The Influence of Repeated Cold Exposure to VFAs Metabolism in Intact Sheep Liver Slices

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Abstract In order to investigate the metabolic use of VFAs in cold-exposed sheep liver, fifteen Merino sheep were repeatedly exposed to an ambient temperature of 2°C. On the 1st, 4th and 7th day of cold exposure, ^{14}C incorporation from ^{14}C acetate, propionate and butyrate into CO_2 , glucose, ketone bodies, cholesterol ester, triglyceride, free cholesterol, NEFA and phospholipid in the liver slices were observed. ^{14}C incorporation from ^{14}C -acetate into CO_2 , glucose, ketone bodies tended to increase and into cholesterol ester, triglyceride, free cholesterol, NEFA tended to decrease on the 1st, 4th or 7th day of cold exposure, but, was changed to decrease into ketone bodies on the 4th day. ^{14}C incorporation from ^{14}C -propionate into CO_2 , glucose, triglyceride, NEFA, phospholipid tended to increase and into cholesterol ester, free cholesterol tended to decrease on the 1st or 4th day of cold exposure, but was changed to increase into free cholesterol on the 4th day. ^{14}C incorporation from ^{14}C -butyrate into CO_2 , glucose, ketone bodies, triglyceride, free cholesterol, NEFA, phospholipid tended to increase and into cholesterol ester tended to decrease on the 1st, 4th or 7th day of cold exposure, but, was changed to increase into cholesterol ester on the 4th day. The results of this experiment indicated that VFAs could be used in various metabolic purpose on cold exposure in sheep liver slices. Further, it was also indicated that each VFA showed their own characteristic ways of metabolic use in such a case of this experiment.

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This study was designed to investigate the metabolic fate of volatile fatty acids in cold-exposed sheep liver in vitro. In the ruminant, much is still unknown about the origin of cold-induced thermogenesis which is the lack of entire energy demand on cold exposure. Recent results indicated that the utilization of NEFA^{3,4)}, VFAs²⁾, glucose^{1,21)} and glycerol¹⁰⁾ increases in cold-exposed ruminant animals, also, these results suggested that the each contribution of these substrates could not be efficient to cold-induced thermogenesis individually. It can be considered that cold-induced thermogenesis should be a result of complex mixture of various metabolic accelerations on cold exposure⁸⁾. In this respect, VFAs, which is the major energy source in ruminant animals^{5,23)}, is supposed to be the minor contribution to cold-induced thermogenesis¹⁵⁾, because of their food depending exogenous character^{7,16)}, however, some intermetabolic character of each VFA should be looked once again. Cold accelerated metabolic increase must need a large quantity of some useful intermetabolites, therefore, we were very interested in the metabolic use of VFAs on cold exposed ruminant animals.

Materials and Methods

Thirty Merino sheep, weighing about 45 kg, were used. The animals were kept with a temperature of 20°C for more than 20 days. Subsequently, the animals were exposed to cold environment with a temperature of 2°C for 1, 4 and 7 days from 12:00 to 24:00 repeatedly in every day. At the end of 1st, 4th and 7th day of cold exposure, the animals were slaughtered at 24:00 in every experiment, and fresh liver was obtained as 0.4 mm thick slices. During the experiment, 600 g of alfalfa hay and 400 g of commercial concentrate were given once a day at 8:00. Water was available *ad libitum*.

Analytical procedures were almost the same as previously reported²². That is, liver slices incubation was carried out in 50 ml Erlenmyer flasks fitted with 2 ml center well. Each flask contained 2 g of liver slices, 10 ml of Krebs-Ringer bicarbonate buffer (pH 7.4), 100 μ moles (1 μ Ci) of 14 C-labelled short-chain fatty acid. The flasks were closed with serum caps after gassing with O₂+CO₂ (95:5). Incubation (3 flasks for each animals: 14 C-glucose, 14 C-ketone bodies and CO₂ assay were carried in different flasks) was carried out for 3 hr. at 38°C during the shaking at 100 strokes/min. The 14 CO₂ assay was made after incubation by injecting 1 ml of Hyamine into the center wells of the Erlenmyer flask 0.5 ml of 10 N H₂SO₄ into the medium. The flasks were shaken for 30 min in ice prior to quantitative removal of Hyamine for estimation of total radioactivity in the trapped CO₂. The glucose was isolated from incubation medium by the method of JONES¹³. Ketone bodies fraction was analyzed by the method of May and FELTS²⁰. Total lipids in the tissue and medium were extracted with a mixture of chloroform and metanol (2:1) by the method of FOLCH, LESS and SLOANE-STANLY¹¹. That is, separation of the lipid fraction was effected on sillicic acid columns. Successive fractions were eluted with 2% ethyl ether in n-hexane (fraction A: cholesterol ester), 10% ethyl ether in n-hexane (fraction B: triglyceride), 50% ethyl ether in n-hexane (fraction C: free cholesterol), 25% methanol in ethyl ether (fraction D: NEFA) and pure methanol (fraction E: phospholipid).

The 14 C activity in each of these fractions was measured by liquid scintillation spectrometer using 15 ml of scintillation solution (toluene composed of 0.01% POPOP and 0.5% PPO). Total nitrogen in the liver slices was determined by the micro-Kjeldahl method, and the results were expressed as mean μ mCi/3hr./g of tissue nitrogen $\pm$ S.D. (standard deviation). Each study involved 5 animals.

Results

1. Acetate metabolism

The representative result of 14 C incorporation from 14 C-acetate into CO₂, glucose, ketone bodies, cholesterol ester, triglyceride, free cholesterol, NEFA and phospholipid on the 1st, 4th and 7th day of cold exposure were shown in Table 1 with their control value respectively.

Table 1. The influence of repeated cold exposure to acetate metabolism in intact sheep liver slices.

Days of exposure	Cold exposure	Transfer of ^{14}C from 1- ^{14}C -acetate into							
		CO_2	Glucose	Ketone bodies	Fraction A*	Fraction B*	Fraction C*	Fraction D*	Fraction E*
1	-	181.2 $\pm$ 7.2**	17.2 $\pm$ 0.6	8.0 $\pm$ 0.3	5.5 $\pm$ 0.2	2.6 $\pm$ 0.1	6.3 $\pm$ 0.2	1.1 $\pm$ 0.0	11.6 $\pm$ 0.5
	+	226.8 $\pm$ 10.3	34.7 $\pm$ 1.5	12.7 $\pm$ 0.5	3.9 $\pm$ 0.1	1.0 $\pm$ 0.0	2.9 $\pm$ 0.1	1.6 $\pm$ 0.0	11.2 $\pm$ 0.6
4	-	203.5 $\pm$ 8.2	23.1 $\pm$ 0.9	11.4 $\pm$ 0.4	4.2 $\pm$ 0.1	2.6 $\pm$ 0.1	4.1 $\pm$ 0.2	1.5 $\pm$ 0.0	11.4 $\pm$ 0.6
	+	203.8 $\pm$ 9.2	33.6 $\pm$ 1.0	8.6 $\pm$ 0.3	4.3 $\pm$ 0.1	2.6 $\pm$ 0.1	3.0 $\pm$ 0.1	0.7 $\pm$ 0.0	11.7 $\pm$ 0.5
7	-	177.4 $\pm$ 9.2	17.5 $\pm$ 0.7	7.9 $\pm$ 0.4	5.5 $\pm$ 0.2	2.5 $\pm$ 0.1	4.5 $\pm$ 0.2	0.9 $\pm$ 0.0	11.2 $\pm$ 0.5
	+	176.6 $\pm$ 9.7	17.8 $\pm$ 0.6	8.2 $\pm$ 0.4	5.4 $\pm$ 0.2	2.6 $\pm$ 0.1	4.6 $\pm$ 0.2	0.2 $\pm$ 0.0	10.9 $\pm$ 0.8

Slices (2g) were incubated in Krebs-Ringer bicarbonate buffer with ^{14}C -acetate (100 μmoles , 1 μCi). * Fraction A, B, C, D and E were composed of cholesterol ester, triglyceride, free cholesterol, NEFA and phospholipid respectively. ** Mean μmCi per 100 mg of tissue $\bar{N} \pm \text{S.D.}$ in 5 animals.

Almost the every change was observed on the 1st day of cold exposure. That is, on the 1st day of cold exposure, ^{14}C incorporation from ^{14}C -acetate into CO_2 , glucose and ketone bodies increased, then ^{14}C incorporation into cholesterol ester, triglyceride, free cholesterol and NEFA decreased. On the 4th day of cold exposure, ^{14}C incorporation into glucose still remained to increase, and ^{14}C incorporation into free cholesterol and NEFA also still remained to decrease. But the increase of ^{14}C incorporation into ketone bodies was changed to decrease, and the decrease of ^{14}C incorporation into cholesterol ester was ceased. On the 7th day of cold exposure, ^{14}C incorporation into NEFA still more remained to decrease and the others were ceased. No other changes were observed so far.

Table 2. The influence of repeated cold exposure to propionate metabolism in intact sheep liver slices.

Days of exposure	Cold exposure	Transfer of ^{14}C from 1- ^{14}C -propionate into							
		CO_2	Glucose	Ketone bodies	Fraction A*	Fraction B*	Fraction C*	Fraction D*	Fraction E*
1	-	402.7 $\pm$ 16.3**	84.3 $\pm$ 3.0	0.3 $\pm$ 0.0	2.2 $\pm$ 0.1	1.0 $\pm$ 0.0	1.5 $\pm$ 0.0	1.1 $\pm$ 0.0	4.0 $\pm$ 0.2
	+	523.1 $\pm$ 21.6	119.6 $\pm$ 4.2	0.3 $\pm$ 0.0	1.6 $\pm$ 0.0	1.9 $\pm$ 0.0	0.7 $\pm$ 0.0	1.7 $\pm$ 0.0	4.9 $\pm$ 0.2
4	-	480.4 $\pm$ 21.0	94.6 $\pm$ 3.7	0.3 $\pm$ 0.0	1.7 $\pm$ 0.0	1.4 $\pm$ 0.0	0.9 $\pm$ 0.0	1.4 $\pm$ 0.0	4.6 $\pm$ 0.2
	+	505.5 $\pm$ 19.5	109.1 $\pm$ 3.5	0.3 $\pm$ 0.0	1.7 $\pm$ 0.0	1.4 $\pm$ 0.0	1.6 $\pm$ 0.0	1.8 $\pm$ 0.0	4.5 $\pm$ 0.2
7	-	394.3 $\pm$ 17.8	86.2 $\pm$ 4.2	0.3 $\pm$ 0.0	2.1 $\pm$ 0.0	1.1 $\pm$ 0.0	1.5 $\pm$ 0.0	1.1 $\pm$ 0.0	3.9 $\pm$ 0.2
	+	402.4 $\pm$ 19.6	88.5 $\pm$ 3.9	0.3 $\pm$ 0.0	2.1 $\pm$ 0.0	1.0 $\pm$ 0.0	1.6 $\pm$ 0.0	1.2 $\pm$ 0.0	4.1 $\pm$ 0.2

Slices (2g) were incubated in Krebs-Ringer bicarbonate buffer with ^{14}C -propionic acid (100 μmoles , 1 μCi). * Fraction A, B, C, D and E were composed of cholesterol ester, triglyceride, free cholesterol, NEFA and phospholipid respectively. ** Mean μmCi per 100 mg of tissue $\bar{N} \pm \text{S.D.}$ in 5 animals.

2. Propionate metabolism

The representative result of ^{14}C incorporation from ^{14}C -propionate into CO_2 , glucose, ketone bodies, cholesterol ester, triglycerid, free cholesterol, NEFA and phospholipid on the 1st, 4th and 7th day of cold exposure are shown in Table 2 with their control value respectively.

Almost the every change was observed on the 1st day of cold exposure as was acetate. That is, on the 1st day of cold exposure, ^{14}C incorporation from ^{14}C -propionate into CO_2 , glucose, triglyceride, NEFA and phospholipid increased, then ^{14}C incorporation into cholesterol ester and free cholesterol decreased. On the 4th day of cold exposure, the decrease of ^{14}C incorporation into free cholesterol was changed to increase, and ^{14}C incorporation into NEFA still remained to increase. The other changes were ceased so far.

3. butyrate metabolism

The representative result of ^{14}C incorporation from ^{14}C -butyrate into CO_2 , glucose, ketone bodies, cholesterol ester, triglyceride, free cholesterol, NEFA and phospholipid on the 1st, 4th and 7th day of cold exposure are shown in Table 3 with their control value respectively.

Table 3. The influence of repeated cold exposure to butyrate metabolism in intact sheep liver slices.

Days of exposure	Cold exposure	Transfer of ¹⁴ C from 1- ¹⁴ C-butyrate into							
		CO ₂	Glucose	Ketone bodies	Fraction A*	Fraction B*	Fraction C*	Fraction D*	Fraction E*
1	—	310.7±11.3**	20.8±0.7	16.2±0.6	2.8±0.1	3.3±0.1	2.9±0.1	13.6±0.4	9.3±0.3
	+	406.8±19.2	49.8±1.9	24.8±1.0	1.6±0.0	5.2±0.2	5.4±0.2	24.9±0.7	16.1±0.5
4	—	310.1± 9.6	32.5±1.3	18.4±0.7	2.5±0.0	4.7±0.2	3.3±0.1	14.1±0.6	12.6±0.4
	+	361.6±10.8	40.6±1.7	21.5±0.8	3.1±0.1	5.5±0.2	4.9±0.2	18.6±0.5	17.1±0.5
7	—	315.2±12.6	27.3±0.8	15.7±0.7	2.7±0.1	3.9±0.1	2.9±0.1	13.5±0.6	10.5±0.4
	+	347.7±11.5	26.7±1.2	16.2±0.8	2.8±0.1	4.3±0.2	3.3±0.1	14.9±0.6	13.3±0.4

Slices (2g) were incubated in Krebs-Ringer bicarbonate buffer with ^{14}C -butyrate (100 μ moles, 1 μCi). *Fraction A, B, C, D and E were composed of cholesterol ester, triglyceride, free cholesterol, NEFA and phospholipid respectively. **Mean μmCi per 100 mg of tissue $\text{N}\pm\text{S.D.}$ in 5 animals.

In contrast with acetate or propionate metabolism, there observed various changes on the each stage of cold exposure, or with substrates. On the 1st day of cold exposure, ^{14}C incorporation from ^{14}C -butyrate into CO_2 , glucose, ketone bodies, triglyceride, free cholesterol, NEFA and phospholipid increased, then ^{14}C incorporation into cholesterol ester decreased. On the 4th day of cold exposure, ^{14}C incorporation into CO_2 , glucose, triglyceride, free cholesterol, NEFA and phospholipid still remained to increase, then the increase of ^{14}C incorporation into ketone bodies was ceased. But, the

decrease of ^{14}C incorporation into cholesterol ester reversed to increase. On the 7th day of cold exposure, ^{14}C incorporation into CO_2 , triglyceride, free cholesterol and phospholipid still remained to increase. The other changes were all ceased so far.

Discussion

In this experiment, normal feeding intact sheep were exposed to cold repeatedly for 7 days. It should be considered that shivering thermogenesis would be dominant to non-shivering thermogenesis during the period of this 7 days of cold exposure⁸⁾. But, it could be also considered that non-shivering thermogenesis plays some parts in cold-induced thermogenesis in this experiment¹²⁾. Among some of the non-shivering thermogenic organs, liver is known to be one of the major heat productive organs, there, some significant changes were expected to be observed with VFAs metabolism in this experiment.

First, ^{14}C incorporation from ^{14}C -VFAs into CO_2 increased in some stage of this experiment. Taking VFAs for direct energy source to cold-induced thermogenesis, the results of this experiment suggested the possibility of contribution from VFAs to cold induced thermogenesis. But, it also suggested that this contribution from VFAs could not be sufficient for paying the lack of entire energy demand on cold exposure, because, the rate of increasing ^{14}C incorporation from ^{14}C -VFAs into CO_2 was much less than the rate of increasing whole body heat production as previously reported⁹⁾. There also observed different pattern of ^{14}C incorporation between acetate, propionate and butyrate. ^{14}C incorporation from ^{14}C -acetate and propionate into CO_2 increased only on the 1st day of cold exposure, however, ^{14}C incorporation from ^{14}C -butyrate increased through the 7 days of cold exposure. This might be a reflection of some different metabolic use of each VFA, but, details would be made obvious by another studies.

Secondly, Different catabolic patterns were observed with acetate and butyrate metabolism. ^{14}C incorporation from ^{14}C -acetate and butyrate into ketone bodies increased on the 1st day of cold exposure, but, it was changed to decrease on the 4th day of cold exposure with acetate. Owing to the great energy demand on cold exposure, the unbalanced energy expenditure by glycolysis and lipolysis produced so much of ketone bodies¹⁸⁾. It was suggested that the formation of ketone bodies from acetate and butyrate would be accelerated by cold exposure.

Thirdly, in contrast with above mentioned oxidative catabolism, VFAs can be glucogenic precursor. ^{14}C incorporation from ^{14}C -VFAs into glucose increased in some stage of this experiment. This result was borne out by previous report of increasing plasma glucose turnover rate in cold exposed sheep^{1,21)}. As for propionate, propionic acid is known to be one of the major glucogenic precursor in the ruminant^{6,17)}. But, it was indicated¹⁰⁾ that propionic acid could not so greatly contribute to cold induced glucose demand^{1,21)} which is almost equal to gluconeogenesis in the ruminant, because of the same reason as VFAs to cold induced thermogenesis. This point of view was confirmed with the result of this experiment. That is, the increase of ^{14}C incorporation into glucose from ^{14}C -propionate was observed only on the 1st day of cold

exposure. On the other hand, acetate and butyrate have been known to be ketogenic substrates, and, these have been supposed not to be a substantial precursor to gluconeogenesis, even if the C-skeleton might be incorporated into glucose in the course of glucose synthesis. As a fact, considerable value of ^{14}C incorporation from ^{14}C -acetate and butyrate was observed on the 1st and 4th day of cold exposure in this experiment. The result of this experiment suggested some possible role of acetate and butyrate in the emergent demand of gluconeogenesis.

Fourthly, it was indicated that lipogenesis was effected by cold exposure. ^{14}C incorporation from ^{14}C -acetate into each of lipid fraction generally decreased without into phospholipid, but, ^{14}C incorporation from ^{14}C -propionate and butyrate tended to increase without into cholesterol ester. This result, reflecting the intermetabolic significance of acetate, suggested that there would be more essential metabolic use of acetate than to be used for lipogenesis on cold exposure. Further, propionate and butyrate seemed to have a still more important lipogenic significance on cold exposure. It is well known that lipogenesis increases in cold-exposed animals as well as lipolysis¹⁹⁾. One of the reason of this phenomenon should be due to increasing heat production and decreasing heat loss. With regard to this point of view, it could be said that acetate might be heat productive and propionate, butyrate might be heat protective. Data of this experiment suggested that this heat protective action of propionate and butyrate would be carried out by the form of NEFA or triglyceride. Cholesterol ester and phospholipid have been known to be used to form some hormones¹⁴⁾ or body structures. Owing to cold accelerated physiological reactions in various organs, the utilization of these substrates might be increased, however, details are still unknown.

Finally, the data of this experiment suggested some possible role of VFAs metabolism in cold exposed sheep. But, to make this problem more cleanly, so many studies are desirable to be performed in the near future.

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めん羊の肝スライスにおける低級脂肪酸代謝に 対する反復寒冷暴露の影響

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寒冷暴露めん羊の熱生産過程に占める低級脂肪酸代謝の役割を検索する目的で, 反復寒冷暴露めん羊の肝スライスにおける ^{14}C 標識低級脂肪酸よりの CO_2 , グルコース, ケトン体, コレステロールエステル, トリグリセリド, 遊離コレステロール, 遊離脂肪酸及びリン脂質への ^{14}C の取込みを観察した. ^{14}C -酢酸よりの CO_2 , グルコース, ケトン体への ^{14}C の取込みは暴露後 1, 4 または 7 日目に増加し, コレステロールエステル, トリグリセリド, 遊離コレステロール, 遊離脂肪酸への取込みは同様に減少した. しかし, 暴露 4 日目にケトン体への取込みの増減は逆転した. ^{14}C -プロピオン酸よりの CO_2 , グルコース, トリグリセリド, 遊離脂肪酸, リン脂質への

^{14}C の取込みは暴露後 1 または 4 日目に増加し, コレステロールエステル, 遊離コレステロールへの取込みは同様に減少した. しかし, 暴露 4 日目に遊離コレステロールへの取込みの増減は逆転した. ^{14}C -酪酸よりの CO_2 , グルコース, ケトン体, トリグリセリド, 遊離コレステロール, 遊離脂肪酸, リン脂質への ^{14}C の取込みは暴露後 1, 4 又は 7 日目に増加し, コレステロールエステルへの取込みは減少した. しかし, 暴露 4 日目にコレステロールエステルへの取込みの増減は逆転した. 本実験の結果は寒冷暴露めん羊の肝における低級脂肪酸の代謝利用の方向を明らかにした.

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