The Influence of Insulin and of Contraction on Glucose Metabolism in the Perfused Diaphragm Muscle from Normal and Streptozotocin-Treated Rats

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1. The metabolism of [U- 14 C]glucose in perfused resting and contracting diaphragm muscle from normal rats and rats made diabetic with streptozotocin was studied in the presence and absence of insulin. 2. The incorporation of [U- 14 C]glucose into glycogen and oligosaccharides was stimulated by insulin under all experimental conditions studied. 3. In the normal perfused resting diaphragm muscle the incorporation of radioactivity from [14 C]glucose into lactate and CO_2 was not affected by insulin. 4. Periodic contractions, induced by electrical stimulation of the perfused diaphragm muscle in the absence of insulin, caused an increased incorporation of ^{14}C into glycogen and hexose phosphate esters, whereas incorporation of ^{14}C into lactate was greatly decreased. Production of $^{14}\text{CO}_2$ in the contracting muscle was not significantly different from that in resting muscle. Addition of insulin to the perfusion liquid caused a further increase in formation of [14 C]glycogen in contracting muscle to values reached in the resting muscle in the presence of insulin. Formation of [14 C]lactate was also stimulated by insulin, to values close to those found in the resting muscle in the presence of insulin. 5. In the diabetic resting muscle the rate of glucose metabolism was very low in the absence of insulin. Insulin increased formation of [14 C]glycogen to the value found in normal muscle in the absence of insulin. Production of $^{14}\text{CO}_2$ and formation of [14 C]hexose phosphate remained unchanged. 6. In the diabetic contracting muscle production of $^{14}\text{CO}_2$ was increased to values approaching those found in normal contracting muscle. Formation of [14 C]lactate and [14 C]glycogen was also increased by contraction, to normal values. Only traces of [14 C]hexose phosphate were detectable. Addition of insulin to the perfusion medium stimulated formation of [14 C]glycogen, to values found in normal contracting muscle. Production of [14 C]hexose phosphate was stimulated by insulin, to approximately the values found in the normal contracting muscle. Production of $^{14}\text{CO}_2$ and [14 C]lactate, however, was not significantly affected by insulin. 7. These results indicate that the defects of glucose metabolism observed in perfused resting diabetic diaphragm muscle can be partially corrected by contraction, and in the presence of insulin the contracting diabetic muscle has a completely normal pattern of glycogen synthesis and lactate production, but CO_2 production remains impaired.

Since the observation of Gemmill (1940, 1941) that insulin stimulated the synthesis of glycogen in the isolated rat diaphragm this preparation has been extensively used for metabolic studies. Studies with the isolated rat diaphragm in this laboratory were concerned with glucose metabolism in insulin deficiency, induced by anti-insulin serum or streptozotocin (Beloff-Chain & Rookledge, 1968).

We have now performed similar studies with a

perfused rat diaphragm preparation, in which the perfusate is recirculated and where periodic contraction can be induced by electrical stimulation, thus making possible a comparison of glucose metabolism in the resting and contracting muscle. This study complements an investigation of glucose metabolism in the normal and insulin-deficient perfused rat heart in working and non-working conditions (Chain, Mansford & Opie, 1969).

EXPERIMENTAL

Animals. The species, age and treatment of the rats was as described by Rookledge (1971). The method of treatment with streptozotocin has also been described (Beloff-Chain & Rookledge, 1968). All streptozotocin-treated rats had blood glucose concentrations greater than 250 mg/100 ml before use.

Materials. Chemicals and enzymes were obtained from the sources listed by Rookledge (1971). Insulin (Isopane quality) was obtained from Burroughs Wellcome and Co., London N.W.1, U.K., and contained less than 0.006 mg of glucagon/unit.

Analytical methods. All the analytical methods used are described by Rookledge (1971) and Beloff-Chain & Rookledge (1968).

Perfusion method and induction of contraction. Cannulation of the diaphragm and its subsequent removal from the animal to the perfusion system was performed as described by Rookledge (1971). The perfusion system was modified to allow recirculation and reoxygenation of the medium (Fig. 1).

The perfusion medium consisted of Krebs-Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) containing 5.55 mM-glucose.

A solution of [$U\text{-}^{14}C$]glucose (about 25 μ Ci in 0.1 ml, specific radioactivity about 3.0 mCi/mmol) was placed into vessel C. After cannulation of the vena cava the tissue was washed out for a period of 30 min by pumping buffer through the tissue and out of the enclosing vessel, and allowing it to run to waste by disconnecting the tubing from vessel I. During the first 20 min of the washing-out period the tissue was kept cool by using perfusate from reservoir D at 0°C. The perfusate was then changed to that from reservoir A at 37°C. The flow during the last 10 min of the washing out period was adjusted to 2 ml/min.

At 5 min before commencement of the perfusion with recirculation, about 10 ml of the effluent perfusate was collected in vessel C containing the solution of radioactive glucose. At the commencement of recirculation, the supply of perfusate was changed over from the reservoir A to vessel C.

Details of the perfusion vessel containing the diaphragm are given by Rookledge (1971). Contraction of the diaphragm was induced with a Palmer Continuously Variable Square-Wave stimulator (London N.W.9, U.K.), one connexion being made through the stainless-steel cannula and the other via the supporting platinum wire attached to the top of the diaphragm (Rookledge, 1971, Fig. 1, L and P respectively). Contraction was induced by 12 stimuli/min at 7–10 V with a pulse duration of 500 μ s. The muscle was connected to a linear displacement transducer and to the electrode by wire P; (Rookledge, 1971, Fig. 1); the magnitude of the contraction was recorded on a chart recorder. Although the pulse stimulating the contraction was somewhat longer than that usually necessary for induction of indirect stimulation, the induced contraction was shown to be essentially indirect as it could be inhibited by curare. Any preparation that showed a large decrease in the magnitude of the contraction was discarded; thus in the experiments reported, a fairly constant degree of contraction was obtained.

Preparation of samples and radioactive measurements. The aqueous ethanolic extraction of the tissue at the end of the perfusion period and quantitative radiochromatography were performed as described by Beloff-Chain *et al.* (1955). The radiochromatograms were scanned by the quantitative automatic computerized scanning device used by Chain, Lowe & Mansford (1970). The glycogen present in tissue extracts and counted on the chromatogram by this method is referred to as 'soluble' glycogen.

Fig. 1. Diagram of perfusion apparatus. A and D, reservoirs holding gassed perfusate at 37°C and 0°C respectively, with sintered glass filters; C, oxygenation chamber gassed with $O_2 + CO_2$ (95:5); B, tube for gassing perfusate; E and J, pumps to circulate perfusate; F and H, bubble traps; I, perfusion vessel containing diaphragm muscle; K, four-way connection controlled by roller clamps; G, pressure gauge.

[^{14}C]Lactic acid in the ethanol extract and perfusate was separated from other labelled intermediates by one-dimensional paper chromatography as described by Beloff-Chain *et al.* (1955). The [^{14}C]lactic acid on the chromatogram was located by radioautography. The area corresponding to [^{14}C]lactic acid was cut into small pieces and, after addition of 1.0 ml of water and 18 ml of scintillant, its radioactivity was counted in the scintillation counter, efficiency being determined by the channels-ratio method of Baillie (1960). Recovery of [^{14}C]lactic acid was between 90 and 100% by this method.

In experiments in which the total incorporation of glucose into glycogen was measured, the glycogen was extracted from the tissue by the method of Walaas & Walaas (1950), and this material is termed 'total' glycogen. The latter was digested with Diazyme (Cambrian Chemicals, London S.E.16, U.K.), one sample of the digest being analysed for glucose and another counted for radioactivity. The initial specific radioactivity of the glucose in the perfusate was used to calculate the results, which are expressed as nmol of glucose incorporated. Samples of the diaphragm muscle were taken in most experiments before and after perfusion, to measure the amount of glycogen initially and finally present in the tissue.

The $^{14}\text{CO}_2$ formed during perfusion was trapped by passing the effluent gas through ethanolamine-methanol (1:1, v/v) and counting the radioactivity of a sample. The efficiency of trapping of the CO_2 was 100%. The dissolved $^{14}\text{CO}_2$ in the perfusate was liberated at the end of the experiment in a sealed vial with 2M-HCl and the CO_2 collected in a small cup containing ethanolamine-methanol; then the contents of the cup were transferred to a vial containing the scintillation liquid and the radioactivity was counted.

RESULTS

Table 1 shows the glucose converted into CO_2 , glycogen and lactate in the same perfused dia-

phragm. The results show that there is a significant insulin effect on the incorporation of glucose into glycogen under all the experimental conditions studied. In the normal perfused muscle, contraction caused a slight but significant increase in the incorporation of glucose into glycogen in the absence of insulin. This effect of contraction on glycogen synthesis was accentuated in streptozotocin-diabetic muscle where the [^{14}C]glycogen values were very low in the absence of insulin in the resting state; in contracting diabetic muscle the [^{14}C]glycogen values approached those obtained in normal muscle in the absence of insulin. The contracting diabetic muscle in the presence of insulin incorporated glucose into glycogen to approximately the same extent as normal muscle under corresponding conditions.

The results given in Table 1 also show that in normal perfused diaphragm muscle, unlike in the perfused heart muscle (Chain *et al.* 1969), neither work nor insulin significantly altered the rate of oxidation of glucose to CO_2 . In the diabetic muscle the rate of oxidation of glucose in the resting state was extremely low, but was increased by contraction, to a value only slightly less than that of normal muscle.

The conversion of glucose into lactate was uninfluenced by insulin in the normal resting muscle. Contraction markedly decreased the conversion of [^{14}C]glucose into [^{14}C]lactate, but production of [^{14}C]lactate in the contracting muscle was stimulated by the addition of insulin. In the resting diabetic muscle the conversion of [^{14}C]glucose into [^{14}C]lactate was very small but contraction increased this value to the same order of magnitude as

Table 1. *Influence of insulin and of contraction on the oxidation of [$\text{U-}^{14}\text{C}$]glucose to $^{14}\text{CO}_2$ and incorporation of [^{14}C]glucose into glycogen and lactate in perfused diaphragm muscle from normal and streptozotocin-diabetic rats*

Results are expressed as nmol of glucose converted into CO_2 , glycogen and lactate/100 mg of tissue after a 30 min perfusion with 6–10 ml of Krebs–Henseleit bicarbonate-buffered medium, pH 7.4, containing 5.55 mM-glucose (approx. 25 μCi of [$\text{U-}^{14}\text{C}$]glucose). Values are the means $\pm$ s.e.m. The number of perfusions is given in parentheses.

Animals	Condition of muscle	CO_2		Glycogen		Lactate	
		No insulin	With insulin	No insulin	With insulin	No insulin	With insulin
Normal	Resting	114 $\pm$ 16 (7)	118 $\pm$ 18 (8)	49.0 $\pm$ 8.0 (10)	333 $\pm$ 73† (12)	1284 $\pm$ 78 (5)	1159 $\pm$ 150 (8)
	Contracting	113 $\pm$ 20 (4)	140 $\pm$ 21 (6)	71.0 $\pm$ 8.0 (12)	360 $\pm$ 65† (11)	367 $\pm$ 40* (9)	871 $\pm$ 111† (10)
Diabetic	Resting	11.9 $\pm$ 2.0‡ (12)	7.6 $\pm$ 2.0 ‡ (12)	12.6 $\pm$ 2.1‡ (12)	58.3 $\pm$ 11.0†‡ (10)	81.3 $\pm$ 17‡ (7)	81.0 $\pm$ 21.0‡ (5)
	Contracting	85.0 $\pm$ 8.0* (5)	73.0 $\pm$ 3.0* (5)	62.0 $\pm$ 17* (5)	295 $\pm$ 45†* (6)	422 $\pm$ 100* (4)	628 $\pm$ 114* (6)

* Significant difference between resting and contracting, $P < 0.05$.

† Significant effect of insulin, $P < 0.05$.

‡ Significant difference between normal and diabetic, $P < 0.05$.

Table 2. Influence of insulin and of contraction on the change in glycogen content and the glycogen turnover in perfused diaphragm muscle from normal and streptozotocin-diabetic rats

Results are expressed as ratios and are calculated from values obtained in some experiments reported in Table 1. Values are the means $\pm$ s.e.m. The number of experiments is given in parentheses.

Animals	Conditions of muscle	Final glycogen content		Final glycogen content (nmol of glucose)		[¹⁴ C]Glycogen content (nmol of [¹⁴ C]glucose converted into glycogen)	
		No insulin	With insulin	No insulin	With insulin	No insulin	With insulin
Normal	Resting	1.04 $\pm$ 0.08 (15)	1.19 $\pm$ 0.06 (25)	32.3 $\pm$ 7.8 (13)	8.7 $\pm$ 1.0† (7)		
	Contracting	0.92 $\pm$ 0.04 (11)	1.25 $\pm$ 0.09† (11)	36.8 $\pm$ 5.3 (12)	15.9 $\pm$ 0.7†* (10)		
Diabetic	Resting	0.79 $\pm$ 0.05† (11)	0.94 $\pm$ 0.06 (12)	88.5 $\pm$ 9.1† (11)	38.2 $\pm$ 9.5†† (10)		
	Contracting	0.83 $\pm$ 0.08 (5)	0.95 $\pm$ 0.08 (6)	8.6 $\pm$ 1.5†* (4)	6.2 $\pm$ 1.9* (5)		

* Significant difference between resting and contracting, $P \leq 0.05$.

† Significant effect of insulin, $P \leq 0.05$.

†† Significant difference between normal and diabetic, $P \leq 0.05$.

that found in the contracting normal muscle, and this was not increased significantly further by insulin.

In some of the experiments reported above and some additional perfusions, the total glycogen content of the diaphragm was determined before and after perfusion. The results obtained in these experiments were then used to calculate (1) the ratio of the final glycogen content to the initial glycogen content, a ratio greater than 1 indicating a net glycogen synthesis, a ratio of less than 1 indicating net glycogenolysis, and (2) the ratio of the final glycogen content to the [¹⁴C]glucose incorporated into glycogen (both values being calculated as nmol of glucose) indicating the proportion of the final glycogen resynthesized, or the glycogen turnover; a ratio of 1 would represent a complete glycogen turnover.

These ratios (Table 2) indicate that there was very little net glycogen synthesis under any of the experimental conditions studied, except in the normal contracting muscle, where there was a significant net increase in the presence of insulin. Some glycogenolysis appeared to occur in the diabetic muscle in all cases. The glycogen-turnover value, however, was markedly increased by insulin in the normal muscle whether in the resting or contracting condition. In the resting diabetic muscle the glycogen turnover was markedly decreased but was increased by insulin. Contraction had a very marked effect in the diabetic muscle, where the glycogen turnover, either in the absence or presence of insulin, was increased to the value obtained in normal muscle with insulin.

Table 3 shows the amounts of the intermediates formed from glucose, extracted in aqueous ethanol and separated by two-dimensional paper chromatography. The results were calculated from a smaller number of experiments than the total number of perfusions performed. This was because in many chromatograms the intermediates were present only in trace amounts that could not be quantitatively measured by the experimental technique. These values were therefore ignored in calculating the means. The results show that under all experimental conditions studied, the perfused muscle in the absence of insulin shows a very low rate of glucose metabolism. This is particularly striking in the resting diabetic muscle. Under all the conditions studied insulin markedly increased the incorporation of glucose into soluble glycogen and into the oligosaccharides. Insulin also significantly increased the conversion of glucose into phosphate esters in the normal resting muscle, but had no effect in the contracting normal muscle. The converse occurred in the diabetic muscle, where insulin had no effect on the phosphate esters in the resting muscle, but a marked effect in the contracting muscle.

Table 3. *Influence of insulin and of contraction on the metabolism of glucose in the perfused diaphragm muscle from normal and streptozotocin-diabetic rats*

Results are expressed as nmol of [14 C]glucose converted and of unchanged substrate/100 mg of tissue after perfusion. Experimental conditions are as given in Table 1. Values are the means $\pm$ s.e.m. The number of perfusions is given in parentheses at the top of the columns. The number of perfusions from which the individual values are calculated is given in parentheses after each result.

	Normal rats				Diabetic rats			
	Resting muscle		Contracting muscle		Resting muscle		Contracting muscle	
	No insulin (11)	With insulin (7)	No insulin (8)	With insulin (9)	No insulin (7)	With insulin (6)	No insulin (5)	With insulin (5)
Soluble glycogen	18.2 $\pm$ 2.1 (7)	199 $\pm$ 45† (5)	8.5 $\pm$ 2.5* (7)	66.6 $\pm$ 12.4*† (9)	5.5 $\pm$ 1.6 (4)	33.6 $\pm$ 16.0 † (5)	19.3 $\pm$ 5.3 (3)	111 $\pm$ 44 (4)
Maltotetraose	12.0 $\pm$ 1.5 (5)	165 $\pm$ 30† (4)	8.7 $\pm$ 2.4 (7)	66.9 $\pm$ 19.8*† (8)	Trace	33.9 $\pm$ 1.8 † (3)	10.7 $\pm$ 3.4 (3)	82.6 $\pm$ 32.2 (4)
Maltotriose	19.0 $\pm$ 6.3 (6)	83.0 $\pm$ 1.50† (7)	11.6 $\pm$ 2.4 (5)	58.1 $\pm$ 11.4† (8)	Trace	20.3 $\pm$ 6.4† (3)	7.0 $\pm$ 2.6 (2)	32.2 $\pm$ 14.7 (3)
Maltose	27.0 $\pm$ 7.8 (4)	35.5 $\pm$ 5.0 (5)	9.0 $\pm$ 3.0 (2)	39.5 $\pm$ 11.6*† (8)	Trace	6.8 $\pm$ 1.7† (3)	Trace	11.3 $\pm$ 3.3 (2)
Glucose in tissue	124 $\pm$ 18 (11)	176 $\pm$ 38 (7)	146 $\pm$ 18 (8)	75.5 $\pm$ 9.2*† (9)	125 $\pm$ 16 (7)	70.3 $\pm$ 6.0 †† (6)	174 $\pm$ 50 (5)	136 $\pm$ 24† (5)
Phosphate esters	12.0 $\pm$ 5.0 (3)	99.0 $\pm$ 13† (6)	36.8 $\pm$ 22 (5)	42.0 $\pm$ 8.3* (7)	6.1 $\pm$ 2.6 (3)	2.8 $\pm$ 0.9† (5)	Trace	31.7 $\pm$ 16.6 (3)

* Significant difference between resting and contracting, $P < 0.05$.

† Significant effect of insulin, $P < 0.05$.

‡ Significant difference between normal and diabetic, $P < 0.05$.

DISCUSSION

The effect of insulin on glucose metabolism in the resting perfused rat diaphragm is essentially the same as that reported with the isolated rat diaphragm (Beloff-Chain & Rookledge, 1968). Only synthesis of glycogen and oligosaccharide is stimulated, whereas production of CO_2 and lactic acid are not affected. It has been shown that the perfused diaphragm muscle preparation is probably hypoxic (Rookledge, 1971) to the same extent as the incubated diaphragm muscle previously used. In both preparations this would account for the relatively low oxidation of glucose to CO_2 as compared with lactate formation. However, the hypoxic condition does not interfere with the very marked effect of insulin on synthesis of glycogen and oligosaccharide. This effect of insulin was much more marked in the perfused preparation than in pieces of incubated diaphragm muscle, probably as a result of the removal of all endogenous insulin during the period of washing out before perfusion with radioactive glucose. The metabolism of glucose in the absence of insulin was so low that in a number of experiments the chromatograms prepared from the aqueous ethanolic extracts of the tissue showed only one major radioactive spot (glucose) on radioautography and all the other intermediates were present in such small concentrations that they were barely visible and could not be determined quantitatively.

In the resting perfused diaphragm from streptozotocin-diabetic rats the incorporation of glucose into soluble glycogen was very much decreased, as was the case with the isolated rat diaphragm (Beloff-Chain & Rookledge, 1968). Addition of insulin stimulated glucose incorporation into soluble glycogen, but to values very much below those obtained in the perfused tissue of normal animals in the presence of insulin.

Contraction, like insulin, increased the incorporation of glucose into total glycogen in both normal and diabetic muscle, but the incorporation was further greatly increased (up to fivefold) by addition of insulin. It is particularly noteworthy that contraction in the presence of insulin in the perfused streptozotocin-diabetic diaphragm preparation increases the incorporation of glucose into glycogen to that of the normal preparation. This is another example of the immediate correction of a specific metabolic defect of diabetes by addition of insulin (Beloff-Chain & Rookledge, 1968; Chain *et al.* 1969). The mechanism of insulin action on glycogen synthesis in muscle remains to be established, and this question has been extensively discussed by Chain *et al.* (1969) in relation to their findings with the perfused heart and in relation to the work of other investigators.

Lactate production from $[^{14}\text{C}]$ glucose was considerably greater in the resting than in the contracting diaphragm muscle from normal rats in the absence of insulin. In the diaphragm preparation from streptozotocin-diabetic rats, however, $[^{14}\text{C}]$ lactate production was greatly decreased in the resting state, but was increased to the values obtained in normal muscle by contraction. No insulin effect on lactate production was observed in the resting muscle, but there was a significant stimulation in the normal contracting muscle, similar to that found in the working heart (Chain *et al.* 1969).

It is difficult to interpret these findings without measuring the specific radioactivity of the lactate and degrading the glycogen formed to see if the $[^{14}\text{C}]$ glucose is incorporated only into the outer chains of the glycogen molecule or also into the core. This combined information would throw light on the origin of the lactate, which could either come from the glucose directly by glycolysis, or via glycogen. Lactate of high specific radioactivity would result both in the former case and in the latter, provided that only the outer chains of glycogen are labelled and rapidly turned over. Labelling in the core and subsequent glycolysis would lead to lactate of lower specific radioactivity because of dilution of the glucose in the glycogen pool. Either of these explanations could account for the differences in radioactivity of the lactate formed in resting and in contracting muscle. The fact that insulin increases lactate production from glucose in both normal and diabetic contracting diaphragm muscle, as it does in the working heart (Chain *et al.* 1969), is evidence that the source of lactate is glycogen. The concept of two pathways of glucose metabolism has been discussed by Chain *et al.* (1969) in relation to results obtained with the perfused heart, and can be equally well applied to the results obtained with the perfused diaphragm muscle.

The perfused diaphragm muscle is not a suitable preparation for the measurement of the specific radioactivities of the metabolic products of glucose, or for glycogen degradation studies, because of the small amount of tissue available.

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