Enhanced Mitochondrial Functions During Hepatocarcinogenesis[1] (35889)

W. L. Banks, Jr., J. J. Terz, and E. S. Higgins

Department of Biochemistry and Division of Surgical Oncology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23219

Augmentation of metabolic coupling of oxidation to phosphorylation in liver mitochondria resulted from treatment of rats with the hepatotoxins, hydrazine or ethanol, or by 67% partial hepatectomy (1). *N*-2-Fluorenylacetamide (2-FAA) produced hepatomas in male Fischer rats following long-term dietary administration of the compound (2). Since carcinogenesis with 2-FAA proceeds also via progressive liver damage, respiratory control ratios (RCR), ADP/O, and phosphorylation rates (PR) were determined in rat liver mitochondria during carcinogenesis resulting from long-term ingestion of this compound. Elevation in respiratory control and phosphorylation rate coincided with liver enlargement. Histologically, the liver showed hyperplastic nodules or areas of regeneration, which suggested that the enhanced mitochondrial functional activities might be a consequence of regeneration or hyperplasia rather than neoplasia. Increased mitochondrial efficiency of energy retention may be a property common to early stages of hepatic neoplasia, as well as to other forms of liver damage.

Materials and Methods. Male Fischer rats were divided into six groups each containing four animals. The initial body weights of control and experimental groups were 239 ± 4 and 233 ± 3 g, respectively. Experimental rats were fed a diet of Purina rat meal with 0.05% *N*-2-fluorenylacetamide (Aldrich Chemical Company) added for a period of 19 weeks; thereafter, the animals were fed the rat meal only. Control groups received Purina rat meal throughout the experimental period. After 41, 45, and 51 weeks, groups of control and experimental animals were anesthetized with sodium pentobarbital (60 mg/kg of body wt) and sacrificed by exsanguination. Two of the carcinogen-fed rats died during the experiment. The livers were removed, representative samples were taken and the mitochondrial fractions were prepared immediately (3).

Respiratory rates were determined polarographically with glutamate as substrate by the method described previously (4). Conventions used in naming respiratory states, respiratory control and ADP/O ratios are those of Chance and Williams (5). Phosphorylation rates (nmoles of ATP synthesized min^{-1} mg^{-1} mitochondrial protein) were estimated by the method of Ozawa *et al.* (6). Mitochondrial protein concentration was determined by a biuret method (7) in the presence of 1% deoxycholate with crystalline bovine serum albumin as standard.

Liver DNA and protein concentrations were estimated on another sample of liver by the methods described by Wannemacher *et al.* (8) following the extraction procedure recommended by Munro and Fleck (9). At the time of sacrifice, a third sample from the livers was fixed in 10% formalin and 4-μ paraffin sections were prepared and stained using hematoxylin and eosin.

A two-tailed Student's t test was used to ascertain significance and a value of $p < .05$ was accepted as the level for significance (10). Means and standard errors of the means (SEM) for each comparison between an experimental and a control group are indicated in Table I; comparisons between experimental groups or between control groups are indicated in the text.

Results. Even though the control and experimental groups of animals were the same size at the start of the experiment, the

[1] Supported in part by an award from the Bureau of Alcohol Studies and Rehabilitation, Department of Health, Commmonwealth of Virginia.

presence of the carcinogen in the diet resulted in lower body weights than those of controls at the three points examined (Table I). Liver size, as measured by actual wet weight or as a proportion of the body weight, was two- to fourfold greater in the three experimental groups than in their respective controls. Relative liver weights (g/100 g of body wt) in the experimental groups were progressively elevated ($p < .05$) at the 45- and 51-week points in comparison to the 41-week level. There was no significant difference in liver DNA concentration between the experimental and control groups at any of the three points examined. Protein concentrations (mg/g of liver) and protein/DNA ratios also were not significantly altered at any of the three points due to carcinogen in the diet. However, the total liver DNA and protein contents in the experimental groups were elevated ($p < .05$) over the control groups at each point as a result of the markedly enlarged liver.

Mitochondrial protein concentration was depressed in the experimental groups to 50–65% of control values (Table I). Respiratory control ratios were significantly elevated in liver mitochondria from experimental groups in comparison to their respective conrols by 44% at week 41, 38% at week 45, and 17% at the final point. RCR was increased ($p < .05$) at week 51 compared to week 45 within the carcinogen-fed groups. Within the control groups, a progressive increase ($p < .01$) in mitochondrial respiratory control was observed when the levels at week 51 were compared with those at weeks 41 and 45. Phosphorylation rates were elevated 35–40% in mitochondria from the livers of the experimentals compared to their corresponding control groups, and the differences reached significance at weeks 45 and 51. Hepatic mitochondrial ADP/O ratios were about 25% greater in the experimental than in control groups at the 41 and 45 week points, but were not significantly different at the 51 week point. An increase was found within the experimentals between the 41 and 45 week groups ($p < .05$) and between the 41 and 51 week groups ($p < .01$) whereas the PR was significantly increased ($p < .01$) within the experimental groups only between weeks 41 and 51. Within the control groups the ADP/O was elevated ($p < .01$) with time, when the 41 and 51 week points were compared, as a result of a significant increase ($p < .05$) between weeks 45 and 51. Similarly, phosphorylation rates were elevated ($p < .01$) within control groups between weeks 45 and 51 which accounted for the significant ($p < .01$) difference between weeks 41 and 51. The change in these parameters within the control groups might be related to maturation or aging of the animal.

Increased RCR levels due to carcinogen in the diet cannot be ascribed to consistently increased state 3 (active state) nor to consistently decreased state 4 (respiration in the absence of phosphate acceptor) respiratory rates. Higher RCR in experimental groups (compared to controls) resulted from an almost significantly elevated state 3 respiration at weeks 41 and 45 accompanied by a significantly reduced state 4 respiration. At week 51, increased RCR due to carcinogen feeding could be ascribed solely to augmented state 3 respiration. The phosphorylation rate is a function of state 3 respiration and the former quantity was elevated in the latter two periods by carcinogen feeding. Therefore, the state 3 respiratory rate appeared to be the more conclusive determinant of the differences noted in the RCR between these groups, even though this quantity was not significant at the earlier periods. However, elevated state 3 respiratory rates at all points might have been significant had there been more animals in the study.

Each liver from 2-FAA-fed animals contained excisable nodules (0.55-1.41 g) and numerous small nodules that were apparent by gross inspection. Histologically, all of the sections from the experimental rats revealed the characteristic hyperplastic nodules that develop during hepatocarcinogenesis by 2-FAA (11). In addition, one hepatoma was seen in each of the experimental groups, which would indicate that the temporal points examined represented a transitional stage in carcinogenesis wherein hepatomas were beginning to develop. There were no differences in mitochondrial functional activi-

ty patterns between the livers with hyperplastic nodules only and those with hepatomas as well.

Discussion. Hepatic mitochondrial respiratory control ratios and phosphorylation rates were found to increase in response to liver damage by chemical means or by ablation of tissue (1). In the present study, these quantities were also augmented by another example of liver damage, destruction of functional hepatocytes during 2-FAA-induced carcinogenesis, since the increases correlated with abnormal growth as seen by the total hepatic enlargement. Although the literature is replete with studies of the relation between mitochondrial activity and tumor growth (12–14), *enhanced* coupling of phosphorylation to oxidation during carcinogenesis has not been reported previously. Indeed, Warburg's early theory postulated that the efficiency of coupling in energy metabolism was depressed in carcinogenesis and led to predominance of anaerobic over aerobic glycolysis in tumor cells (15). The RCR is generally considered to be a more sensitive estimate of the degree of coupling than are the ADP/O or P/O (16). Hence, the discrepancy between our findings (with isolated mitochondria) and those reported previously may be due partly to the fact that earlier conclusions were based upon P/O or ADP/O ratios in tumors and control tissues (not always homologous). However, we did find a transient increase in the ADP/O as well.

The enhanced coupling of mitochondrial oxidative phosphorylation that was observed may be due to its elevation either in neoplastic cells, remaining functional (hyperplastic and normal) cells, or both. Previous studies with isolated tumor tissue of various types would tend to implicate uncoupling or no alteration in coupling (12, 13). Thus, the increased RCR and PR which were observed might better be attributed to the remaining functional hepatocytes since the livers in our study were probably at a transitional stage in carcinogenesis and contained mitochondria from several types of cells. Moreover, elevation of the RCR and ADP/O was most pronounced and significant in the 41-week group and least apparent in the 51-week group (Table I); the proportion of normal and hyperplastic cells would be expected to decrease during carcinogenesis (11). In addition, the histological evidence delineated areas of hyperplasia or regeneration at all three points studied. Since both RCR and PR were elevated in regenerating liver (1), the increase of these quantities in livers from carcinogen-fed animals might be better ascribed to the hyperplastic rather than to normal or neoplastic cells. Neoplasia, due to the carcinogen, could reduce the effective functional capacity of the liver sufficiently to set in motion the same mechanisms as are activated by partial hepatectomy.

Elevated mitochondrial functional activity reported previously (1) was also accompanied by a fall in mitochondrial protein content and occurred during a stage of regeneration prior to that for increased protein synthesis or cell division (17, 18). In this study, as in others (12), neither the cellular protein concentrations, DNA concentrations, nor protein/DNA ratios were altered by carcinogen feeding. This result may be a reflection of "average" values for the several diverse cell types present since ultrastructural changes in hyperplastic areas have been linked to enhanced protein synthesis (19, 20). Yet, mitochondrial protein concentration was lowered in the carcinogen-fed animals similarly to that observed by others for various tumor tissues and carcinogens (14). Depression in liver mitochondrial protein during hepatocarcinogenesis could be a reflection of reduction in number (14) and size (20) of mitochondria and/or loss of specific mitochondrial proteins (14). Evidence has been cited (1) for the postulation that elevated mitochondrial functional activities, coincident with liver damage and regeneration, could be related to decreased number of mitochondria per cell, to permeability changes and/or to increased turnover of the organelles.

Azo-dye carcinogenesis in mice is also accompanied by reduced numbers of hepatic mitochondria; whereas hamsters, which are not susceptible to carcinogenesis by these compounds, exhibit no changes in hepatic mitochondrial populations (21). Thus, elevated oxidative phosphorylation may: (a) be an

TABLE I. Hepatic Responses to *N*-2-Fluorenylacetamide Feeding.

Week:	41		45		51	
	Control	Tumor	Control	Tumor	Control	Tumor
n:	4	4	4	4	3	2
Body wt (g)	358 ± 9	323 ± 7[a]	355 ± 6	314 ± 14[a]	379 ± 19	310 ± 4[a]
Liver wet wt (g)	9.88 ± 0.32	21.27 ± 2.04[b]	10.43 ± 0.32	26.36 ± 1.38[b]	10.20 ± 0.64	37.18 ± 3.84[b]
Relative liver wet wt (g/100 g of body wt)	2.76 ± 0.05	6.59 ± 0.67[b]	2.94 ± 0.05	8.38 ± 0.19[b]	2.69 ± 0.04	12.04 ± 1.42[b]
Liver DNA conc (mg/g of liver)	2.61 ± 0.24	3.36 ± 0.37	2.84 ± 0.35	3.10 ± 0.28	3.56 ± 0.37	4.21 ± 0.79
Liver protein/DNA (mg/mg)	46.2 ± 6.7	38.4 ± 2.5	42.6 ± 2.8	39.7 ± 3.9	30.2 ± 3.1	25.4 ± 0.6
Mitochondrial protein (mg/g of liver)	24.5 ± 1.0	15.6 ± 0.5[b]	23.9 ± 1.6	15.5 ± 0.8[b]	21.6 ± 0.9	10.9 ± 0.6[b]
State 4 respiration (n-atom O_2 min^{-1} mg^{-1} of mitochondrial protein)	21.3 ± 0.6	17.0 ± 1.4[a]	19.2 ± 1.0	15.4 ± 0.9[a]	15.3 ± 0.7	16.6 ± 0.6
State 3 respiration (n-atom O_2 min^{-1} mg^{-1} of mitochondrial protein)	53.0 ± 3.6	58.8 ± 3.0	54.9 ± 3.7	61.9 ± 4.4	58.5 ± 3.0	73.3 ± 2.8[b]
Respiratory control ratio	2.49 ± 0.14	3.58 ± 0.41[b]	2.89 ± 0.23	3.99 ± 0.15[b]	3.84 ± 0.03	4.51 ± 0.09[a]
ADP/O	1.51 ± 0.09	1.91 ± 0.07[a]	1.81 ± 0.09	2.21 ± 0.08[a]	2.17 ± 0.08	2.35 ± 0.03
Phosphorylation rate (nmoles of ATP min^{-1} mg^{-1} of mitochondrial protein)	80.7 ± 9.5	113 ± 9.6	98.1 ± 4.7	136 ± 13.1[a]	127 ± 1.6	172 ± 4.6[b]

[a] $p < .05$.
[b] $p < .01$.

event in the carcinogenic sequence; (b) arise from a stimulus similar to that operative in liver regeneration; or (c) reflect a regenerative activity of nonprecancerous hepatocytes during hepatocarcinogenesis. It is not possible to distinguish definitely between these alternatives from the present results.

Summary. Hepatic mitochondrial functional activities, as determined by ADP/O, respiratory control ratio, and phosphorylation rate with glutamate as substrate, were augmented in male Fischer rats between 22–32 weeks following ingestion of the carcinogen, 2-*N*-fluorenylacetamide (2-FAA). Histologically, the livers from the carcinogen-fed group all contained hyperplastic nodules characteristic of areas of regeneration; and hepatomas were found in one animal in each of the three periods studied. Previously, increased mitochondrial activities of this type were reported for animals treated with the hepatotoxins, hydrazine and ethanol, as well as during regeneration following partial hepatectomy. This stage of hepatocarcinogenesis following the long-term feeding of 2-FAA represents an additional instance wherein these mitochondrial functional activities were elevated coincident with significant liver damage. In this case, enhanced mitochondrial activities might be an expression of the presence of the hyperplastic nodules. Whether these increases were part of the pathological chain of events during carcinogenesis or exposed a phenomenon common to both carcinogenesis and regeneration could not be ascertained from these experiments.

The authors thank Mrs. Brenda Cox and Mr. Wilson Friend for their excellent assistance in conducting these experiments.

1. Higgins, E. S., and Banks, W. L., Jr., Biochem. Pharmacol. **20**, 1513 (1971).

2. Dunning, W. F., Curtis, M. R., and Madsen, M. E., Cancer Res. **7**, 134 (1947).

3. Schneider, W. C., and Hogeboom, G. H., J. Biol. Chem. **183**, 123 (1950).

4. Banks, W. L., Jr., Kline, E. S., and Higgins, E. S., J. Nutr. **100**, 581 (1970).

5. Chance, B., and Williams, G. R., Advan. Enzymol. Relat. Areas Mol. Biol. **17**, 65 (1956).

6. Ozawa, K., Kitamura, O., Ohsawa, T., Araki, H., Murata, T., and Honjo, I., J. Biochem. (Tokyo) **67**, 149 (1970).

7. Gornall, A. C., Bardawill, C. J., and David, M. M., J. Biol. Chem. **177**, 751 (1949).

8. Wannemacher, R. W., Jr., Banks, W. L., Jr., and Wunner, W. H., Anal. Biochem. **11**, 320 (1965).

9. Munro, H. N., and Fleck, A., *in* "Mammalian Protein Metabolism," (H. N. Munro, ed.), Vol. 3, p. 481. Academic Press, New York (1969).

10. Bailey, N. T., "Statistical Methods in Biology," pp. 23, 45–49. Wiley, New York (1959).

11. Epstein, S., Ito, N., Merkow, L., and Farber, E., Cancer Res. **27**, 1702 (1967).

12. Reid, E., "Biochemical Approaches to Cancer," pp. 7–15, 74–76, 92–93, 104. Pergamon, London (1965).

13. Wenner, C. E., Advan. Enzymol. Relat. Areas Mol. Biol. **29**, 321 (1967).

14. Aisenberg, A. C., "The Glycolysis and Respiration of Tumors," p. 101, Academic Press, New York (1961).

15. Warburg, O., Science **123**, 309 (1956).

16. Lehninger, A. L., "The Mitochondrion," p. 132, Benjamin, New York (1964).

17. Bucher, N. L. R., N. Engl. J. Med. **277**, 686, 738 (1967).

18. Bucher, N. L. R., Int. Rev. Cytol. **15**, 245 (1963).

19. Flaks, B., Eur. J. Cancer **4**, 297 (1968).

20. Merkow, L., Epstein, S. M., Farber, E., Pardo, M., and Bartus, B., J. Nat. Cancer Inst. **43**, 33 (1969).

21. Price, J. M., Miller, J. A., and Miller, E. C., Cancer Res. **11**, 523 (1951).

Received March 19, 1971. P.S.E.B.M., 1971, Vol. 138.