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FLAVASPIDIC ACID: EFFECTS ON CELL RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN ISOLATED HEPATOCYTES

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Summary: Flavaspidic acid-N-Methyl-glucaminate caused a concentration-dependent increase in oxygen consumption and decrease in ATP concentration, and an increased incorporation of ^{14}C-oleate, ^{14}C-octanoate and ^{14}C-glucose into $^{14}CO_2$ in isolated rat hepatocyte suspensions. Mitochondrial respiratory control ratio and P:O ratio were decreased by flavaspidic acid, indicating an uncoupling of oxidative phosphorylation. Although this agent remains a useful probe for the study of hepatic transport and metabolism of long chain fatty acids and other organic anions, conditions under which it is employed must be designed so as to avoid the potentially confusing effects of its adverse influence on cell energetics.

INTRODUCTION

Flavaspidic acid is a phloroglucinol derivative which competes in vitro with the binding of fatty acids to the 12,000 MW cytosolic fatty acid binding protein (FABP) of liver and intestine (1,2). This property of flavaspidic acid has been used to advantage in studies of the role of FABP in the uptake and utilization of long chain fatty acids in rats and dogs in vivo (3,4) and in vitro in everted intestinal sacs (2), perfused rat liver (3), and isolated rat hepatocytes (5,6). During studies of the effect of flavaspidic acid on utilization of albumin-bound ^{14}C-oleate by isolated hepatocytes, we noted a seemingly paradoxical increase in $^{14}CO_2$ production under conditions in which total ^{14}C-oleate uptake and utilization was inhibited (5). Since similar increases in substrate oxidation may reflect uncoupling of oxidative phosphorylation, this observation prompted the present studies of the effect of flavaspidic acid on respiration and energy production in isolated liver cells and rat liver mitochondria. Our findings demonstrate that flavaspidic acid, at moderate concentration, uncouples oxidative phosphorylation in isolated hepatocytes. This effect may in part explain conflicting results of studies in which flavaspidic

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acid has been employed in the investigation of the role of FABP in hepatic utilization of long chain fatty acids.

MATERIALS AND METHODS

Materials. 1-(^{14}C)-oleic acid 1-(^{14}C)-sodium octanoate, and ^{14}C-glucose were purchased from New England Nuclear, Boston. Unlabeled oleic acid was purchased from Calbiochem, San Diego, CA, and was more than 99% pure by gasliquid chromatography. Octanoic acid, glucose, tris buffer, triethanolamine, ATP, ADP and firefly-lantern luciferase were obtained from Sigma Chemical Co., St. Louis, MO. Fatty acid free albumin, purchased from Sigma, was found to contain less than 0.02 nmol fatty acid per nmol albumin. Flavaspidic acid-N-methyl-glucaminate (hereafter, flavaspidic acid) was generously provided by Dr. A. Aho, Turku, Finland. Male, Sprague-Dawley rats, 250-300 g, maintained on standard laboratory chow (Feedstuffs Processing Company, San Francisco, CA) were used in all experiments.

Studies utilizing isolated liver cells. Suspensions of isolated rat liver cells were prepared by a modification of the method of Berry and Friend (7). Livers were perfused with Ca^{++} free Hanks medium, gassed with 95% O_2, 5% CO_2, containing .05% collagenase (Type I, Sigma Chemical Co., St. Louis, MO). Cells were suspended in Ca^{++} free, biocarbonate-free Hanks with 10 mM sodium phosphate buffer, pH 7.4, and were incubated at 37° C in a Dubnoff shaker in 25 ml siliconized Erlenmeyer flasks in a final volume of 2 ml. Incubations contained 6-14 mg cell protein, and albumin at a final concentration of 1.5 g/100 ml. More than 85% of cells excluded trypan blue at the end of the longest incubations. Incubations of cells prepared from one liver were done in duplicate or triplicate, and the mean taken as the value for that experiment (n = 1).

Hepatocyte oxygen consumption was measured over 1-3 minutes at 37° C in a Gilson oxygraph with a 1.5 ml chamber, utilizing a calibrated Clarke electrode.

Hepatocyte ATP levels were measured by adding an aliquot of the incubated cell suspension directly to ethanol in a dry ice/acetone bath. The ATP was extractred into the ethanol, and its concentration was measured by the luciferase method as described by Holmsen et al (8).

In studies of $^{14}CO_2$ production, cells were incubated in flasks containing a center well. At the end of the incubation, 0.2 ml Hydroxide of Hyamine (Packard Instrument Co., Inc., Downers Grove, IL) was added to the center well and 0.2 ml of 70% perchloric acid to the contents of the flask. $^{14}CO_2$ was collected during an additional 60 min incubation, after which the contents of the center well were assayed for radioactivity in Liquifluor toluene solution (New England Nuclear) containing 10% Biosolv (Beckman Instruments, Inc., Fullerton, CA) in a Brinkman Liquid Scintillation System Model LS-250.

Preparation of isolated mitochondria from rat liver. Rats were killed by decapitation and livers perfused with ice-cold 0.25 M sucrose. Livers were then homogenized in 2 volumes of 0.25 M sucrose in 10 mM phosphate, pH 7.4. The homogenate was diluted to 10:1 with the same solution and centrifuged at 600 x g for 10 minutes. The supernatant was then spun at 8500 x g for 10 minutes, and the mitochondrial pellet was resuspended in cold 0.25 M Sucrose, 10 mM Tris-HCl, pH 7.4.

Studies of mitochondrial respiration were carried out in a Gilson oxygraph utilizing a calibrated Clarke electrode. The respiration medium consisted of 225 mM sucrose, 10 mM phosphate, pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, and 20 mM Triethanolamine.

Statistical methods. Significance of differences among experimental groups was determined by the paired "t" test.

TABLE I

Effect of Flavaspidic Acid on Oxygen Consumption and ATP Concentration in Isolated Hepatocytes

	Oxygen Consumption	ATP Concentration
	nmol/mg prot/min	nmol/mg/prot
Control	5.4 ± 0.3	8.2 ± 0.8
Flavaspidic Acid		
0.2 mM	5.5 ± 0.4	8.5 ± 0.6
0.05 mM	5.7	-
0.10 mM	6.7	-
0.44 mM	9.2 ± 0.7*	5.1 ± 0.8†
1.0 mM	10.8	-

Isolated hepatocytes were incubated for 4 minutes in medium containing 0.22 mM albumin, ± 0.44 mM flavaspidic acid. Subsequently, 0_2 consumption and ATP concentration were measured as described in Methods. Mean ± S.E., n=3; a single value indicates the mean of duplicate or triplicate incubations from a single rat liver.
* $p < .01$ vs. controls
† $p = .02$ vs. controls

RESULTS

Effect of Flavaspidic acid on oxygen consumption and ATP concentration in isolated hepatocytes. Addition of flavaspidic acid in moderate concentrations to rat hepatocyte suspensions significantly increased hepatocyte oxygen consumption in a dose-dependent manner, as shown in Table I. This effect was also seen following addition to the incubation of the classical uncoupler of oxidative phosphorylation, 2, 4- dinitrophenol (results not shown). Concurrent with this effect on cell respiration, there was a significant decrease in hepatocyte ATP concentration so that, 4 minutes after the addition of 0.44 mM flavaspidic acid, ATP levels had fallen to 62% of control values.

Effects of flavaspidic acid on CO_2 production. Flavaspidic acid significantly increased production of $^{14}CO_2$ from ^{14}C-oleate by isolated liver cells, as shown in Table II. This effect did not reflect increased entry of fatty acid into the hepatocytes, since in separate experiments it has been found that uptake of oleate was inhibited under these conditions (5). These effects on oxygen consumption and CO_2 production from oleate were similar to those seen when 2, 4- dinitrophenol was added to hepatocyte suspensions, both in our own laboratory, and in experiments of other workers (9). Flavaspidic acid also significantly

TABLE II

Effect of Flavaspidic Acid on $^{14}CO_2$ Production from 1-^{14}C-oleate and 1-^{14}C-octanoate in Isolated Hepatocytes

	^{14}C Incorporated into $^{14}CO_2$ (pgram atoms/mg prot/4 min)	
	1-(^{14}C)-oleate	1-(^{14}C)-octanoate
Control	117 ± 6	424 ± 26
Flavaspidic Acid	435 ± 23*	870 ± 23†

Isolated hepatocytes were incubated for 4 minutes in media containing 0.2 mM albumin, and 0.44 mM oleate or 0.22 mM octanoate, ± 0.44 mM flavaspidic acid. $^{14}CO_2$ was collected as described in Methods. Mean ± SEM; for oleate, n=7; for octanoate, n=3.
* $p < .001$ vs. controls
† $p < .01$ vs. controls

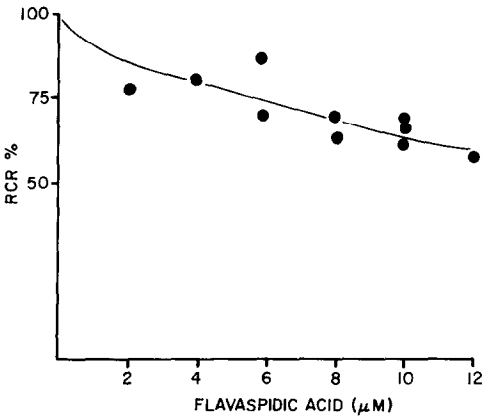

Figure 1. Effect of flavaspidic acid on oxygen consumption by rat liver mitochondria. Mitochondrial oxygen consumption was measured as described in Methods in the presence or absence of flavaspidic acid. Substrate consisted of 5 mM malate and 5 mM pyruvate. State 3 conditions were produced by the addition of .3 mM ADP, and the Respiratory Control Ratio (RCR) calculated as the ratio of oxygen consumption in state 3 to that in state 4 (no phosphate acceptor present). Results are those from three separate experiments. The results are expressed as % of control values.

increased $^{14}CO_2$ production from ^{14}C-octanoate by isolated hepatocytes (Table II), and from 5 mM U-^{14}C-glucose (466 vs. 203 pmole/mg protein; mean of duplicate 15 min incubations).

Effect of flavaspidic acid on hepatic mitochondrial respiration. Flavaspidic acid has been

previously shown to decrease P:O in isolated rat mitochondria (10). We confirmed this effect in these studies. As shown in Fig. 1, flavaspidic acid also decreased the Respiratory Control Ratio (state 3/state 4) in isolated liver mitochondria, almost entirely reflecting increased state 4 respiration. The ADP:O ratio was decreased to the same extent as the RCR.

DISCUSSION

Earlier studies showed that flavaspidic acid acted as an uncoupler of oxidative phosphorylation in isolated mitochondria (10). The present studies confirm this but in addition demonstrate that this agent also produces similar effects in intact hepatocytes, at concentrations in the range of .05 to .10 mM. Our results differ with recently published studies which suggest that flavaspidic acid does not uncouple oxidative phosphorylation in liver (11). The reasons for these conflicting conclusions are unclear. Only a single, higher concentration of flavaspidic acid (5mM) was employed in those studies; the effect of flavaspidic acid on hepatocyte ATP concentration was not studied. The effects of flavaspidic acid on CO_2 production from long chain fatty acids are similar to those seen with the classical uncoupler, 2,4- DNP.

Our finding is of significance because flavaspidic acid has been employed in numerous studies as a probe to investigate the role of certain cytosolic proteins in the hepatocellular metabolism and transport of long chain fatty acids and other organic anions (2,3,4,5,6). In these studies, the effects of flavaspidic acid generally have been attributed to competition between flavaspidic acid and fatty acids or the other organic anions for binding to putative cytosolic acceptor proteins. This interpretation seems justified when experimental conditions are such that uncoupling of oxidative phosphorylation is excluded directly, by measurement of oxygen consumption and ATP concentration (12), or indirectly is rendered less likely by decreased rather than increased substrate oxidation (2). However, when high or uncertain concentrations of flavaspidic acid are employed (4,6), interpretation of the experimental data is particularly difficult.

In addition to flavaspidic acid, fatty acids (13,14), sulfobromophthalein (15) and unconjugated bilirubin (16) uncouple oxidative phosphorylation in isolated mitochondria. Since albumin or ligandin have been shown to protect against these effects, in vitro (16),

and since the presence of intracellular binding proteins might be expected to afford similar protection in the intact cell, it might be concluded that, in the liver cell and in vivo, uncoupling by this class of compounds, i.e., the "organic anions," may not be significant. However, the present studies show that uncoupling does occur if the extracellular concentration is sufficient, and this factor must be considered in the design and interpretation of experiments in which these agents are employed.

REFERENCES

1. Mishkin, S., Stein, L., Gatmaitan Z. and Arias, I.M. (1972) Biochem. Biophys. Res. Commun. 47, 997-1003.
2. Ockner, R.K., Manning, J.A. (1976) J. Clin. Invest. 53, 632-641.
3. Mishkin, S., Stein, L., Fleischner, G., Gatmaitan, Z. and Arias, I.M. (1975) Am. J. Physiol. 228, 1634-1640.
4. Goresky, C.A., Daly, D.S., Mishkin, S., Arias, I.M. (1978) Am. J. Physiol. 34, E542-E553.
5. Burnett, D.A., Ockner, R.K. (1976) Clin. Res. 24, 432A.
6. Wu-Rideout, M.Y.C., Elson, C., Shrago, E. (1976) Biochem. Biophys. Res. Commun. 71, 809-816.
7. Berry, M.N., Friend, D.S. (1969) J. Cell. Biol. 43, 506-520.
8. Holmsen, H., Holmsen, I., Bernhardsen, A. (1966) Anal. Biochem. 17, 456-473.
9. Ontko, J.A. (1972) J. Biol. Chem. 247, 1788-1800.
10. Runeberg, L. (1962) Biochem. Pharm. 11, 237-242.
11. Wu-Rideout, M.Y.C., Woldegiorges, G., Shrago, E., Elson, C. (1978) Biochem. Pharm. 27, 2425-2429.
12. Burnett, D.A., Lysenko, N., Manning, J.A., Ockner, R.K. (1979) Gastro. Accepted for publication.
13. Pressman, B.C., Lardy, H.A. (1956) Biochim. Biophys. Acta. 21, 458-466.
14. Lehninger, A.L., Remmert, L.F. (1959) J. Biol. Chem. 234, 2459-2464.
15. Killenberg, P.G., Hoppel, C.L. (1974) Mol. Pharm. 10, 108-118.
16. Kamisaka, K., Gatmaitan, Z., Moore, C.L., Arias, I.M. (1975) Pediat. Res. 9, 903-905.