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EFFECTS ON GROWTH AND METABOLISM OF RAT LIVER BY HALOTHANE AND ITS METABOLITE TRIFLUOROACETATE

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Abstract—Rats were treated with trifluoroacetate (TFA) in doses which correspond approximately to the amount of TFA formed during a 5 hr period of Halothane narcosis.

Trifluoroacetate which is the chief metabolite of Halothane causes as much as 40 per cent enlargement of liver within 4–5 days.

Liver enlargement induced by TFA conforms to a co-ordinated growth of hepatic cells including the protein content and some enzyme activities. There is a marked decrease in the activity of pyruvate kinase and an increase of glycerol 1-phosphate oxidase. Adrenalectomy potentiates the increase in the glycerol 1-phosphate oxidase activity during TFA treatment. TFA causes a decrease in phosphorylated trioses common to glycolysis and gluconeogenesis, but an increase in certain tricarboxylic acid cycle intermediates.

Experiments with isolated perfused rat livers show that TFA has an immediate effect on liver metabolism. It changes the steady state of intracellular metabolites common to glycolysis and gluconeogenesis and increases the turnover of lactate (and pyruvate).

SEVERAL of those substances which bring about enlargement of the liver, have been found to induce certain enzymes in the endoplasmic reticulum which are responsible for the biological transformation of drugs.[1–6] These observations have led to the concept that liver enlargement is directly related to this increase of the endoplasmic reticulum. However certain drugs can enlarge liver without a concomitant increase in enzyme activities in the endoplasmic reticulum.[7] Halothane, the most widely used volatile anaesthetic, may be included in this latter group of substances. In liver, Halothane is metabolized to its product trifluoroacetate (TFA). Stier[8] has reported studies on the formation and excretion of this compound in animals. The effects of Halothane on liver metabolism in mice have already been reported.[9,10] In this study the metabolic effects of Halothane and its metabolite TFA are compared by studying rat liver under *in vivo* conditions and in isolated perfused liver preparations. Various key regulatory enzymes and substrates concerned with the glycolytic pathway and tricarboxylic acid cycle have been measured, as well as protein and neutral fat. The results indicate that most of the effects observed after Halothane treatment may be attributed to TFA.

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MATERIALS AND METHODS

Animals

Male Wistar strain rats weighing 200–260 g were used and fed *ad lib.* on a standard commercial rat diet ("Altromin", Lage–Lippe, Germany).

Reagents

All chemicals and enzymes were obtained from Merck, Riedel-de Häen, Fluka and Boehringer. Halothane was obtained from Imperial Chemical Industries and trifluoroacetate from Fluka. For alkaline hydrolysis of fat, glycerol-free KOH supplied by Merck was employed.

Halothane treatment

The rats were exposed to an atmosphere of 0·8–1·0% Halothane in O_2 for 60 min daily. No signs of abnormal behaviour were observed during the weak narcosis. Analysis of substrate content and enzyme activities were carried out on the fourteenth to seventeenth days of treatment. For the analysis of substrates, samples of liver were rapidly removed by clamps pre-cooled in liquid nitrogen. The rest of the liver was taken out, weighed and used for determination of enzymes and protein.

TFA treatment

TFA was administered with the drinking water for 5–6 days. The concentration of TFA, neutralized with NaOH, was such as to provide the rats with approximately 130 μmoles TFA/100 g body wt./24 hr, assuming normal water consumption. Every day, two to three rats were removed from the experimental series and killed under ether anaethesia. The series were repeated and the data quoted in the tables represent mean values established on four to six animals per day.

Adrenalectomy and thyroidectomy

Two additional series of rats which had undergone adrenalectomy or thyroidectomy, were also given TFA. The time interval between the operation and treatment with TFA was at least 10 days.

Liver perfusion

The method of liver perfusion used was exactly as described previously.[11,12] In order to observe direct effects of TFA, livers from fed rats were used without any pretreatment. After 60 min equilibration of perfusion, TFA was infused at the rate of 200 μmoles/hr to 100 ml of perfusion medium. The final concentration of TFA at the end of 2 hr infusion thus amounted to a theoretical maximum of 4 mM.

ANALYTICAL METHODS

Analysis of fluorine content

Total fluorine content was determined as follows; plasma was dried in a current of air on filter paper and liver tissue was dried in an oven at 150°. The residues were then ashed according to the method of Schöninger.[13] The inorganic fluoride thereby obtained was determined by the method of Hall, with minor modifications. The

concentration of buffer was increased four-fold and that of alizarin complex and lanthane nitrate solution two-fold.[14,15] Further details have been given previously.[16] The amount of inorganic fluoride in plasma and liver was also determined by the same procedure with omission of ashing.

Biochemical procedures

The procedure described previously[11] was used for obtaining liver samples and for preparation of extracts used for determination of substrates. Substrates were determined enzymatically. ATP was determined by the method of Lamprecht *et al.*,[17] ADP and AMP by the method of Adam,[18] glucose by the method of Slein,[19] malate, α-glycerophosphate and lactate by the method of Hohorst,[20] pyruvate as described by Bücher *et al.*,[21] α-ketoglutarate by the method of Bergmeyer and Bernt[22] and dihydroxyacetone phosphate by the method of Bücher and Hohorst.[23] 3-phosphoglycerate was determined with phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase.[24] For determination of enzyme activities liver samples cooled in an ice bath were homogenized in 0·15 M phosphate buffer pH 7·2 by means of an Ultraturrax homogenizer. The supernatants obtained after centrifugation at 80,000 *g* for 10 min were used for assay of enzyme activities. The extracts for assay of glycerol 1-phosphate oxidase were prepared as described before. Appropriate precautions were taken to prepare homogenates under cold conditions.[10] Enzyme activities were assayed by standard procedures.[17] Protein was measured according to the Biuret method (readings multiplied by the factor of 17·5).[25] The method of Kreutz[26] was used to determine neutral fat by measuring the amount of glycerol enzymatically.

RESULTS

Experiments in vivo *with Halothane and TFA*

The results of Halothane and TFA treatment are presented in Tables 1–3, together with the results of control animals.

TABLE 1. RELATIVE LIVER WEIGHT AND CONTENT OF PROTEIN, GLYCOGEN AND NEUTRAL FAT IN LIVERS OF RATS TREATED WITH TRIFLUOROACETATE OR HALOTHANE COMPARED WITH UNTREATED ANIMALS (DOSAGE AND EXPERIMENTAL CONDITIONS SEE TABLE 2)

Constituents	Untreated	Treated with Trifluoroacetate (4–5 days treatment)	Halothane (14–17 days treatment)
Soluble protein (mg/g wet wt.)	88 ± 1·6	93	86
Glycogen (μmoles glucose/ g wet wt.)	301 ± 25	230	240
Neutral fat μmoles/g wet wt.)	10 ± 0·9	11	18
% liver wt. to body wt.	3·7	5·3	4·9

TABLE 2. ACTIVITIES OF SOME IMPORTANT ENZYMES IN RAT LIVERS, TREATED WITH TRIFLUOROACETATE OR HALOTHANE AND COMPARED WITH LIVERS OF UNTREATED ANIMALS

Enzymes (IU × 10^2/g wet wt.)	Untreated†	Experimental conditions* Trifluoroacetate treatment (5 days)	Halothane treatment (14–17 days)
Pyruvate kinase	1200 ± 50	700	710
Phosphoglycerate kinase	14400 ± 600	13000	12300
Glycerol 1-phosphate oxidase	16 ± 1·4	36	30
Glycerol-phosphate dehydrogenase	5600 ± 130	5400	5300
Malic enzyme	85 ± 10	88	150
Glucose 6-phosphate dehydrogenase	240 ± 10	200	210
Glyceraldehyde 3-phosphate-dehydrogenase	9300 ± 600	8900	7000
Enolase	2800 ± 140	2600	
Malate dehydrogenase	29000 ± 1400	26000	32000
Isocitrate dehydrogenase	2000 ± 7	1800	1900
NADPH–oxidase	15	16	

* Trifluoroacetate; 130 μmoles/100 g rat/24 hr contained in the drinking water; Halothane; 0·8–1 per cent in O_2 atmosphere, 1 hr daily. (The selection of enzymes measured in these series followed the results in Ref. 10.)

† Values represent means ± S.E.M.

TABLE 3. CONTENT OF SOME IMPORTANT SUBSTRATES IN LIVERS OF UNTREATED RATS COMPARED WITH THOSE AFTER TREATMENT WITH TRIFLUOROACETATE OR HALOTHANE

Substrate (n moles g/wet wt.)	Untreated	Halothane	Treated with trifluoroacetate 1 day	Treated with trifluoroacetate 4 and 5 days	Per cent changes (untreated against TFA 4 and 5 days)
Lactate	1280 ± 73	530	600	415 ± 35	−71
Pyruvate	128 ± 15	34	32	23 ± 3	−85
Glycerol 1-phosphate	248 ± 10	220	230	172 ± 18	−36
Dihydroxy acetone phosphate	35 ± 1·6	24	23	21 ± 2	−45
3-phosphoglycerate	242 ± 13	180	137	160 ± 23	−32
Glucose 6-phosphate	377 ± 18	230	310	250 ± 22	−20
Glucose	8200 ± 200	7000	7500	7000 ± 300	−15
α-ketoglutarate	82 ± 11	75	72	135 ± 22	+60
Malate	440 ± 68	600	575	930 ± 100	+110
Lactate–Pyruvate	10	16	19	18	
Glycerophosphate–dihydroxy acetone–phosphate	7	9	10	8	
ATP–ADP	3·3	2·8	3·6	3·5	
Adenine nucleotides (sum)	3600	3700	3100	3360	

* For experimental conditions see text.
Values represent means ± S.E.M.

Halothane. The nature of the changes effected by Halothane is similar to results reported earlier for mice.[10] The relative liver weight increased by 30 per cent. There was no change in protein content. The content of neutral fat increased by 75–80 per cent, whereas the glycogen content was slightly diminished by about 20 per cent.

The most striking changes in enzyme activities were a 40 per cent decrease in pyruvate kinase, an 80 per cent rise in malic enzyme and a 100 per cent rise in glycerol 1-phosphate oxidase activity. The activity of the other enzymes assayed did not change significantly compared with controls. There was a decrease in the content of glycerol 1-phosphate, dihydroxyacetone phosphate and 3-phosphoglycerate and especially of lactate and pyruvate. No significant change in the content of glucose, α-ketoglutarate, total adenine nucleotides or in the ATP–ADP ratio was found, whereas malate content increased by 35 per cent.

Trifluoroacetate. TFA. As seen from Tables 1–3, TFA exerts changes in rat liver similar to those observed after Halothane, even though the rats were treated for a considerably shorter time. After 5 days of TFA treatment the relative liver wt. had increased by 43 per cent. As with Halothane treatment protein content did not change, but a 20 per cent decrease in glycogen content was noted. However, in contrast to Halothane treatment no change in neutral fat was observed. The effect of TFA on enzyme activities (Table 2) resembled the results of Halothane treatment with the exception of malic enzyme, which remained the same as control values. The 42 per cent fall in pyruvate kinase activity and the 125 per cent rise in glycerol 1-phosphate oxidase activity were remarkably similar in extent to the changes induced by Halothane.

It is noteworthy that TFA did not augment the activity of NADPH oxidase, the enzyme usually taken as microsomal marker. This is in contrast to the general stimulation of this enzyme by many other drugs which cause liver enlargement.[27,28] Table 3 shows the results of substrates estimated in liver after rats had received TFA for 1, 4 or 5 days. As in experiments with Halothane there was a fall in the level of triose phosphates, with greater decreases in lactate and pyruvate.

These changes in substrate levels were apparent after only 1 day of TFA administration, indicating its rapid action on liver. In some of the treated animals a rise in the intracellular content of malate and α-ketoglutarate in liver was noted after the fifth day of treatment. The content of glucose and total adenine nucleotides and the ATP–ADP ratio did not change.

Amount of fluorine in liver and plasma (TFA treatment)

The amount of organically bound fluorine in plasma and liver reached a steady level on the second day with a 1:1 ratio of liver to plasma concentration, which declined on days 4 and 5 (Table 4). Inorganic fluoride amounted to less than 1 per cent

TABLE 4. CONTENT AND CONCENTRATION OF TRIFLUOROACETATE IN LIVERS AND PLASMA OF RATS AFTER A DAILY DOSE OF 130 μmoles TRIFLUOROACETATE/100 g RAT/24 HR GIVEN IN DRINKING WATER. (SINGLE ESTIMATIONS ON TWO DIFFERENT ANIMALS)

	Content of trifluoroacetate (μmoles/g liver or ml blood)				
Days of treatment	1	2	3	4	5
Liver	0·9	1·0	1·3	1·1	1·5
	0·75	1·0	1·2	1·0	1·4
Plasma	0·7	0·8	1·1	1·06	1·3
	0·75	1·0	1·25	0·6	1·1

of total fluorine. The average daily amount of TFA taken up from the drinking water by a rat weighing 200 g is about 260 μmoles. Thus the fluorine content of the liver or of the plasma represents about 10 per cent of the administered amount of TFA. Part of TFA may be stored or may form a complex with other compounds in the tissues. Stier has described two different *k*-values for the elimination of TFA.[8]

Effects of trifluoroacetate on isolated perfused rat liver

Because of the rapid action of TFA, its direct effect on liver *in vitro* was investigated by the technique of liver perfusion. Sixty min after the start of perfusion, 200 μmoles TFA (diluted in 0·9% NaCl solution and neutralized) were infused per hr into 100 ml of perfusion medium. About 10 min after starting the TFA infusion, the levels of lactate and pyruvate in the medium decreased. Because the pyruvate content of the medium decreased faster than that of lactate the lactate–pyruvate ratio increased with time (Fig. 1). There was only a slight increase in the level of glucose under the influence of TFA, whereas the content of urea did not change.

Metabolites in liver and perfusion medium. The immediate decrease in the content of lactate and pyruvate in the perfusion medium is due to a higher rate of uptake and higher turnover of these substrates in the presence of TFA. This could be demonstrated in experiments where 500 μmoles of lactate per 60 min was infused together

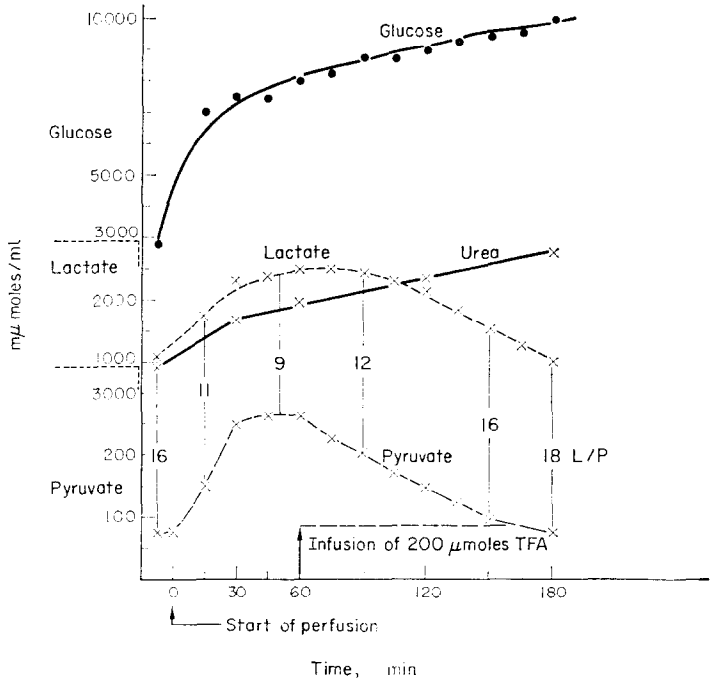

FIG. 1. Changes of substrate content in the perfusion medium during liver perfusion (see Methods). After equilibration of 60 min, 200 μmoles of trifluoroacetate are infused continuously/hr/100 ml medium.

with TFA. The rate of uptake of lactate was in the order of 40 μmoles/g of liver/hr. This is in contrast to control experiments (normal fed rats without TFA infusion), where the lactate uptake is about 20 μmoles/g/hr. At the end of perfusion (180 and

TABLE 5. THE CONTENT OF SOME METABOLITES IN ISOLATED PERFUSED LIVERS INFLUENCED BY TRIFLUOROACETATE (TFA) UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Experimental conditions	Lactate	Pyruvate	Glycero-phosphate	Malate	Keto-glutarate	L/P	G/D	ADP/ATP
				PART A				
Fed rats								
Control	2150	230	370	203	300	10	8	3·4
TFA (200)	1100	73	490	233	300	15	10	3·8
TFA (400)	1000	38	380	187	255	29	11	3·1
TFA pretreated	358	40	373	237	410	9	6	3
Control + lactate (500)	4100	340	590	305	360	12	10	3·1
TFA (200) + lactate (500)	2000	154	580	670	470	13	9	3·2
TFA pretreated + lactate (500)	817	63	306	308	408	13	7	2·7
				PART B				
Starved rats								
Control	588	112	45	80	115	5·3	3	2·2
Control + lactate (1000)	2254	203	247	211	608	11	8	3·0
TFA (400) lactate (1000)	1380	29·5	280	240	668	47	9	2·5
Hyperthyreosis	970	93	145	165		10·8	3·5	2·3

Part A, fed rats; Part B, starved rats. (Direct effects by TFA infusion of 200 or 400 μmoles/hr or after pretreatment, see Methods). Lactate infusion; 500 or 1000 μmoles/hr. Time of starvation: 16–20 hr. Content of substrates in nmoles/g wet wt. (Number of experiments, control n = 25; different experimental conditions n = 4–10.)

120 min after starting infusions) a sample of the perfused liver was removed with clamps, pre-cooled in liquid nitrogen for the analysis of the content of some intracellular metabolites. The content of lactate was in the same range as in the medium (comparing μmoles/g liver and/ml medium respectively). This demonstrates that not only the uptake of lactate, but its turnover was also enhanced.

Table 5 shows the content of those substrates in liver which are markedly influenced by TFA. Values for controls are also given and these results were obtained from rats in the same metabolic state but receiving no TFA. In addition, the substrate content of

TABLE 6. INTRACELLULAR CONTENT OF CERTAIN PHOSPHORYLATED INTERMEDIATES IN PERFUSED RAT LIVERS

Experimental conditions	3-Phosphoglycerate	2-Phosphoglycerate	Phosphoenol pyruvate	3-P-Glycerate/2-P-glycerate
Fed control	154	15	58	10
Fed + TFA	88	10·4	33	8·4
Fed + lactate (600 μmoles/60 min) + TFA	240	19·1	64	12·6
Starved rat + lactate	289	28·4	142·4	10·2
Starved rat + lactate (1 mmole/60 min) + TFA	114	9·2	47	12·2
Starved rat + pyruvate (1 mmole/60 min)	491	52	308	9·4
Starved rat + TFA + pyruvate (1 mmole/60 min)	291	26·2	162	11·1

400 μmoles trifluoroacetate (TFA)/60 min infused after 15–30 min equilibration.
Substrates infused after 60 min of start of perfusion.
Results expressed as nmoles/g wet wt.

livers from hyperthyroid rats and starved rats are included, since the lactate uptake and turnover is greatly stimulated under these conditions.[29,30] The predominant effect of TFA was on enhancement of uptake and turnover of lactate and pyruvate. This increased turnover results in low levels of these two substrates in liver as well as in perfusion medium, even during lactate infusion. Owing to the greater diminution of pyruvate content, the lactate to pyruvate ratio is higher than in the controls. This is clearly seen in the perfusion experiments with livers from starved rats. After starvation, cytoplasmic substrates in liver are generally more oxidized and the lactate to pyruvate ratio is low. Infusion of L-lactate brings this ratio within the normal range (i.e. 10–11). However infusion of TFA together with L-lactate in starved rats results in more than a four-fold increase in lactate–pyruvate ratio (Table 6).

In contrast to the sharp decrease in the intracellular content of lactate and pyruvate, α-glycerophosphate, malate and α-ketoglutarate levels are constant and approximate the levels of these substrates found in control experiments. However the level of these intermediates is high in relation to very low levels of lactate and pyruvate. TFA also causes a small but significant lowering of 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate levels in the liver. The diminution of these intermediates

is due to depletion of key gluconeogenic substrates such as lactate and pyruvate. Infusion of lactate or pyruvate brings the levels of triose phosphates to normal or even higher values than controls. Glycogen breakdown was unaffected by TFA and it could not maintain or replenish the levels of these intermediates.

Effect of TFA on mitochondrial metabolism

It is well established that the metabolism of liver mitochondria is under the control of at least two endocrine glands, the thyroid gland and the adrenal glands. Thyroidectomy almost completely abolishes the activity of the mitochondrial enzyme, glycerol 1-phosphate oxidase.[31] A single injection of triiodothyronine into these rats then stimulates mitochondrial metabolism, including a pronounced elevation of the activity of glycerol 1-phosphate oxidase. In contrast, after adrenalectomy the activity of this enzyme is about 60 per cent higher.[32]

As seen in Table 2, the most striking effect of TFA on enzyme activities is the rise in glycerol 1-phosphate oxidase. In order to determine if TFA acts exclusively on mitochondrial metabolism, rats were adrenalectomized before TFA treatment. The activities of glycerol 1-phosphate oxidase and pyruvate kinase as well as the content of lactate, pyruvate and malate were determined in livers of these rats, since TFA also alters these parameters. The only major effect of both adrenalectomy and thyroidectomy on TFA treatment was the alteration in the activity of glycerol 1-phosphate oxidase (Table 7). Adrenalectomy augmented the enzyme activity, while thyroidectomy prevented the rise in activity seen after TFA treatment.

TABLE 7. CHANGES OF THE ACTIVITIES OF PYRUVATE KINASE AND α-GLYCEROPHOSPHATE OXIDASE IN LIVERS OF ADRENALECTOMIZED OR THYROIDECTOMIZED RATS DURING TREATMENT OF TRIFLUOROACETATE (TFA)

Experimental conditions	Enzymes (IU × 10^2 per g wet wt.) α GPox	PK
Untreated	16	1200
TFA alone	36	700
Adrenalectomy	25	1200
Adrenalectomy + TFA	75	700
Thyroidectomy + TFA	<5	1050

TFA given in drinking water, see Methods.

DISCUSSION

The main purpose of this investigation was to clarify that the metabolite of Halothane, trifluoroacetate (TFA),[33,34] is responsible for the main effects of Halothane on liver growth and metabolism. TFA produces the same changes in liver metabolism as Halothane, except that neutral fat content and malic enzyme activity were unaffected. However the effects of TFA were more immediate than those of Halothane and perfusion experiments demonstrated that TFA can directly influence liver metabolism.

Two main effects of TFA observed in the present study were a significant increase in glycerol 1-phosphate oxidase activity and an enhancement in the turnover of lactate and pyruvate in liver. These changes were noted after the first day of TFA treatment. TFA caused about 100 per cent stimulation in the turnover and uptake of lactate. This stimulation in metabolism of lactate and pyruvate by TFA is surprising since fluorinated compounds are usually associated with inhibition of metabolic processes,[35,36] rather than acceleration. TFA does not appear to depress the glycolytic or gluconeogenic capacity of the liver, but increases the oxygen consumption. Although there are reports that Halothane in higher concentrations uncouples oxidative phosphorylation,[37] it appears unlikely that the results reported in our studies were due to uncoupling of oxidative phosphorylation. The constancy of the ATP–ADP ratio after TFA treatment both in the perfusion and *in vivo* experiments supports this view.

There have been various reports suggesting that Halothane in low concentrations inhibits state III respiration of rat liver mitochondria in a reversible manner.[38,39] Recent studies by Harris *et al.*[37] suggest that Halothane inhibits oxidation of NAD linked substrates by inhibiting the enzymic activity of complex I of the electron transfer chain (NADH,–CoQ reductase).

Although it is rather difficult to make a direct comparison of the effects noted after Halothane treatment of isolated mitochondria, perfusion or *in vivo* studies, it appears unlikely that Halothane or its metabolite TFA suppressed the oxidation of NAD linked substrates in our studies. No significant changes in some of the tricarboxylic acid cycle intermediates such as citrate, α-ketoglutarate or malate were noted in perfusion studies. Supplying substrates such as pyruvate and lactate did not result in uneven accumulation of either of these substrates in the presence of TFA. Since mitochondria were exposed to a rather high concentration of Halothane in the above mentioned studies, it could cause a conformational change in mitochondrial structure which may be detrimental to enzymic activity.[37]

The enhancement of lactate and pyruvate turnover and uptake by TFA without apparent stimulation of the tricarboxylic acid cycle or glucose synthesis is interesting. TFA also caused a decrease in the intracellular content of 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolypyruvate. This suggests an increase in gluconeogenic flux for the synthesis of dihydroxyacetone phosphate, which is then converted into α-glycerophosphate. The increase in the activity of α-glycerophosphate oxidase, noted after 24 hr treatment of rats with TFA, could be due to the continuous supply of α-glycerophosphate to mitochondria. The supply of reducing equivalents to mitochondria via α-glycerophosphate in the presence of TFA may be due to an increased oxidation of hydrogen by mitochondria. It can also result from the operation of a secondary electron transfer mechanism as a consequence of TFA treatment. The increased oxygen consumption by liver may also be associated with increased mitochondrial metabolism caused by TFA.

In vivo studies on rats pre-treated for various lengths of time with TFA show that α-glycerophosphate content is lowered to the same extent as in hyperthyroid rats. This is due to increased activity of α-glycerophosphate oxidase and also possibly due to substrate exchange with various pools in the whole animal. However in the perfusion experiments where TFA is infused, the activity of α-glycerophosphate oxidase is not

enhanced during this short time. There is also no possibility of substrate exchange to the same extent as in the whole animal. This may explain the higher levels of α-glycerophosphate generally noted in perfusion experiments as compared to *in vivo* studies. Contrary to Platt and Cockrill,[40] our results have shown that Halothane and its metabolite TFA exert their metabolic effects on mitochondria. Our studies also show the importance of hydrogen transfer via the "α-glycerophosphate oxidase system" under the stress of various pharmacological compounds or different (i.e. hormonal) metabolic states.

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