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EFFECT OF HYDRAZINE ON PLASMA FREE FATTY ACID TRANSPORT*

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Abstract—Effects of intravenously injected hydrazine (1·15–1·20 mmoles/kg) on plasma free fatty acid (FFA) transport were studied in one-day fasted, anesthetized rats. Two hours after hydrazine injection, blood glucose levels were depressed, and plasma FFA concentration and pool size were elevated to twice the control level. When, at this time, the plasma free fatty acid pool was labeled with palmitic or oleic acid-1-^{14}C, its fractional turnover rate appeared to be little affected by hydrazine. However, an increase was observed in the fraction of the labeled fatty acids which, on leaving the blood, appeared in the liver. The chief component of plasma FFA transport to the liver was accelerated to more than twice the rate in control rats.

FOR more than 50 years, hydrazine has been known to cause fat to accumulate in the mammalian liver.[1] In the fasting rat, this effect with sublethal dosages is rapid and reversible[2] and is accompanied by an elevation of plasma free fatty acids (FFA),[3] which are considered to be the chief source of fat for the liver in the fasted state.[4]

The aim of the present study is to determine whether hydrazine increases significantly the rate of plasma FFA transport to the liver. The size of the plasma FFA pool, its fractional turnover rate, and the fraction of the FFA turnover representing transport to the liver have been estimated.† The extent to which hydrazine accelerates free fatty acid transport to the liver was estimated and compared with the observed effect of hydrazine on liver total fatty acid concentration.

MATERIALS AND METHODS

Condition of animals studied

Animals used in these studies were fasted, anesthetized male rats from the Holtzman Co., Madison, Wis. Having been deprived of food for 20–26 hr, these rats, weighing 280–360 g, were anesthetized with 45 mg pentobarbital sodium (Nembutal)/kg and tracheotomized.[3] A dilute solution of hydrazine in 0·9% NaCl was injected into a tail vein of test animals, while controls received the same volume (5 ml/kg) of isotonic saline. Additional small volumes of pentobarbital were given at intervals as needed to maintain a surgical level of anesthesia up to the time of sacrifice.

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† Experiments were conducted according to the *Principles of Laboratory Animal Care*, of the National Society for Medical Research.

Dosage and purity of the administered hydrazine

Hydrazine was obtained commercially as the liquid free base (Eastman, 95%). For injection, 0·2 ml of commercial hydrazine was made up to a 25-ml volume with isotonic saline on each day of an experiment. The undiluted hydrazine was stored between experiments in a brown bottle at 4° and was discarded after 3 months of use.

Since hydrazine is known to undergo auto-oxidation, the purity of fresh and used bottles was tested at intervals by the colorimetric method of Dambrauskas and Cornish,[5] with hydrazine sulfate used as the standard. Results indicated that the commercial hydrazine used in these studies was 91–95% pure. As tested with the Conway microdiffusion technique, the hydrazine was found to contain less than 0·6% ammonia. When diluted with CO_2-free water, it showed a pH slightly higher than was expected on the basis of the known pK of hydrazine (7·93); there was, therefore, no evidence of acidic contaminants. The chief impurity was believed to be water.

The dosage of hydrazine used in these studies was 1·15–1·20 mmoles/kg. This is about 60% of the LD_{50} dosage as determined for nonfasted, unanesthetized rats.[6]

Analytical procedures

Plasma volume was estimated as the Evans blue dye dilution space. In this procedure, dye was injected with a Hamilton syringe into the external jugular vein, and blood samples were taken 3, 10, and 20 min later. Plasmas were diluted with 15 parts by volume of water before extinction was measured at 620 mμ.

In determining plasma FFA concentration, care was taken to obtain only approximately 3 ml of the blood first shed after the animal was sacrificed by guillotine. Bloods were collected in heparinized tubes, placed immediately in an ice bath, and later spun in a refrigerated centrifuge. Plasma FFA was determined by a modification[7] of the Dole procedure.[8]

Plasma FFA pool size was calculated as the product of FFA concentration and plasma volume.

Blood glucose concentrations were determined by the method of Dubowski.[9] Liver total fatty acids were determined[3] by titration of the fatty acids obtained by saponifying an aliquot of liver total lipid.

Isotopic procedures

Isotope studies were carried out with commercially prepared oleic, palmitic, and stearic acids-1-^{14}C, having specific activities, respectively, of 19, 2·1, and 1·6 mc/mmole. A Tri-Carb liquid scintillation spectrometer was used for the ^{14}C counting of samples, which were first dissolved in a toluene solution containing, as phosphors, 4 g/liter PPO (2,5-diphenyloxazole) and 100 mg/liter POPOP [1,4-di-2-(5-phenyloxazolyl)benzene]. A counting efficiency of about 57% was achieved.

Prior to injection, the labeled fatty acid was converted to the sodium soap, dissolved in an isotonic solution containing rat albumin, and filtered through Whatman 50 filter paper. In preliminary studies, the sodium soap was dissolved in a dialyzed solution of rat plasma proteins from which the lipoproteins had been removed after ultracentrifugation in concentrated NaCl–KBr (density 1·20).[10] In the main studies with palmitic and oleic acids-1-^{14}C, the injection vehicle was 0·9% NaCl containing 1·2% rat albumin (from Pentex Corp., Kankakee, Ill.). A 0·6-ml volume of the

solution, containing 0·4–0·9 μc, was injected into the external jugular vein, and, at a specified interval, the whole liver or a heparinized blood sample was removed.

In preparing liver samples for ^{14}C analysis, total lipid was extracted and also saponified, for phospholipids do not dissolve readily in toluene. The procedure for isolating the total fatty acids for ^{14}C counting was essentially that used for their titrametric determination.

In a first study of the plasma FFA turnover rate, ^{14}C was measured in the plasma FFA which had been extracted by the method of Dole[8] and isolated from other lipids by the method of Borgström.[11] In the main ^{14}C studies, a single blood sample was taken within 3 min after the injection of the isotope and before ^{14}C had time to reappear in the plasma triglyceride.[12] Plasma was extracted with a 2 : 1 v/v mixture of chloroform and methanol, and the extracted lipid was saponified according to the procedure of Albrink.[13] This procedure for isolating the plasma total fatty acids was also used in measuring the activity of solutions of albumin-bound ^{14}C-labeled fatty acids used for injection.

The fractional turnover rate (K) of the plasma FFA pool was estimated by determining the fraction of the labeled fatty acids leaving the blood in a measured time interval. Plasma FFA-^{14}C (disintegrations/min/ml or in the total plasma volume) was represented at times T_1 and T_2, respectively, as A_1 and A_2; and K was calculated to be $\ln(A_1/A_2)/(T_2 - T_1)$. This approach requires caution, however, for the estimates of K obtained depend in large part on the intervals after the isotope injection when A_1 and A_2 are measured.

In this confusing situation, a key fact is the demonstration that in man, K values obtained from the 2–4 min interval after palmitic acid-1-^{14}C injection approximate the actual mean fractional turnover rate of the whole plasma FFA pool.[14] In rats, the fractional turnover rate of plasma FFA is so great that the fraction of i.v. injected ^{14}C-labeled fatty acids remaining in the blood after 1 min is similar to that remaining after 3 min in man. Ideally, in estimating K in rats, the slope of the isotope disappearance curve should be determined at about 1 min after the injection of labeled fatty acids.

In a first attempt to detect possible effects of hydrazine on the fractional turnover rate of plasma FFA, blood was sampled first at 1–1·3 min (T_1) and again at 2–2·6 min (T_2) after palmitic acid-1-^{14}C injection. This method of estimating K deals with a portion of the isotope disappearance curve believed to give low values for K and has the further limitation that the interval between blood withdrawals is difficult to measure accurately.

In subsequent studies a known quantity of palmitic or oleic acid-1-^{14}C was injected, and only one blood sample was taken 2–3 min later, usually by decapitation. The quantity of isotope injected was considered to be the quantity of ^{14}C in the total blood plasma at time zero; i.e. to be A_1 at $T_1 = 0$ min. The ^{14}C concentration of the subsequent plasma sample was measured, and, on the basis of body weight, the plasma volume and its ^{14}C content (A_2) were estimated. This second method of estimating K appeared to yield greater reproducibility and a more precise basis for detecting changes in K.

The fraction of the FFA turnover representing transport to the liver was estimated by injecting the labeled fatty acids into the external jugular vein and determining what fraction of the ^{14}C on leaving the blood could be recovered from the liver.

In one experiment rats were sacrificed 5·5–6 min after receiving stearic acid-1-^{14}C. In subsequent work with palmitic and oleic acids-1-^{14}C animals were sacrificed within 2·8 min after the isotope injection; in calculating the fraction of labeled fatty acids migrating to the liver a correction was made for the 5–8% of injected ^{14}C remaining in the blood plasma.

Experimental design

All experiments were set up as matched studies, and each experimental animal was studied on the same day with one or more controls having similar body weights. As indicated in the tables, some of the data were treated as paired; on the other hand, in cases when data were lost, the variances for the treatment groups were pooled in estimating the standard error of mean difference.

The same animals were used for determining the large effect of hydrazine on plasma FFA concentration (Table 1) and on the total fatty acid content of the liver (Table 6). Separate groups of rats were used for estimating the plasma volume, the fractional turnover rate of the plasma FFA pool, and the fraction of plasma FFA which, on leaving the blood, is extracted by the liver.

RESULTS

Table 1 shows the results of a study to determine the time sequence of changes in plasma FFA and blood glucose concentrations after the injection of hydrazine. The

TABLE 1. TIME COURSE OF HYDRAZINE EFFECTS ON THE BLOOD

Time after injections	Plasma FFA (μmoles/ml)			Blood glucose (mg/ml)		
	Hydrazine-treated	Controls	Difference*	Hydrazine-treated	Controls	Difference
(hr)						
1	1·08 (9)†	0·83 (9)	0·25 ± 0·103‡	0·46 (6)	0·55 (6)	0·09 ± 0·026
2	1·45 (7)	0·71 (7)	0·74 ± 0·084	0·42 (8)	0·54 (8)	0·12 ± 0·024
3	1·08 (9)	0·72 (9)	0·36 ± 0·051	0·50 (6)	0·56 (6)	0·06 ± 0·056

* Plus or minus S.E.M., calculated by pooling variances from two groups.

† Numbers in parentheses indicate the animals in each group.

‡ This treatment effect also becomes significant at $P < 0·01$, if data are treated as paired.

maximal elevation of plasma FFA and the greatest depression of blood glucose were observed after 2 hr, when the FFA concentration was twice the control level. Subsequent experiments were conducted at the 2-hr interval after hydrazine administration.

Determination of plasma volume showed that at the 95% confidence level there was no more than a 5% difference between those animals receiving hydrazine and their controls. Therefore, as presented in Table 2, the plasma pool of FFA was estimated to be approximately doubled by the hydrazine treatment.

Results summparzed in Table 3 indicate that, at 2 hr after the injection of hydrazine, the fractional turnover rate of the palmitic acid portion of the plasma FFA pool was slightly reduced, whereas that of the oleic acid portion was not affected. The

observation that hydrazine doubled plasma FFA pool size but had liitle effect on its fractional turnover rate implies that the total FFA turnover rate, i.e. $K \times$ pool size, is nearly twice as great in hydrazine-treated animals as in controls.

Data from Table 4 indicate that the livers of hydrazine-treated animals extracted a larger fraction of the labeled fatty acids leaving the blood than did those of controls.

TABLE 2. EFFECT OF HYDRAZINE ON PLASMA FFA POOL

	Plasma FFA conc. (μmoles/ml)	Plasma volume (ml/100 g body wt.)	Plasma FFA pool* =(μmoles plasma FFA /100 g body wt.)
Hydrazine-treated	1·45 ± 0·062 (7)	3·66 ± 0·077 (9)	5·31 ± 0·253
Controls	0·71 ± 0·056 (7)	3·67 ± 0·091 (9)	2·61 ± 0·215
Difference ± S.E. (based on paired data)		0·01 ± 0·075	
Difference ± S.E. (based on pooled variance)	0·74 ± 0·084	0·01 ± 0·119	2·70 ± 0·332

* Plasma FFA concentration and plasma volume were determined in separate groups of anesthetized rats 2 hr after the injection of hydrazine or saline. Plasma FFA pool size is calculated as the product of FFA concentration ($\bar{x}$), and plasma volume ($\bar{y}$). The S.E. for ($\bar{x}\bar{y}$) for test animals and for the control group is estimated as $\{[\bar{x}.\text{S.E.}(\bar{y})]^2 + [\bar{y}.\text{S.E.}(\bar{x})]^2\}^{1/2}$; the S.E. of the calculated effect of treatment on FFA pool size is estimated from the pooled variance of ($\bar{x}\bar{y}_T$) and ($\bar{x}\bar{y}_C$).

TABLE 3. EFFECT OF HYDRAZINE ON FRACTIONAL TURNOVER RATE OF PLASMA FFA

Compound injected	Times of estimating plasma-^{14}C 1 (min)	2 (min)	Calculated K Hydrazine-treated group	Control group	Difference	P
Palmitic acid-1-^{14}C	1–1·3	2–2·6	0·68 ± 0·040 (6)	0·87 ± 0·041 (6)	− 0·19 ± 0·057	<0·01
Palmitic acid-1-^{14}C	0	2–3	0·88 ± 0·019 (10)	0·96 ± 0·021 (10)	− 0·08 ± 0·029	<0·02
Oleic aicd-1-^{14}C	0	2·65–2·89	0·96 ± 0·031 (7)	1·01 ± 0·012 (7)	− 0·05 ± 0·036	not sig.

The fractional turnover rate, K, of the plasma FFA pool was estimated by measuring the fraction of i.v. injected ^{14}C-labeled fatty acids which disappeared from the blood in a measured time interval. In obtaining the first estimates of K, blood was sampled at two intervals after the isotope injection. In subsequent studies, the ^{14}C content of the total plasma volume at time zero was considered to be the quantity of ^{14}C injected. The fraction of the injected ^{14}C-labeled fatty acid later remaining in the blood was estimated by measuring the ^{14}C concentration in blood plasma and estimating plasma volume from body weight. See text for further details.

This effect of hydrazine appears to have been greatest for the oleic acid portion of the plasma FFA pool, although no effect on the fractional turnover rate of plasma free oleic acid was observed. These facts imply that livers of hydrazine-treated rats extracted per unit time an increased fraction of a presumably increased pool of plasma free oleic acid. A similar effect on the hepatic extraction of plasma free palmitic acid was not clearly demonstrated.

A rough calculation of the rate at which livers of control and hydrazine-treated animals extract FFA is shown in Table 5. It is known that the fractional turnover

rate of the various plasma free fatty acids are not all identical.[15] It is uncertain which of the estimates of K in Table 3 was most representative of the whole plasma FFA pool. It is further uncertain which estimate in Table 4 best approximated the fraction of plasma FFA turnover representing transport to the liver. The figures transposed from these tables to Table 5 are, of course, somewhat arbitrary. It is tentatively

TABLE 4. EFFECT OF HYDRAZINE ON THE FRACTION OF INJECTED ^{14}C-LABELED FATTY ACIDS RECOVERED IN THE LIVER

Compound injected	Fraction of injected ^{14}C recovered in livers		
	Hydrazine-treated	Controls	Difference
Stearic acid-1-^{14}C	0·30 ± 0·004 (6)	0·25 ± 0·009 (6)	0·05 ± 0·009*
Oleic acid-1-^{14}C	0·27 ± 0·009 (7)	0·18 ± 0·009 (7)	0·09 ± 0·012*
Palmitic acid-1-^{14}C	0·26 ± 0·11 (6)	0·20 ± 0·010 (6)	0·06 ± 0·015*

* S.E. of mean difference was calculated from pooled variance.

The ^{14}C in liver total fatty acids was measured shortly after the i.v. injection of known quantities of ^{14}C-labeled fatty acids. Rats receiving stearic acid-1-^{14}C were sacrificed within 5–6 min after the isotope injection. Rats receiving oleic or palmitic acid-1-^{14}C were killed within 2–3 min, and the quantity of ^{14}C remaining in the blood and that extracted by the liver were determined. The fraction of the injected labeled fatty acids which, on leaving the blood, was taken up by the liver, was calculated.

TABLE 5. ESTIMATED EFFECT OF HYDRAZINE ON RATE OF HEPATIC UPTAKE OF PLASMA FFA

	Hydrazine-treated	Controls
1. Plasma FFA pool, μmoles/100 g body weight	5·31	2·61
2. Fractional turnover rate, fraction/min	0·88	0·96
3. FFA turnover rate, μmoles/min/100 g body weight	4·67	2·51
4. FFA turnover rate, μmoles/hr/100 g body weight	280	151
5. Fraction of 4 representing transport to liver	0·27	0·20
6. Hepatic FFA uptake, μmoles/hr/100 g body weight	75·6	30·2
7. Liver weight, g/100 g body weight	3·1	3·1
8. Hepatic FFA uptake, μmoles/hr/g liver	23·4	9·4

estimated that 2 hr after the injection of hydrazine or saline, livers extracted plasma FFA at a rate, respectively, of 23 and 9 μmoles/hr/g tissue. It is concluded that hydrazine increased by more than 100% the rate of the chief component of FFA transport to the liver.

Correlated with the above procedures to estimate the effect of hydrazine on the rate of plasma FFA transport to the liver were further measurements to estimate the effect of hydrazine on the total fatty acid content of the liver. The aim, of course, was to compare the magnitude of the two effects and to determine to what extent the effect on FFA transport might account for the effect on liver fat content.

In the study in which liver total fatty acids were measured, animals were sacrificed 1 and 3 hr after the injection of hydrazine or saline. In this and other studies, the

ratio of liver wet weight to total body weight was carefully studied. No effect of treatment on liver weight was observed during the short duration of these studies; therefore, liver total fatty acid content was expressed as μmoles/g liver. It is observed from Table 6 that hydrazine had no significant effect on liver total fatty acids during

TABLE 6. EFFECTS OF HYDRAZINE ON LIVER TOTAL FATTY ACIDS

Time after injection (hr)	Liver total fatty acids (μmoles/g tissue)	
	Hydrazine treated	Controls (saline-injected)
3	180	138
1	146	149
Net change	34	−11
	per 2 hr	per hr
Effect of hydrazine and anesthesia	34	17
Effect of anesthesia	−11	−5·5
Effect of hydrazine alone ± S.E.*	45 ± 6·54	22·5 ± 3·27

* On each experimental run, two hydrazine-treated rats were sacrificed, one at 1 hr and one at 3 hr after this injection; and two control animals were also killed, one at each of these two intervals after receiving saline. From the four resulting measures of liver total fatty acid concentration was obtained one estimate of the effect of hydrazine. The mean and S.E. of nine independent estimates of the effect of hydrazine on liver total fatty acid concentration were determined from the analysis of 36 rat livers.

the first hour. During the next 2 hrs, livers of hydrazine-treated rats accumulated 34 μmoles total fatty acid/g tissue and showed an average increase of 17 μmoles/hr/g. Reference to the data of Table 5 shows that at the midpoint of this interval, i.e. 2 hr after hydrazine injection, livers were calculated to be extracting 23 μmoles FFA/hr/g tissue. It is concluded that, in hydrazine-treated animals, the rate of FFA uptake by the liver exceeded the rate of total fatty acid accumulation in that organ.

Interpretation of Table 6 is complicated by the previously reported[3] fact that livers of fasted control animals show a net loss of total fatty acids during continued anesthesia with pentobarbital. Hydrazine prevented the loss of 5·5 μmoles/hr/g of liver total fatty acids brought about by anesthesia and induced a gain of 17 μmoles/hr/g in liver total fatty acids. Hydrazine is therefore considered to have exerted a total effect of 22·5 μmoles/hr/g on liver total fatty acids. Yet, 2 hr after its injection, hydrazine was calculated to increase the rate of FFA transport to the liver by only 14 μmoles/hr/g above control levels. The hydrazine-induced acceleration of hepatic uptake of plasma FFA appeared to be insufficient to account for the observed effects on liver total fatty acids, but, for reasons to be discussed later, this conclusion is still open to question.

DISCUSSION

The conceptual framework of these studies warrants some comment, It has been established that a part of the FFA which is released by adipose tissues is re-extracted by adipose tissues and undergoes no net transport.[16] When the turnover rate of plasma

FFA is calculated as the product of pool size and fractional turnover rate, it includes this circular migration of fatty acids. FFA turnover may be considered a mathematically unsatisfactory concept unless it is defined merely as the rate at which FFA leaves and enters the blood. This limited definition is meaningful for the purpose of this paper when one has also determined approximately what fraction of the FFA, on leaving the blood, is taken up by the liver, as opposed to other organs, including adipose tissues. The product of this fraction and the FFA turnover rate constitutes an estimate of the rate of FFA uptake by the liver.

There are, however, indications of a second route of FFA transport to the liver which is not detected by the methods of the present studies. Hirsch *et al*[17] have shown that in the fed rabbit, portal vein blood has a higher plasma FFA concentration than arterial blood. Apparently, tissues which drain into the portal vein, perhaps the fatty tissues of the mesenteries, may release FFA. In the fed dog, there is little net difference in plasma FFA concentration between arterial and portal venous blood.[18] Shoemaker and associates present evidence to interpret this fact to mean not that there is no FFA release, but that release is balanced by removal in other parts of the circuit.[19] In the present studies with fasted rats, it is not known what portion of the portal venous FFA is derived from splanchnic tissues. The major part of the FFA released into the portal vein blood passes through the liver, enters the general FFA pool, and provides no special problems. However, that portion extracted during the initial passage through the liver has essentially no effect on the estimation of the size of the plasma FFA pool, its fractional turnover rate, or on the estimated fraction of the FFA turnover representing transport to the liver. Consequently, FFA obtained by the liver through this second route is undetected, and the calculated rates of hepatic uptake of plasma FFA are underestimated in the present study by an unknown amount.

The calculation that hydrazine accelerated the hepatic uptake of plasma FFA by 14 μmoles/hr/g liver above the control level is, as previously stated, quite inexact. If, as appears likely, hydrazine stimulated the release of FFA also by tissues draining directly into the portal vein, this calculation probably underestimated the actual difference in hepatic uptake rate between hydrazine-treated and control rats. It is clear that the hydrazine-induced acceleration of hepatic uptake of plasma FFA was great enough to account for at least a major part of the observed effect on liver total fatty acid contents (45 μmoles/g liver in 2 hr). It is emphasized, however, that even if the effect on FFA transport were shown to equal that on liver total fatty acid contents, this result would not rule out the possibility of important direct effects on fat metabolism in the liver.[20] A variety of experimental approaches will be required to determine to what extent effects of hydrazine on FFA transport govern the effect on liver total fatty acid contents.

Nevertheless, the present studies do establish several links in the chain of events by which hydrazine brings about fat accumulation in the liver. Hydrazine has long been known to bring about hypoglycemia,[21] an effect known to elevate plasma FFA. However, the hypoglycemia has usually been considered a delayed effect.[21] Actually, hydrazine is a highly irritating substance, and its i.p. or s.c. injection produces an initial elevation of blood glucose.[6, 22] It is noteworthy that, in the present studies with i.v. injected hydrazine, a depression of blood glucose was observed after only 1 hr, and the time of maximal depression of blood glucose appeared to coincide

with the time of maximal elevation of plasma FFA. Probably the hypoglycemia is a major factor in elevating the plasma FFA.

Plasma FFA concentration is a major factor governing the rate of FFA uptake by the liver. In perfused livers, the rate of uptake is proportional to FFA concentration in the perfusion medium over an extremely wide range.[23] It was to be expected that a hydrazine-induced elevation of plasma FFA would be accompanied by an increased rate of FFA transport to the liver.

However, the rate of hepatic uptake of at least the oleic acid component of the plasma FFA pool was increased in hydrazine-treated animals even more than its plasma concentration. A similar effect has been noted when insulin is infused into the portal vein,[24] but an accelerated release of endogenous insulin in hypoglycemic, hydrazine-treated rats appears unlikely. These experiments do not explain why hydrazine causes an increased fraction of at least part of the plasma FFA pool to be extracted per unit time by the liver.

It is concluded that hydrazine increases the rate of FFA transport to the liver chiefly by increasing plasma FFA pool size; however, hydrazine also brings about the hepatic uptake of an increased fraction of the plasma FFA leaving the blood.

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