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MITOCHONDRIAL ACTIVITY AND CYTOTOXICITY OF VITAMIN A (RETINOL) IN YEAST AND HUMAN CELL CULTURES

PROTECTIVE EFFECT OF ANTIOXIDANTS

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Abstract—Vitamin A inhibited the growth of yeast and human cells in a dose-dependent but selective manner in cultures utilizing a non-fermentable carbon and energy source. At sub-inhibitory concentrations in yeast cultures (~100 μg/mL), the vitamin had a stimulatory effect on the mitochondrial system, foreshortening the lag phase in the adaptation to non-fermentable substrate. At inhibitory concentrations, vitamin A depressed mitochondrial protein synthesis relative to cytoplasmic protein synthesis and induced the mitochondrial mutation *petite* but had little or no mutagenicity with respect to nuclear genes at the concentrations used. The vitamin showed a dose-dependent cytotoxicity (lethality) in both yeast and human cells. All of these deleterious effects were overcome to a large extent by the presence of antioxidants implicating free-radical metabolites in much of the toxicity.

Vitamin A is a factor in the proliferation and differentiation of animal cells but the underlying mechanisms are complex and largely unknown. The compound can also suppress growth and show manifest toxicity if administered in excessive amounts. Emphasis has been placed on the membranolytic properties of retinol [1] leading to the conclusion that reaction with membrane systems may be the basis of much of the cellular effects. At physiological concentrations, stabilization/promotion of membrane function may explain the beneficial effects, at least in part, while excessive intake may labilize membranes with deleterious results. An example of the latter may be seen in the report that hypervitaminosis A in the rat leads to aberration of mitochondrial membrane structure [2]. Following up this finding, *in vitro* studies reported herein indicate that mitochondria are primary targets of the vitamin.

The existence of an inducible microsomal system for the oxidative metabolism and degradation of vitamin A has also been reported in the literature [3]. In the present study an assessment is made of the extent to which free radical metabolites may be involved in retinol toxicity.

MATERIALS AND METHODS

Yeast

Haploid strains of the yeast *Saccharomyces cerevisiae* from the authors' laboratory were used. These organisms are eukaryotic with mitotic and meiotic cycles and with mitochondria similar to those of mammalian cells in all fundamental respects.

Media. Medium contained 1% yeast extract (Difco) and either 2% glucose (YED) or 4% glycerol (YEG) as respective carbon and energy sources. For solid medium, 2% agar (Difco) was added.

Chemicals. Vitamin A (retinol), glutathione and vitamin E (*dl*-α-tocopherol) were obtained from the Sigma Chemical Co. (Poole, U.K.). Vitamin C (L-ascorbic acid) was obtained from BDH (Poole, U.K.). Stock solutions of glutathione and vitamin C were made up in water, and vitamin A and vitamin E in ethanol.

Growth studies. Growth curves were obtained from shake cultures using an EEL colorimeter to measure turbidity at suitable intervals.

Absorption spectra. Cells were grown to stationary phase in YED liquid medium, washed and resuspended in cuvettes at a high concentration (approx. 10^9 cells/mL). Recordings were made in a Beckman DU-7 spectrophotometer.

Toxicity. Cell death was used as the criterion. This was measured as the number of colonies that developed expressed as a percentage of cells plated on YED medium.

Mutation studies. Cells were treated with vitamin A at various concentrations for 2 hr in YED medium, washed and plated. The frequency of the well-known mitochondrial mutation to respiratory deficiency known as *petite* was scored by plating the cells on YED medium. The high spontaneous frequency of this mutation (about 10^{-2} in most strains) and the readily recognizable small white colonies into which *petite* cells develop makes scoring unambiguous. In a number of cases, verification of the *petite* phenotype of colonies was obtained by velvet pad transfer to the non-fermentable (YEG) medium on which the respiratory deficient cells cannot grow.

Nuclear mutation was scored as reversal of methionine auxotrophy in strain 6–81 by plating treated and untreated cells on a synthetic medium lacking methionine and counting the number of colonies that appeared. The spontaneous frequency

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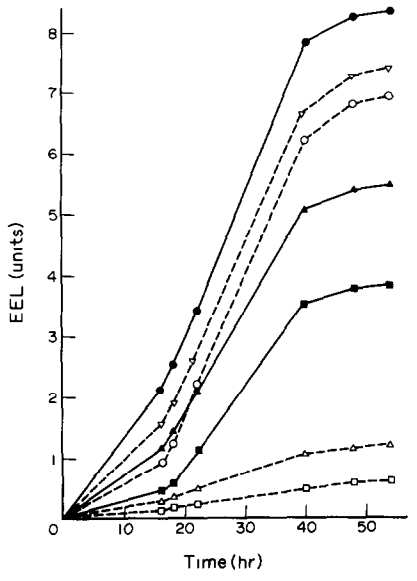

Fig. 1. Inhibition of growth of yeast cells (strain D4) by vitamin A at high concentration (1 mg/mL) in glucose medium (YED) and glycerol medium (YEG) respectively and the protective effect of glutathione (GSH, 10 mM): stimulatory effect of low concentration of vitamin A (0.1 mg/mL) in YEG medium. Key: solid lines and symbols, YED cultures; broken lines and open symbols, YEG cultures. (●,○) Controls; (■,□) vitamin A (1 mg/mL); (▽) vitamin A (0.1 mg/mL); (▲,△) vitamin A (1 mg/mL) + GSH.

of reversal to prototrophy in this methionine-requiring strain is about 10^{-6}.

Effects of antioxidants. Vitamin A and antioxidants were added together at time zero to shake cultures. Aliquots were withdrawn at appropriate intervals, plated on YED medium and viability and *petite* frequency scored. Turbidity measurements during growth of cultures were used to construct growth curves.

Human cells

Skin fibroblasts from a normal adult female were routinely cultured in Eagle's Essential Minimal Medium (Flow Laboratories, Irvine, U.K.) containing Earle's salts supplemented with 0.03% sodium bicarbonate, 10% foetal bovine serum, 20 mM Hepes, 2 mM L-glutamine, 100 units % penicillin and 100 μg % streptomycin. Glucose (2%) or glutamine (2 mM) were added as respective carbon and energy sources to the above medium which was obtained free of added glucose from Flow Laboratories. Cells were inoculated into polystyrene flasks at a seeding density of approx. 5×10^4/mL. Cells were incubated overnight to allow attachment before addition of vitamin A. Toxicity was measured as cell death which was indicated firstly by alteration in morphology to a rounded form, secondly by counting the number of cells which had become detached into the medium and finally by suspending all cells following trypsinization, washing, resuspending and assessing viability by the standard test of trypan blue (0.4%) staining.

Table 1. Toxicity and mitochondrial mutagenicity of vitamin A (vit A, 4 mg/mL) in yeast cells: protective effect of glutathione (GSH, 10 mM), vitamin E (vit E, 5 mM) and vitamin C (vit C, 10 mM) (at these concentrations of antioxidants, maximum protection was achieved)

Treatment	Viability (%)	*Petite* (%)
Control	87.9 ± 0.3	1.2 ± 0.2
vit A	7.8 ± 1.8	18.1 ± 0.6
vit A + GSH	60.2 ± 2.1	7.9 ± 0.7
vit A + vit E	41.3 ± 2.4	8.6 ± 0.9
vit A + vit C	53.1 ± 1.7	8.5 ± 0.5

Cells of strain D4 were treated for 2 hr in the presence and absence of vit A with and without the antioxidants in glucose-containing medium (YED), washed and plated on YED medium. Results are the average of four separate experiments with standard deviations.

RESULTS AND DISCUSSION

Yeast

The effect of vitamin A at concentrations ranging from 0.1 to 1.0 mg/mL on the growth of haploid yeast strains D4, D18 and D75 in fermentable (YED) and non-fermentable (YEG) medium was investigated. A typical series of growth curves with strain D4 using a high and a low concentration are shown in Fig. 1. In the YEG medium it can be seen that the vitamin at low concentration had a stimulatory effect foreshortening the lag phase. Although small, this effect was a consistent feature in repeat experiments with all three strains. In the glucose-containing medium a stimulatory effect was not detected at the low concentration in any of the three strains and growth curves were little or no different from controls. At the high concentration, vitamin A inhibited growth in YED medium by about 60% but inhibited almost totally in the non-fermentable medium. Similar results were obtained with strains D18 and D75. The presence of the vitamin in excess of 0.2 mg/mL inhibited the growth of the three strains in a dose-dependent manner but, again, cells growing in the YEG medium were more sensitive than cells in YED medium. These results indicated that vitamin A primarily affected mitochondrial function promoting adaptation to the mitochondrial substrate at low dose and inhibiting its utilization at relatively high doses. It is also apparent from Fig. 1 that the inhibitory effects were to some extent reversed by the presence of glutathione, an aspect investigated in greater depth in subsequent experiments.

Toxicity and mitochondrial mutagenicity. Yeast cells treated with retinol at comparatively high concentrations ranging from 0.5 to 4 mg/mL showed loss of viability (toxicity) and increased rate of mutation to the *petite* condition in a dose-dependent manner. The possibility that the increase in the mitochondrial mutation among surviving cells was

Table 2. Comparative mutagenicity of vitamin A (4 mg/mL) in the nucleus and the mitochondrion in the yeast strain 6–81

Treatment	Viability (%)	Nuclear mutation per 10^7 viable cells	*Petite* (%)
Control	83.6 ± 0.3	19 ± 1.2	1.1 ± 0.2
vit A	9.2 ± 2.7	23 ± 3.1	16.1 ± 1.8

Results are the average of three experiments with standard deviations. Approximately 1000 colonies were scored in each case for viability and *petite* frequency. A total of approximately 10^8 untreated cells and 10^9 treated cells were plated in petri dishes containing selective medium in scoring nuclear mutation. See Materials and Methods for details of procedure.

due to a selective advantage of *petite* cells was discounted in tests which showed that spontaneous *petite* mutants were not less susceptible to lethal damage than normal cells. Results with strain D4 using a high concentration of the vitamin are recorded in Table 1. Exposure was of 2 hr duration but treatment for 4 hr did not alter significantly the effects produced. From the table it can be seen that the presence of the antioxidants glutathione, vitamin E and vitamin C reversed significantly both toxicity and *petite* induction ($P = <0.01$ in each case). In these experiments, a range of antioxidants up to 50 mM was used but maximum efficiency was achieved at the concentrations listed in Table 1. These results indicate that the cellular activity of vitamin A at relatively high concentrations was due in large measure to the production of toxic free radicals. When these were removed by the free-radical scavengers the residual activity of the vitamin was due presumably to the reactivity of the intact retinol molecule. The findings would seem to preclude the possibility that the antioxidants detoxified the vitamin by reacting directly with it in the medium since it would be expected that the greater the amounts of antioxidants the less the activity of the vitamin. The antioxidants also protected against the toxicity and mitochondrial mutagenicity in strains D18 and D75 but to a different degree in each case. This is not unexpected in view of the variation in our strains in their inherent ability to produce the mixed function oxidase cytochrome P450 mainly responsible for the metabolism of toxic compounds [4].

The results are recorded in Table 2 of a comparative study of the mutagenic effects of vitamin A on mitochondria and nucleus, respectively. The increase in nuclear mutation was not significant while the frequency of the *petite* mutation was increased about 15-fold. It must be pointed out that whereas reversion to prototrophy is due to forward mutation in a nuclear gene, the mechanism of the *petite* mutation is not fully understood. The heritable change apparently arises from a flaw in the replication of the yeast mitochondrial chromosome leading to extensive deletions in the circular DNA molecule.

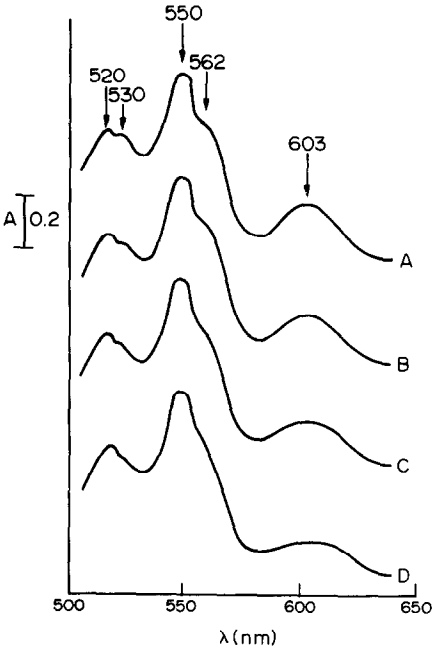

Fig. 2. Absorption spectra of yeast cells (strain D4): inhibitory effect of vitamin A on the synthesis of cytochromes aa_3 and *b* relative to cytochrome *c* and the protective effect of GSH. α-Peaks of cytochromes *c*, *b* and aa_3 occur at 550, 562 and 603 nm, respectively, and β-peaks of *c* and *b* at 520 and 530 nm, respectively. (A) Control; (B) vitamin A (0.2 mg/mL); (C) vitamin A (1 mg/mL) + GSH (10 mM); (D) vitamin A (1 mg/mL).

Table 3. Toxicity of vitamin A in human cell cultures utilizing glucose and glutamine as respective carbon and energy sources: protective effect of the antioxidants glutathione and vitamin E

Treatment	Viability (%)	
	Glucose medium	Glutamine medium
Control	96.6 ± 0.2	96.4 ± 0.4
vit A (50 μg/mL)	20.4 ± 2.9	13.5 ± 4.6
vit A (100 μg/mL)	8.3 ± 0.9	3.3 ± 0.7
GSH	97.5 ± 0.2	95.9 ± 0.4
vit E	97.3 ± 0.1	98.7 ± 0.3
vit A (50 μg/mL) + GSH	83.7 ± 4.5	41.3 ± 3.0
vit A (50 μg/mL) + vit E	76.6 ± 3.3	31.7 ± 4.9
vit A (100 μg/mL) + GSH	42.1 ± 4.4	16.4 ± 7.5
vit A (100 μg/mL) + vit E	36.3 ± 7.6	10.3 ± 4.1

A minimum of 1000 cells was scored in each culture. Concentrations of GSH and vit E were 2.5 and 5 mM, respectively. At these concentrations of antioxidants maximum protection was obtained. Results are the average of three separate experiments with standard deviations.

Compounds reacting directly or indirectly with DNA replication will usually increase the frequency of the aberration, sometimes dramatically [5]. Although lacking a respiratory system, *petite* cells can grow in

glucose-containing medium since the organism is a facultative anaerobe with cellular requirements for ATP being met by glycolysis in the absence of oxidative phosphorylation. This attribute is useful in identifying drugs with primary anti-mitochondrial activity: a mitochondrial inhibitor will preclude growth when the energy source is a mitochondrial substrate such as glycerol but would permit growth in glucose medium.

Absorption spectra. Further evidence of selective anti-mitochondrial activity was obtained from absorption spectra of cells grown in glucose medium to stationary phase in the presence of relatively high concentrations of vitamin A. Tracings of spectra are shown in Fig. 2 in which it can be seen that the vitamin depressed the synthesis of cytochromes aa_3 and *b* while the synthesis of cytochrome *c* was unaffected. The conclusion of selective inhibition of the mitochondrial system follows from the fact that cytochrome *c* is coded by a nuclear gene and is synthesized on cytoplasmic ribosomes whereas aa_3 and *b* are coded by mitochondrial genes and translated on mitochondrial ribosomes. Since the vitamin induces the *petite* condition it is more likely to be inhibiting organellar protein synthesis at the level of transcription than interfering directly with the function of mitochondrial ribosomes. The inhibitory effect was largely overcome by the presence of supplementary glutathione again implicating free radicals as a major factor.

Human cells

Toxicity (lethality) of vitamin A was assessed at concentrations ranging from 10 to 100 μg/mL. Degree of toxicity was dose-related and results of treatment at concentrations of 50 and 100 μg/mL are recorded in Table 3 in fermentable and non-fermentable medium, respectively. It can be seen that the vitamin is more toxic in the latter medium, particularly at the higher concentration. This selective effect was apparent in the first instance on microscopic investigation of cultures in which a change in morphology to a rounded form (impending cell death) occurred in less than 24 hr in the glutamine medium but appeared later in the glucose medium. These results indicated that mitochondria were primary targets of the vitamin placing cells utilizing glutamine under stress sooner than cells in which the glycolytic pathway was operative. Compounds known to have primary anti-mitochondrial activity behave in this way whereas cellular inhibitors such as cyloheximide do not discriminate against cultures utilizing glutamine [6]. In the experiments with yeast cells, the evidence for primary effects of vitamin A on mitochondria is strong particularly with respect to mitochondrial biogenesis but is circumstantial in the human cultures. Extrapolation from yeast to humans is controversial but the yeast cell is a good model of the eukaryotic cell with mitotic and meiotic cycles and mitochondria similar in all essential features to those in mammalian cells: respiratory chain, ATPase complex, TCA cycle, antibiotic-sensitive ribosomes, an intrisic circular chromosome with virtually the same complement of genes. Furthermore, the mitochondrial inhibitor that only affects yeast mitochondria is not known.

The role of mitochondria in the activity of vitamin A is not clear at this point either in a beneficial or deleterious context. Blockage of the organelle *per se* could arrest the growth of human cells and perhaps this aspect of the anti-proliferative action may be a factor in the use of the vitamin in cancer chemotherapy, an application currently under test. If toxic amounts of vitamin A are administered in cancer treatment it may by useful to consider administering antioxidants at the same time assuming that free radical metabolites are the main cause of toxic side effects while anti-mitochondrial activity of the vitamin molecule is responsible for the anti-cancer effect. The possible usefulness of anti-mitochondrial agents in the treatment of cancer is discussed by us elsewhere [7].

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REFERENCES

1. Roels OA, Anderson OR, Lui NST and Shah DO, Vitamin A and membranes. *Am J Clin Nutr* **22**: 1020–1032, 1969.
2. Goodhall AH, Fisher D and Lucy JA, Cell fusion, haemolysis and mitochondrial swelling induced by retinol and derivatives. *Biochim Biophys Acta* **595**: 9–16, 1980.
3. Leo MA and Lieber CS, New pathways for retinol metabolism in liver microsomes. *J Biol Chem* **260**: 5228–5236, 1985.
4. King DJ, Wiseman A and Wilkie D, Genetic regulation of cytochrome P-450 in *Saccaromyces*. *Mol Gen Genet* **192**: 466–470, 1983.
5. Bernardi G, The petite mutation in yeast. *Trends Biochem Sci* **4**: 197–201, 1979.
6. Patel R, Hola M, Riley PA and Wilkie D, Detection of antimitochondrial activity of drugs in cultured mammalian cells utilizing glutamine as the carbon and energy source. *Exp Cell Biol* **52**: 176–182, 1984.
7. Wilkie D, Antimitochondrial drugs in cancer chemotherapy. *J Roy Soc Med* **72**: 599–601, 1979.