodiazepine derivatives have been shown to bind with both alpha- and beta-RARs and thereby inhibit

TABLE 4. Effect of vitamin A deficiency on [^{3}H]Ro-4864 binding to PBRs in subcellular fractions of guinea pig lung

Fractions	Control			Pair-fed control			Vitamin A deficient		
	Specific binding (pmol/mg protein)	Total binding (pmol/g tissue)	Distribution of receptors (%)	Specific binding (pmol/mg protein)	Total binding (pmol/g tissue)	Distribution of receptors (%)	Specific binding (pmol/mg protein)	Total binding (pmol/g tissue)	Distribution of receptors (%)
Homogenate	1.7 ± 0.1	181.3 ± 3.0		1.5 ± 0.1	178.5 ± 5.4		0.8 ± 0.1	110.9 ± 7.4	
Nuclear	3.2 ± 0.1	33.4 ± 0.3	18.4	2.1 ± 0.1	30.8 ± 0.4	17.2	1.4 ± 0.1	16.0 ± 0.6	14.4
Mitochondrial	7.0 ± 0.3	131.9 ± 2.2	72.7	5.5 ± 0.2	120.0 ± 3.2	67.7	2.5 ± 0.2	68.3 ± 3.1	61.6
Microsomal	1.6 ± 0.1	9.5 ± 0.4	5.2	1.1 ± 0.1	8.5 ± 0.7	4.9	1.0 ± 0.1	4.6 ± 0.1	4.1

Values are the means ± SEM of four different experiments. In each experiment, tissues were taken from either 3 or 4 animals. Binding capacity was measured at a ligand concentration of 4 nM. The binding capacity of subcellular fractions of lung was decreased significantly ($P < 0.01$ to 0.001) in the vitamin A-deficient group when compared with the control and pair-fed control group.

the differentiation of HL-60 cells [44]. Kato *et al.* [45] reported that retinol depletion causes a significant decrease in beta-RAR mRNA levels in various tissues (maximum effect in lung) without any significant effect on either alpha- or gamma-RAR mRNA levels. It has been suggested by Curran and Morgan [18] that PBRs facilitate expression of protooncogene c-*fos* by nerve growth factors, which are also stimulated by retinoic acid [46], whereas RARs antagonize the activity of cellular oncogene c-*jun*/c-*fos* [47], which are well known to stimulate cell proliferation leading to cancer. The metabolic interaction between RARs and c-*jun*/c-*fos* leads to inhibition of c-*jun*/c-*fos* activities. This inhibition is strongly dependent on the concentration of RARs and is affected significantly by the cellular levels of RAR expression.

Since RA affects the expression of RARs in tissues (maximum effects in lung) of retinol-deficient rats [47], it may be that the decreased level of retinol in lung of vitamin A-deficient guinea pigs leads to a lower level of RARs, thereby activating the oncogene c-*jun*/c-*fos*, and these actions are facilitated by the decreased number of PBRs. Therefore, vitamin A actions on cell proliferation and differentiation are mediated by modulating the levels of several retinoid receptors that belong to the steroid hormone receptor superfamily [48] and/or by modulating the levels of PBRs; this could open up a novel molecular mechanism of action of the micronutrient vitamin A. To explore the precise mechanisms responsible for the suggested changes reported in this study, we must also consider the age at which these animals were studied. There are significant changes in the densities of lung receptors to many hormones and neurotransmitters during maturation, especially at puberty. Thus, differences in receptor profiles could also be due to effects of vitamin A on these maturational events.

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