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Oxygen Consumption in Perfused Skeletal Muscle

EFFECT OF PERFUSION WITH AGED, FRESH AND AGED–REJUVENATED ERYTHROCYTES ON OXYGEN CONSUMPTION, TISSUE METABOLITES AND INHIBITION OF GLUCOSE UTILIZATION BY ACETOACETATE

Neil B. RUDERMAN,*† Friedrich W. KEMMER,‡ Michael N. GOODMAN* and Michael BERGER‡

**Division of Diabetes and Metabolism, University Hospital, Evans Department of Medicine and Department of Physiology, Boston University Medical Center, Boston, MA 02118, U.S.A., and ‡Department of Medicine E and Diabetes Forschungsinstitut, University of Düsseldorf, D-4000 Düsseldorf, German Federal Republic*

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1. O_2 consumption, glucose metabolism and the energy status of skeletal muscle were compared in isolated rat hindquarters perfused with aged (21–35 days), fresh and aged–rejuvenated human erythrocytes. 2. The age of the erythroeytes did not affect O_2 consumption, glucose utilization or lactate release either at rest or during exercise. The concentrations of ATP, phosphocreatine and lactate within the muscle were also unaffected by the use of aged erythrocytes. 3. Perfusion with acetoacetate did not inhibit glucose utilization; but, it caused a marked increase in the tissue concentration of citrate in the soleus, a slow-twitch red muscle, and a smaller increase in the gastrocnemius, which contains fast-twitch red and white fibres. Results were similar in hindquarters perfused with aged and aged–rejuvenated erythrocytes. 4. These findings suggest that perfusion with aged human erythrocytes does not cause major alterations in the metabolic performance of the isolated rat hindquarter.

The isolated perfused rat hindquarter has been a useful tool for the study of fuel and amino-acid metabolism in skeletal muscle (reviewed by Ruderman *et al.*, 1977). The preparation originally described by Ruderman *et al.* (1971) was perfused with aged human erythrocytes, both because such cells are readily available and because their rate of glycolysis is lower than that of fresh cells. Furthermore, aged erythrocytes had previously been shown to maintain the viability during perfusion of rat liver, a tissue with a much higher metabolic rate than muscle (Ross *et al.*, 1967).

Aged erythrocytes are deficient in 2,3-bisphosphoglycerate (Bunn *et al.*, 1969) and therefore have a diminished ability to transfer O_2 at a given pO_2 (Valtis & Kennedy, 1954). Nevertheless, the metabolism of the rat hindquarter perfused with such cells has been shown closely to resemble that of muscle in an intact rat. Tissue concentrations of ATP, phosphocreatine and AMP, and the lactate/pyruvate ratio, are maintained, K^+ is not lost by the preparation during a perfusion, and its rate of O_2 consumption and lactate release are similar to those of the hindquarter of an intact rat (Ruderman *et al.*, 1971, 1979). In addition, the tissue appears morphologically normal under the electron microscope after 45 min of perfusion (Ruderman *et al.*, 1971).

Rennie & Holloszy (1977) have suggested that hindquarters perfused with aged human erythrocytes are hypoxic. They reported that O_2 consumption at rest is doubled, and during exercise quadrupled, when aged cells are preincubated with inosine, adenine, and glucose to restore 2,3-bisphosphoglycerate. In addition, they observed that free fatty acids inhibited glucose uptake and glycolysis, just as they do in heart (Randle *et al.*, 1966), when rejuvenated erythrocytes were used. In contrast, both they and we (Goodman *et al.*, 1974; Berger *et al.*, 1976) observed no such inhibition in hindquarters perfused with aged cells, and other groups of workers have failed to observe it when fresh bovine erythrocytes (Jefferson *et al.*, 1972) or erythrocyte-free media (Reimer *et al.*, 1975) were used.

Because of these disparate results, we investigated systematically the effects of perfusion with different types of erythrocytes on the metabolism of

† To whom correspondence should be addressed.

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the rat hindquarter. The present paper compares the effects of perfusion with aged, fresh and aged–rejuvenated human erythrocytes and fresh rat erythrocytes on O_2 consumption in resting and exercising muscle. In addition, the effect of acetoacetate on glucose metabolism was evaluated in hindquarters perfused with aged and aged–rejuvenated cells.

Materials and Methods

Animals

Female Osborne–Mendel rats from the stock of the Diabetes Research Institute of the University of Düsseldorf were used for all studies carried out in Düsseldorf, and male Sprague–Dawley rats from Charles River Laboratories (Wilmington, MA, U.S.A.) for those carried out in Boston. All rats were maintained on Purina Laboratory Chow. They were fed *ad libitum* and weighed 180–200g at the time of study, unless otherwise noted. All procedures were carried out in rats anaesthetized with pentobarbital (5mg/100g body wt.) administered intraperitoneally.

Materials

Inosine, adenine and ethyl acetoacetate were obtained from Sigma, and Pentex bovine serum albumin (Cohn fraction V, 0.8mg of fatty acid/g of albumin) from Miles Corp., Kankakee, IL, U.S.A. or Slough, Bucks., U.K. Sodium acetoacetate, free of ethanol, was prepared from its ethyl ester as described by Krebs & Eggleston (1945).

Perfusion medium

Details of the preparation of the perfusate have been described elsewhere (Ruderman *et al.*, 1971; Berger *et al.*, 1976). Aged human erythrocytes were taken from the donor 21–36 days before use, and fresh human erythrocytes were taken 4 days before use. The fresh cells were stored in ACD (aged citric acid/sodium citrate/glucose) and the aged cells either in ACD (Düsseldorf) or in CPD (citrate/phosphate/glucose; Boston). Fresh rat erythrocytes were taken from 300g male rats 1 day before use and were washed with saline and Krebs–Henseleit solution (Krebs & Henseleit, 1932) as described previously (Ruderman *et al.*, 1971).

Rejuvenation of erythrocytes was carried out by the method of Valeri (1974) as described by Rennie & Holloszy (1977). The half-saturation pressure of the aged erythrocytes increased from 16 to 40mmHg (2.13 to 5.33kPa) (corrected to pH 7.2) following rejuvenation. The standard perfusion medium contained Krebs–Henseleit solution, washed erythrocytes (7–8g of haemoglobin/100ml), 5.5 or 6.6mM-glucose, 1.6mM-lactate, and 0.15mM-pyruvate. Insulin was not routinely added. The medium was gassed with O_2/CO_2 (19:1, v/v) throughout the perfusion. The initial pH of the medium was 7.4; after 45min of perfusion, with the hindquarter at rest, it was between 7.25 and 7.35. The half-saturation pressure of the aged erythrocytes did not change during the perfusion.

Perfusion procedure

Details of the perfusion apparatus and the operative procedure were as described previously (Ruderman *et al.*, 1971; Berger *et al.*, 1976). The apparatus used in the experiments with the Sprague–Dawley rats was modified in that a Silastic coil was used in place of a bulb oxygenator (Goodman & Ruderman, 1978). Use of a coil simplifies the perfusion apparatus and diminishes haemolysis; it does not appear to affect O_2 consumption or alter the metabolism of the preparation, however.

Hindquarters were cyclically perfused unless otherwise noted. Measurements were begun after a 10-min period of equilibration. Muscle contraction was induced by electrical stimulation of the sciatic nerve with 10ms pulses at a rate of 5Hz with a Grass Model SD9 stimulator. Intense contractions were maintained for 15min (Berger *et al.*, 1976). Tension was not measured in these studies; however, in later experiments in which a double pulse was used, muscle tension was approximately 59g (E. Richter, personal communication). The flow rate was monitored with a Gilmont flow meter (A. H. Thomas Co., Philadelphia, PA, U.S.A.). When the flow rate was maintained at 11ml/min, pressure in the tubing leading to the aortic catheter was 8.0–9.5kPa (60–70mmHg).

Sampling of perfusate and tissue

In the studies carried out in Boston, the initial volume of perfusate was 150ml. The first 20ml of medium that passed through the tissue was discarded, and thereafter the medium was recycled. After 10min, a sample was collected for the determination of zero-time values; thereafter, samples of perfusate were taken every 30min for 1h. In some experiments, at the end of each perfusion, the right soleus and gastrocnemius muscles were quickly removed and frozen between clamps pre-cooled in liquid N_2. Muscles were stored in liquid N_2 until processed. The experiments carried out in Düsseldorf were performed in a similar manner, except that the medium was recycled as soon as the preparation was placed in the perfusion apparatus.

Analytical methods

Methods for deproteinization and neutralization of tissue and perfusate and for analysis of glucose, lactate, pyruvate, citrate, glucose 6-phosphate, ATP and phosphocreatine were as described previously (Goodman *et al.*, 1974; Berger *et al.*, 1975). Total

O_2 content of arterial and venous blood was determined with a Lex-O_2-Con apparatus (Lexington Instruments, Lexington, MA, U.S.A.). The pO_2 was measured in arterial and venous perfusate using an Instrumentation Laboratory Blood Gas Analyzer (Instrumentation Laboratory, Watertown, MA, U.S.A.).

Calculations

Rates of O_2 consumption were calculated from the arteriovenous difference for O_2 and the flow rate. Rates of glucose uptake and lactate release were determined from changes in their concentrations in the perfusate (Ruderman *et al.*, 1971). All data are expressed per g of muscle perfused. Earlier studies have shown that the muscle portion of the perfused hindquarter is equivalent to one-sixth of the total body weight in rats weighing 180–270g (Ruderman *et al.*, 1971) and that a similar relationship holds in much larger rats (N. B. Ruderman, F. W. Kemmer, M. N. Goodman and M. Berger, unpublished work). The quantity of O_2 dissolved in plasma was determined from the expression:

$$[O_2](\mu mol/ml) = [(pO_2/760) \times 0.024 \times 1000]/22.4$$

where 0.024 is the absorption coefficient for O_2 in plasma at 37°C. O_2 bound to haemoglobin was determined by subtracting this value from the measured total O_2. Statistical analyses were done by Student's *t* test.

Results

Previous studies

Rates of O_2 consumption previously reported for hindquarter preparations perfused with different types of erythrocytes and with erythrocyte-free media are listed in Table 1. Values of 20–27 μmol/h per g of muscle have generally been obtained with aged cells except in one small series reported by Berger *et al.* (1976) and in the study of Rennie & Holloszy (1977). Perfusate flow rate was lower in the latter experiments; however, as will be shown later, this could only partially account for the low rate of O_2 consumption in this study. The reason for the low rate of O_2 usage in the initial study by Berger *et al.* (1976) is not clear.

O_2 consumption by hindquarters perfused with fresh human, rat and bovine erythrocytes, and with

Table 1. *O_2 consumption in the isolated rat hindquarter perfused with different types of erythrocytes and without erythrocytes*

Results are the means of the indicated number of experiments. The hindquarters preparation described by Ruderman *et al.* (1971) was used in all studies and muscle was in the resting state. The standard perfusing media contained 3–4% bovine albumin, 5–10mM-glucose and physiological concentrations of lactate and pyruvate. The data of Rennie & Holloszy (1977) were recalculated taking into account the fact that the gluteal and lower back musculature comprise one-third of the soft tissue of the hindquarter (see Ruderman *et al.*, 1971; Goodman & Lowenstein, 1977; Karlsson *et al.*, 1975). Perfusate flow rate and O_2 used are expressed per g of muscle perfused.

Type of erythrocytes	State of rat	*n*	[Haemoglobin] (g/100ml)	Insulin in medium	Perfusate flow rate (ml/min per g)	O_2 used (μmol/h per g)
Aged human						
Ruderman *et al.* (1971)	Starved	23	7.5	–	0.33	20
	Starved	7	7.5	+	0.33	25
Berger *et al.* (1976)	Fed	4	7.5	–	0.33	15
Berger *et al.* (1978)	Fed	4	7.5	–	0.31	22
Rennie & Holloszy (1977)	Fed	8	8	+	0.16	13
Kemmer *et al.* (1979)	Fed	10	7.5	+	0.33	24
Fresh bovine						
Karlsson *et al.* (1975)	Fed	6	7.5	–	0.19	24
Lewis *et al.* (1977)	Fed	6	11	+	0.34	18
Fresh rat						
Goodman & Lowenstein (1977)	Fed	4	6.5	+	0.37	25
Fresh human						
Rennie & Holloszy (1977)	Fed	2	12	+	0.16	27
Rejuvenated human						
Rennie & Holloszy (1977)	Fed	16	12	–	0.16	27
No erythrocytes						
Strohfeldt *et al.* (1974)	Fed	6		–	1.37	31
	Fed	7		+	1.37	38
Reimer *et al.* (1975)	Fed	5		–	1.10	38
	Fed	5		+	1.10	40

aged–rejuvenated human cells, has generally been similar to that reported for aged human erythrocytes. Strict comparisons are difficult, however, because insulin, which frequently causes a 10–30% increase in O_2 consumption (Ruderman *et al.*, 1971; Karlsson *et al.*, 1975; see Table 1), was used in some studies and not others. Also, the effects of different preparations and concentrations of albumin on O_2 consumption have not been systematically evaluated, nor has the effect of perfusate flow rate. The highest rates of O_2 consumption reported for the hindquarter preparation at rest have been noted when it is perfused with an erythrocyte-free medium at a very high flow rate (see Table 1).

Because of the many variables in these studies, it was decided to compare systematically the effects of aged, fresh and aged–rejuvenated cells on O_2 consumption and glucose metabolism by the hindquarter preparation. Also, to diminish the possible contribution of inter-laboratory variability, investigations were carried out in two laboratories, by two different sets of investigators. Perfusions in which aged and fresh human erythrocytes were compared were carried out at the Diabetes Research Institute of the University of Düsseldorf, German Federal Republic, by Drs. Kemmer and Berger, and all others at Boston University Medical Center, U.S.A. by Drs. Ruderman and Goodman.

Perfusate flow rate

In hindquarters perfused with aged human erythrocytes, O_2 consumption did not increase when flow was increased from the initial value of 0.45 ml/min per g of muscle to 0.66 ml/min per g (Table 2). Only when the flow rate was diminished to 0.15 ml/min per g was there a statistically-significant decrease in O_2 usage. Thus, it appears that the preparation is adequately oxygenated at the standard flow rate of approx. 0.33 ml/min per g. A very similar pattern was seen in hindquarters

Table 2. *Effect of perfusate on O_2 consumption by hindquarters of Osborne–Mendel rats perfused with aged and fresh human erythrocytes*

Fresh cells were used within 4 days after withdrawal from the donor and aged cells after 28–36 days. Results are means ± S.E.M. with the numbers of experiments in parentheses. Values for several flow rates were obtained in the same rat. Flow rates were changed at 10-min intervals during the perfusion by altering the arterial pressure. O_2 consumption was measured twice at each flow rate and the mean of the two values is reported. The order of the different flow rates was varied from one rat to another except that perfusion at the lowest rate was always carried out last. *Values significantly higher than those obtained with a flow rate of 0.15 ml/min per g ($P<0.05$). **Significantly different from values in hindquarters perfused at the same flow rate with aged erythrocytes ($P<0.05$).

		O_2 consumption (μmol/h per g of muscle)					
Erythrocytes	Perfusate flow rate (ml/min per g) ...	0.15	0.23	0.30	0.45	0.53	0.66
Aged		18.4 ± 2.2 (5)	23.0 ± 1.4* (6)	21.2 ± 1.2 (8)	23.6 ± 1.8* (6)	24.4 ± 2.2* (6	24.4 ± 2.4* (5)
Fresh		19.6 ± 1.0 (6)	19.6 ± 1.2** (6)	21.8 ± 1.4 (10)	24.4 ± 2.0* (6)	25.4 ± 1.8* (6)	25.4 ± 1.6* (6)

Table 3. *Effect of isometric exercise on glucose uptake, lactate release, O_2 consumption and tissue metabolites in isolated hindquarters of Osborne–Mendel rats perfused with aged and fresh human erythrocytes*

Results are means ± S.E.M. for six rats in each group. Studies were carried out after a 10-min equilibration period. Separate rats were used for the rest and exercise experiments. Exercise was induced by electrical stimulation of the sciatic nerve at a rate of 5 Hz and was maintained for 20 min. O_2 consumption was measured at 10 and 20 min and the mean of the two values was used. See legend to Table 2 and the Materials and Methods section for additional details.

		Metabolite and O_2 exchange rates (μmol/h per g of muscle)			Tissue concentration (μmol/g of muscle)				
Type of erythrocytes	Exercise	Glucose uptake	Lactate release	O_2 used*	Glucose	Lactate	Pyruvate	ATP	Phospho-creatine
Fresh	–	0.4 ± 1.4	2.0 ± 0.6	20.2 ± 1.0	1.3 ± 0.1	1.1 ± 0.1	0.15 ± 0.01	5.8 ± 0.3	18.9 ± 0.9
Aged	–	−1.8 ± 2.0	1.8 ± 0.6	19.4 ± 1.0	1.1 ± 0.04	1.0 ± 0.1	0.10 ± 0.01	5.6 ± 0.2	17.8 ± 0.7
Fresh	+	6.2 ± 1.0	20.0 ± 1.8	37.8 ± 1.6	1.8 ± 0.1	11.0 ± 0.1	0.15 ± 0.01	4.2 ± 0.3	9.8 ± 2.1
Aged	+	7.2 ± 0.6	19.8 ± 2.8	38.4 ± 3.2	2.0 ± 0.1	12.0 ± 1.8	0.10 ± 0.01	4.0 ± 0.4	10.9 ± 0.9

* Based on means of two independent determinations for each individual experiment.

Table 4. *Effect of increasing flow rate on O_2 consumption and tissue metabolites during intense exercise*
Hindquarters of fed Sprague–Dawley rats were used. Flow rate was increased by increasing the arterial pressure from 9.3 to 17.3 kPa (70 to 130 mmHg). Isometric exercise was induced by electrical stimulation of the sciatic nerve at a rate of 5 Hz for 15 min. Results are means ± S.E.M. of four to six experiments. Adapted from Berger *et al.*, 1975.

Flow rate (ml/min per g)	O_2 used (μmol/h per g)	Tissue concentration (μmol/g) of		
		Lactate	ATP	Phosphocreatine
0.33	46.8 ± 4.8	11.8 ± 1.2	3.7 ± 0.2	5.3 ± 0.8
0.82	64.8 ± 3.0	6.5 ± 1.1	4.2 ± 0.2	7.0 ± 0.9

Table 5. *O_2 consumption of hindquarters perfused with aged and aged–rejuvenated human erythrocytes and fresh rat erythrocytes*
Results are means ± S.E.M. for four rats in each group. Exercise was induced by electrical stimulation of the sciatic nerve at a rate of 4 Hz for 15 min. Hindquarters had previously been perfused for 15 min with the muscle at rest. Flow rates were 12 ml/min (0.30 ml/min per g) for the 200 g rats and 13 ml/min for the 450–500 g rats. Male Sprague–Dawley rats fed *ad libitum* were used. See the Materials and Methods section for details.

Wt. of rats (g)	Type of erythrocyte	O_2 consumption (μmol/h per g of muscle)	
		Rest	Exercise
200	Aged human	26.4 ± 1.2	48.6 ± 2.4
	Rejuvenated human	27.2 ± 1.0	46.6 ± 3.8
	Fresh rat	25.4 ± 0.8	50.6 ± 2.4
450–500	Aged human	16.4 ± 1.2	
	Rejuvenated human	16.8 ± 1.6	

perfused with fresh human erythrocytes; at most flow rates, O_2 consumption was nearly the same as with the old cells.

Aged and fresh human erythrocytes

The age of the erythrocytes did not affect O_2 consumption, glucose utilization or lactate release either at rest or during intense isometric contraction (Table 3). The concentrations of ATP and phosphocreatine within the muscle were also not affected by the use of aged cells, although tissue concentrations of pyruvate tended to be somewhat lower. Overall, these findings indicate that even in the markedly hypoxic state of the perfused tissue during strenuous exercise (see Berger *et al.*, 1975), fresh erythrocytes do not offer any advantage with respect to oxygenation of the contracting tissue. On the other hand, as shown in Table 4, increased O_2 consumption does occur when perfusate flow rate is increased during intense exercise. Thus, O_2 delivery is not optimal at standard flow rates when the demands of the msucle cell are markedly increased. Parenthetically, in all of these experiments, the perfusion medium was recycled. In other studies, we found no difference between aged and fresh erythrocytes when a flow-through technique, i.e., medium not recycled, was used (data not shown).

Aged and rejuvenated human erythrocytes

Rates of O_2 consumption by the hindquarter were almost identical when perfusions were carried out using aged or aged–rejuvenated human erythrocytes or fresh rat erythrocytes (Table 5). The use of fresh or rejuvenated cells also did not increase O_2 consumption during intense exercise.

Similar findings were noted when older, larger rats were used. The latter results are noteworthy in that O_2 consumption was the same with aged and fresh cells, even though the absolute extraction of O_2 per total hindquarter at rest was nearly 50% greater in the older rats. (This is because the weight of the hindquarter of a 450–500 g rat is 2.3–2.5 times that of a 200 g animal. This more than compensates for the lower rate of O_2 consumption per g of muscle perfused.) Increments in flow rate produced only a small, but variable increase in O_2 consumption in the older rats. Thus, O_2 consumption per g of tissue was lower than in the younger rats, in keeping with the lower basal metabolic rate of these animals (Spector, 1957).

Fractional O_2 extraction

In six hindquarters perfused with aged human erythrocytes, the pO_2 in arterial plasma was 330 ± 18 mmHg (44.0 ± 2.4 kPa) and in venous plasma 48 ± 2 mmHg (6.4 ± 0.27 kPa). Comparable values with rejuvenated cells were 343 ± 37 mmHg (45.7 ± 4.9 kPa) and 58 ± 6 mmHg (7.7 ± 0.8 kPa) ($n = 3$). By using these data, together with measurements of the total O_2 content of arterial and venous blood and the fact that haemoglobin would be nearly 100% saturated at arterial pO_2, it is possible to calculate the percentage haemoglobin saturation in venous blood. The results shown in Table 6 indicate that haemoglobin was approx. 70% saturated regardless of whether aged or rejuvenated cells were used. This probably occurred at a somewhat lower venous

Table 6. *Extraction of O_2 from haemoglobin by hindquarters of 200g male Sprague–Dawley rats perfused with aged and aged–rejuvenated human erythrocytes*

Values for O_2 bound to haemoglobin were calculated by subtracting the O_2 dissolved in perfusate water (calculated from pO_2) from the total O_2 content of the perfusate. It was assumed that the absorption coefficient for O_2 in the perfusate was 0.024 and that all of the O_2 not dissolved in perfusate water was bound to haemoglobin. It was also assumed that haemoglobin was 100% saturated in arterial blood. See the text for details.

Type of erythrocytes	O_2 in perfusate water (μmol/ml)		O_2 bound to haemoglobin (μmol/ml of perfusate)		Haemoglobin saturation in venous blood (%)
	Arterial	Venous	Arterial	Venous	
Aged	0.44	0.07	3.66	2.63	72
Rejuvenated	0.46	0.08	3.74	2.72	73

Table 7. *Effect of acetoacetate on glucose uptake and O_2 consumption in isolated rat hindquarters perfused with aged and rejuvenated human erythrocytes*

Results are means ± S.E.M. with numbers of observations in parentheses. Hindquarters of Sprague–Dawley rats were perfused for 60min with standard medium containing 10mM-glucose and 10m-i.u. of insulin/ml. The concentration of unesterified fatty acid in the medium was approximately 100μM at the start of each perfusion. and 150μM at its completion.

Additions to perfusate	Type of erythrocytes	Glucose uptake (μmol/h per g of muscle)	O_2 used (μmol/h per g of muscle)
None	Aged	20.9 ± 1.3 (6)	27.2 (2)
	Rejuvenated	21.8 ± 3.1 (5)	30.4 ± 2.2 (4)
3mM-Acetoacetate	Aged	19.9 ± 1.7 (6)	35.4 (2)
	Rejuvenated	21.8 ± 1.6 (5)	40.4 ± 3.2 (5)

Table 8. *Effect of acetoacetate on tissue metabolites in soleus and gastrocnemius muscles of isolated rat hindquarters perfused with aged and aged–rejuvenated erythrocytes*

Results are means ± S.E.M. with numbers of observations in parentheses. The soleus was freeze-clamped following excision: the gastrocnemius was freeze-clamped *in situ*. See Table 7 and the Materials and Methods section for additional details.

Type of erythrocytes	Additions to perfusate	Muscle ...	Tissue concentration (nmol/g) of: Citrate		Glucose 6-phosphate
			Gastrocnemius	Soleus	gastrocnemius
Aged	None (3)		114 ± 6	116 ± 25	226 ± 41
	3mM-Acetoacetate (3)		148 ± 12	488 ± 21	194 ± 12
Rejuvenated	None (5)		143 ± 4	182 ± 5	190 ± 19
	3mM-Acetoacetate (5)		213 ± 12	429 ± 38	187 ± 31

pO_2 with the aged cells; however, the data are not sufficient to determine this with certainty.

Perfusion with acetoacetate

Rennie & Holloszy (1977) reported that oleate inhibits glucose uptake and glycolysis and increases muscle citrate concentration in the isolated rat hindquarter when it is perfused with aged–rejuvenated human erythrocytes. They did not observe an effect when untreated aged cells were used. We, therefore, repeated these experiments using acetoacetate in place of the fatty acids. Earlier studies have shown that acetoacetate has the same effect on glucose metabolism as free fatty acids in the perfused rat heart (Randle *et al.*, 1966) and incubated soleus muscle (Maizels *et al.*, 1977; Cuendet *et al.*, 1975). As shown in Table 7, acetoacetate did not diminish glucose utilization under these conditions, nor did it increase the concentration of glucose 6-phosphate in the gastrocnemius. On the other hand, it caused a marked increase in muscle citrate (Table 8), which was particularly evident in the soleus. The increment in the soleus was the same whether aged or aged–rejuvenated cells were used. Additional studies are needed to determine whether in the absence of acetoacetate, muscle citrate is higher with rejuvenated cells. In keeping with previous findings (Ruder-

man *et al.*, 1971), O_2 consumption was 25–30% greater in hindquarters perfused with acetoacetate. The reason for this is not known.

Discussion

The data indicate that aged human erythrocytes are able to maintain O_2 consumption in the perfused rat hindquarter as well as fresh and aged–rejuvenated erythrocytes. These findings are consistent with most earlier reports (Table 1), and with the finding that rates of lactate release and the tissue concentrations of ATP and phosphocreatine are similar when aged and fresh cells are used in the perfusion medium (Table 3). In addition, it has been shown that the tissue concentrations of ATP, ADP, AMP and phosphocreatine, and the lactate/pyruvate ratio, are very similar in hindquarters perfused with aged erythrocytes and in the same hindquarter muscle taken directly from an anaesthetized rat (Ruderman *et al.*, 1979).

When the muscle was at rest, haemoglobin saturation in venous blood draining from the hindquarter was approximately 70% when both aged and rejuvenated erythrocytes were used. The venous pO_2 was 58 mmHg (7.7 kPa) with the rejuvenated cells and 48 mmHg (6.4 kPa) with aged cells, suggesting that maintenance of O_2 delivery with aged cells required a lower pO_2. Although this is the likely reason why rates of O_2 consumption were maintained when aged cells were used, more data are needed to confirm this. Both haemoglobin saturation and venous pO_2 values in the perfusate are similar to that found in venous blood draining muscle *in vivo*.

In contrast to the data reported here, Rennie & Holloszy (1977) observed that O_2 consumption was nearly 50% lower in hindquarters perfused with aged human erythrocytes than in hindquarters perfused with fresh or aged–rejuvenated erythrocytes. For reasons that are unclear, we have generally found rates of oxygen consumption 70–100% higher than those reported by Rennie & Holloszy (1977) when we perfuse with aged human erythrocytes (see Tables 1–3 and 5). Their studies differed from those reported here in that the haemoglobin concentration was increased from 8 to 12 g/100 ml when switching from aged to fresh or rejuvenated cells. Also, when the muscle was at rest, perfusions were carried out at a lower flow rate per g of tissue (see Table 1). One explanation for their findings is that the increased haemoglobin concentrations compensated for the low flow rate and, therefore, maintained O_2 consumption. This is most unlikely, since it would imply that a 50% increase in haemoglobin concentration could produce a 100% increase in oxygen consumption. In addition, flow rates of the magnitude used in the studies of Rennie & Holloszy are associated with only a 10–20% decrease in O_2 consumption in hindquarters perfused with 8 g of haemoglobin/100 ml (Table 2). Rates of O_2 consumption during exercise are not comparable in the two studies because of differences in the frequency and intensity of contraction.

The term 'glucose–fatty acid cycle' was coined by Randle and his coworkers (1963, 1966) to describe the inverse relationship between fatty acid and glucose metabolism in heart and other tissues. In experiments with the perfused rat heart, they and others (review by Randle *et al.*, 1966) noted that unesterified fatty acids and ketone bodies can replace glucose as a fuel; more specifically, they observed that increases in the concentration of unesterified fatty acids or one of the ketone bodies led to decreased rates of glucose uptake, glycolysis and pyruvate oxidation and an increase in tissue glycogen concentration. They also observed changes in intracellular metabolites, which suggested that glycolysis was inhibited at phosphofructokinase, due to an increase in citrate, and that glucose oxidation was inhibited at pyruvate dehydrogenase, at least in part due to an increase in acetyl-CoA relative to CoA. Subsequently, similar effects of fatty acids and/or ketone bodies have been described in mammary tissue, the submaxillary gland, and possibly brain (see Ruderman *et al.*, 1979). The effect of these lipid fuels on skeletal muscle is less clear. Most investigators have been unable to demonstrate inhibition of glucose uptake or glycolysis by fatty acids or ketone bodies in the perfused hindquarter, although inhibition of pyruvate oxidation has been described (see Berger *et al.*, 1976; Ruderman *et al.*, 1979). On the other hand, exactly the same inhibitory pattern has been observed in the incubated rat soleus (Maizels *et al.*, 1977; Cuendet *et al.*, 1975), a slow-twitch red muscle, and in slow and fast-twitch red muscle in the intact rat following exercise (Rennie *et al.*, 1976).

In contrast with the earlier reports, Rennie & Holloszy (1977) were able to demonstrate inhibition of glucose uptake by oleate in the perfused rat hindquarter and the noted inhibition of phosphofructokinase and a marked increase in citrate in slow-twitch red and fast-twitch red muscles. They suggested that better oxygenation due to the use of aged–rejuvenated erythrocytes (and a higher haemoglobin concentration) may have allowed them to demonstrate these effects. The present study partially confirms these findings, in that perfusion with acetoacetate caused a 3–4-fold increase in citrate concentration in the soleus. On the other hand, the results differ in that acetoacetate did not decrease glucose uptake and the same changes in tissue metabolites occurred whether aged or aged–rejuvenated erythrocytes were used. Rennie & Holloszy discarded their perfusate after a single pass, where-

as most earlier investigators assessing the effects of lipid substrates on glucose metabolism recycled it. This led them to suggest that glucose utilization and glycolysis might be already attenuated in the recycled system due to the accumulation of fatty acid and this would negate any effect of additional lipid substrate (Rennie & Holloszy, 1977). The finding that the concentration of citrate in the soleus increased 200–400% following the addition of acetoacetate in the present study, whereas fatty acids caused only a 30–60% increase in Rennie's experiments, suggests this explanation is most unlikely. In addition, the muscle citrate concentration in our control preparations was lower than that reported by Rennie & Holloszy, and the unesterified fatty acid concentration increased only from 100 to 150 μmol/litre during a perfusion. Finally, in an earlier study (Goodman *et al.*, 1974) we were unable to demonstrate inhibition of glucose utilization by oleate, even when the perfusate was discarded after a single pass.

The results presented here indicate that conditions for maintaining optimal oxygenation of the hindquarter should be re-established whenever the O_2 demands of the preparation are increased. Thus, during intense exercise, when flow rate was increased, O_2 consumption was enhanced and tissue lactate, phosphocreatine and ATP concentrations were better maintained. This suggests that O_2 delivery had been rate-limiting for energy generation at the standard flow rate. Possibly, increasing the haemoglobin concentration would have had the same effect as increasing the flow rate; however, this was not assessed. Finally, although we found no disadvantage in using aged human erythrocytes, the report of Rennie & Holloszy (1977) suggests that some precautions shold be observed if they are used. In particular, the aged erythrocytes available at each institution should initially be compared with fresh or rejuvenated cells for their ability to maintain O_2 consumption by the hindquarter.

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