The Pentose Phosphate Pathway in Regenerating Skeletal Muscle

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1. The activities of the oxidative enzymes (glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) and of the non-oxidative enzymes (transaldolase, transketolase, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase) of the pentose phosphate pathway were measured at various times during the first 24 h of skeletal-muscle regeneration after administration of Marcaine, a myotoxic local anaesthetic.
2. The activities of the oxidative enzymes increased after Marcaine injection and rose to 9 times control activities by 24 h.
3. The activities of all non-oxidative enzymes were increased after Marcaine administration, but to a much smaller extent than the oxidative enzymes (1.1–1.7-fold).
4. Histochemical analysis localized glucose 6-phosphate dehydrogenase activity within muscle fibres of control and Marcaine-treated muscles.
5. Cycloheximide or actinomycin D prevented the increase in oxidative enzyme activities, suggesting a requirement for synthesis of protein and RNA.

The activity of the pentose phosphate pathway in skeletal muscle is enhanced during embryogenesis (Beatty *et al.*, 1966) and in a number of experimental situations, including denervation (Turner & Manchester, 1972; Koski & Max, 1974; Ilyin *et al.*, 1975; Robbins & Hall, 1976), ischaemia (Archangeli *et al.*, 1973) and administration of Marcaine (bupivacaine, 1-*n*-butyl-DL-piperidine-2-carboxylic acid 2,6-dimethylanilide hydrochloride) (Rifenberick *et al.*, 1974; Wagner *et al.*, 1977b), a myotoxic local anaesthetic that causes acute degeneration of muscle fibres followed by rapid and complete regeneration (Benoit & Belt, 1970). At 1 day after local application of Marcaine, fibres of the rat extensor digitorum longus muscle undergo degeneration characterized morphologically by disappearance of the plasma membrane, alteration of mitochondria and the sarcotubular system, dissolution of myofibrils, and degeneration of myonuclei (Jirmanova & Thesleff, 1972). At 2 days after application of Marcaine early signs of regeneration, notably the presence of presumptive myoblasts, are discernible (Jirmanova & Thesleff, 1972). If Marcaine is injected intramuscularly in combination with hyaluronidase, widespread muscle damage is observed and muscle fibres appear to undergo regeneration in a synchronous manner (Hall-Craggs, 1974). In an effort to identify early biochemical responses to the regenerative stimulus, i.e. those changes that might be involved in the initiation of regeneration, we injected Marcaine and hyaluronidase intramuscularly and assayed the enzymes of the pentose phosphate pathway during the first 24 h of skeletal-muscle regeneration.

Since regeneration is accompanied by an inflammatory response (Hall-Craggs, 1974) involving small

phagocytic cells that contain high activities of acid hydrolases (Steinman & Cohn, 1974), we also measured the activity of β -glucuronidase, a typical lysosomal acid hydrolase, to assess the contribution of the inflammatory response to changes in enzyme activities in Marcaine-treated muscles.

The data below demonstrate dramatic early enhancement of the rate-limiting enzymes of the pentose phosphate pathway, mediated via a process that is blocked by inhibitors of RNA and protein synthesis. Some of these results have been presented in preliminary form (Wagner *et al.*, 1977a,c).

Experimental

Materials

Actinomycin D, cycloheximide, hyaluronidase (type III), glucose 6-phosphate, 6-phosphogluconate, ribose 5-phosphate, NADH, NADP⁺, α -glycero-phosphate dehydrogenase (EC 1.1.1.8), triose phosphate isomerase (EC 5.3.1.1), ribose 5-phosphate isomerase (EC 5.3.1.6), transketolase (EC 2.2.1.1), D-ribulose 5-phosphate 3-epimerase (EC 5.1.3.1), fructose 6-phosphate, erythrose 4-phosphate and 4-methylumbelliferyl β -D-glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was obtained from Boehringer Mannheim (New York, NY, U.S.A.). Marcaine (bupivacaine) is a product of Winthrop Laboratories (New York, NY, U.S.A.). Male rats (Charles River CD strain) weighing 175–200 g were purchased from the Charles River Breeding Labs (Wilmington, MA, U.S.A.), and fed on water and regular laboratory chow (Ralston Purina Co., St. Louis, MO, U.S.A.) *ad libitum*.

Treatment of muscles

Marcaine (2.0mg) and hyaluronidase (300 NF units) in 0.9% NaCl were given in a single injection into the anterior tibial muscle (Hall-Craggs, 1974) to evaluate initial responses to the myotoxic drug. This procedure is a modification of the three-daily-injection regimen of Hall-Craggs (1974). The present regimen of Marcaine plus hyaluronidase causes a massive degenerative response, although not all fibres are involved, as appears to be the case after three daily Marcaine injections (Hall-Craggs, 1974; Hall-Craggs & Singh Seyan, 1975).

The contralateral anterior tibial muscle was not injected, and served as the control. In early experiments, injection of control muscles with 0.9% NaCl produced a small increase in glucose 6-phosphate dehydrogenase activity. At various times after a single injection of Marcaine plus hyaluronidase, muscles were excised, weighed, minced with scissors and then homogenized (1:10, w/v) by hand with a TenBroeck homogenizer (four to six strokes) in 50mM-Tris/HCl, pH 7.6, containing 0.5mM-dithiothreitol. Homogenates were centrifuged at 0–4°C for 20min at 18000g. The resulting supernatants were used for enzyme assays.

Inhibition of protein and RNA synthesis

Cycloheximide and actinomycin D were injected intraperitoneally at respective dosages of 50mg/kg and 1.5mg/kg. Cycloheximide was administered at the time of Marcaine injection, or at various times thereafter (see below); actinomycin D was administered 3h before injection of Marcaine. Control rats received 0.9% NaCl intraperitoneally. Rats were decapitated at 6 or 8h after drug treatment. Muscles were removed and treated as described above.

Enzyme assays

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49). This was assayed by a modification of the method of Glock & McLean (1953). The medium for the assay contained 50mM-Tris/HCl, pH 8.1, 3.0mM-glucose 6-phosphate, 0.5mM-NADP⁺ and 0.1 unit (1 μ mol/min) of 6-phosphogluconate dehydrogenase, in a total volume of 1.0ml.

6-Phosphogluconate dehydrogenase (EC 1.1.1.44). This was assayed in a medium containing 50mM-Tris/HCl, pH 8.1, 3.0mM-6-phosphogluconate and 0.1mM-NADP⁺ in a final volume of 1.0ml.

Absorbance changes were also assessed in the absence of substrate and this blank rate was subtracted from the rate with substrate. Inclusion of Marcaine in assay cuvettes at 0.77 or 1.54mM (estimated Marcaine concentration in injected muscles)

had no effect on glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase activities.

The non-oxidative enzymes were assayed by modifications of the coupled assay systems described by Novello & McLean (1968). Incubation medium for each enzyme comprised 50mM-Tris/HCl, pH 7.5, 0.2mM-NADH, 2 units of α -glycerophosphate dehydrogenase and 14.4 units of triose phosphate isomerase, with the additions described below, in a final volume of 1.0ml.

Transaldolase (EC 2.2.1.2). This was assayed by adding 20mM-fructose 6-phosphate and 1.0mM-erythrose 4-phosphate to the reaction medium.

Transketolase (EC 2.2.1.1). This was assayed by adding 1.0 unit of D-ribulose 5-phosphate 3-epimerase, 1.0 unit of ribose 5-phosphate isomerase and 5mM-ribose 5-phosphate to the reaction medium.

Ribose 5-phosphate isomerase (EC 5.3.1.6). This was assayed by adding 5mM-ribose 5-phosphate, 0.25 unit of transketolase and 0.25 unit of D-ribulose 5-phosphate 3-epimerase to the reaction medium.

D-Ribulose 5-phosphate 3-epimerase (EC 5.1.3.1). This was assayed by adding 5mM-ribose 5-phosphate, 0.1 unit of transketolase, and 0.1 unit of ribose 5-phosphate isomerase to the reaction medium.

β -Glucuronidase (EC 3.2.1.31). This was assayed with 4-methylumbelliferyl β -D-glucuronide as substrate in 0.1M-sodium acetate buffer (pH 3.75) in the presence of 0.2% (v/v) Triton X-100. The assay was carried out as described by Max *et al.* (1971).

Enzymes were assayed by monitoring the change in A_{340} in a Beckman model 25 recording spectrophotometer at 25°C. Rates were linear with respect to time (up to 5min) and protein concentrations. Substrate concentrations were saturating. Addition of Mg²⁺ had no effect on enzyme activities. Transketolase activity was not influenced by the addition of thiamin pyrophosphate to the incubation medium; presumably sufficient amounts of Mg²⁺ and thiamin are present in supernatants. Enzyme specific activities were calculated as nmol/min per mg of supernatant protein and the data are presented as means $\pm$ S.E.M. Specific activities of the oxidative and non-oxidative enzymes agree with published values (Tan & Wood, 1969; Turner & Manchester, 1972; Kauffman *et al.*, 1976; Heinrich *et al.*, 1976).

Protein determinations

Supernatant fractions were analysed for protein by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. Non-collagen protein was determined by diluting homogenates with 9 vol. of 0.1M-NaOH (Lilienthal *et al.*, 1949) and leaving the resulting mixture overnight at 25°C. After centrifugation at 800g, non-collagen protein was determined in the resulting supernatants by the method of Lowry *et al.* (1951).

Histochemical and histological procedures

Control and Marcaine-treated muscles were mounted on cryostat chucks and quenched in a Dewar flask containing acetone cooled by solid CO₂. Sections (10 μ m) were cut at -15°C, air-dried and stained for glucose 6-phosphate dehydrogenase or Nissl substance as described previously (Kauffman *et al.*, 1974).

Statistical analysis

The activities of the non-oxidative enzymes of the pentose phosphate pathway were compared by two-factor analysis of variance with repeated measures on one factor (Winer, 1962).

Results and Discussion

The activities of glucose 6-phosphate dehydrogenase (Fig. 1) and 6-phosphogluconate dehydrogenase (Fig. 2) are dramatically elevated in skeletal muscle during regeneration after muscle destruction caused by Marcaine, rising to six times control values by 8 h and nine times control values by 24 h.

Administration of cycloheximide at the time of Marcaine injection or actinomycin D 3 h before Marcaine injection prevented the increase in glucose 6-phosphate dehydrogenase (Table 1) and 6-phosphogluconate dehydrogenase (Table 1). That these dosages were adequate to inhibit protein and RNA synthesis has been shown in a number of studies. For example, cycloheximide administration to rats (1 mg/kg) inhibited amino acid incorporation into skeletal-

muscle protein by 80% (Narayanan & Eapen, 1973). Further, Grampp *et al.* (1972) reported a 50–60% decrease in uridine incorporation into acid-precipitable material by actinomycin D at a dose of 0.5 mg/kg. Thus protein and RNA synthesis seems to be required for elevation of the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Although administration of cycloheximide at the time of Marcaine injection prevented the increase of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, cycloheximide injection at 2 or 4 h after Marcaine did not (results not shown). This result suggests that activation of protein synthesis occurred shortly after treatment with Marcaine, after which time enzyme activities continued to increase even in the presence of the inhibitor.

To determine whether the presence of a stimulatory factor or absence of an inhibitory factor caused enhanced glucose 6-phosphate dehydrogenase activity in regenerating muscles, supernatants of Marcaine-treated and control muscles were mixed and the mixture was assayed for glucose 6-phosphate dehydrogenase. The activities of the mixed supernatants were additive at 2, 6 and 24 h after Marcaine (results not shown). Therefore no stimulatory substance is present. Similarly, normal muscles do not appear to contain inhibitory substances that would have to be destroyed before an increase of glucose 6-phosphate dehydrogenase activity could occur. We conclude that glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are rapidly induced in skeletal muscle in response to Marcaine.

Fig. 1. Glucose 6-phosphate dehydrogenase activity of Marcaine-treated (●) and control (▲) rat anterior tibial muscles

At indicated times after intramuscular injection of Marcaine, rat anterior tibial muscles were excised, homogenized and centrifuged. The supernatant fractions were assayed for glucose 6-phosphate dehydrogenase as described in the text. Values shown are means of four determinations $\pm$ S.E.M.

Fig. 2. 6-Phosphogluconate dehydrogenase activity of Marcaine-treated (●) and control (▲) rat anterior tibial muscles

At indicated times after intramuscular injection of Marcaine, rat anterior tibial muscles were excised, homogenized and centrifuged. The supernatant fractions were assayed for 6-phosphogluconate dehydrogenase activity as described in the text. Values shown are means of four determinations $\pm$ S.E.M.

Table 1. *Effect of cycloheximide and actinomycin D on glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in Marcaine-treated and control muscles*

Marcaine was injected intramuscularly into rat anterior tibial muscles as described in the text. Rats were either not treated or injected intraperitoneally with cycloheximide (50mg/kg) at the time of Marcaine injection or actinomycin D (1.5mg/kg) 3 h before Marcaine injection. At 8 h after Marcaine injection rats were decapitated, and muscles were removed, homogenized and centrifuged. The supernatant fractions were assayed for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase as described in the text. Each value is the mean $\pm$ S.E.M. for the number of determinations shown in parentheses.

Treatment of rats	Enzyme activities (nmol/min per mg of supernatant protein)			
	Glucose 6-phosphate dehydrogenase		6-Phosphogluconate dehydrogenase	
	Marcaine-treated muscles	Control muscles	Marcaine-treated muscles	Control muscles
Not treated	8.67 $\pm$ 1.02 (8)	1.92 $\pm$ 0.20 (8)	7.04 $\pm$ 0.43 (8)	2.97 $\pm$ 0.49 (8)
Cycloheximide	1.84 $\pm$ 0.08 (4)	2.01 $\pm$ 0.12 (4)	2.07 $\pm$ 0.21 (4)	2.27 $\pm$ 0.24 (4)
Actinomycin D	2.29 $\pm$ 0.44 (3)	1.56 $\pm$ 0.14 (3)	2.74 $\pm$ 0.25 (3)	2.96 $\pm$ 0.25 (3)

A possible complication of these results resides in the phagocytic-cell infiltration, which is a prominent feature in the early phases of muscle regeneration (Hall-Craggs, 1974). It is unlikely, however, that phagocytic-cell infiltration contributed significantly to the increase in glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase during the first 8 h after Marcaine injection. Histochemical studies performed at 8 h localized glucose 6-phosphate dehydrogenase almost exclusively within muscle fibres (Plate 1b), and not within interstitial cells. Although infiltration of muscle by phagocytic cells was just discernable at 8 h, by 24 h Nissl staining revealed infiltration of the muscle by large numbers of phagocytic cells (Plate 1c). Phagocytic cells probably contributed to the increase of glucose 6-phosphate dehydrogenase activity observed at this time, since localization of the glucose 6-phosphate dehydrogenase indicated that enzyme activity was present in these cells as well as muscle fibres (Plates 1b, 1c). Cycloheximide, which prevented the increase in glucose 6-phosphate dehydrogenase 7 h after Marcaine, did not alter invasion of muscle by phagocytic cells, which still stained histochemically for the enzyme (results not shown).

Comparison of the time course of glucose 6-phosphate dehydrogenase with that of β -glucuronidase, which might be enriched in phagocytic cells, showed that the activities did not increase in parallel (Fig. 3). There was no substantial increase in β -glucuronidase until 6–8 h after Marcaine injection, whereas glucose 6-phosphate dehydrogenase activity increased about 3-fold during this period (Fig. 3). By using histological techniques similar to those in the present study, Smith (1965) and Snow (1973) also showed that glucose 6-phosphate dehydrogenase was localized within muscle cells in regenerating muscle. Thus it appears that enhanced enzyme activity resides in muscle fibres themselves for at least the first 6–8 h

Fig. 3. *Glucose 6-phosphate dehydrogenase and β -glucuronidase in Marcaine-treated and control rat anterior tibial muscles*

At indicated times after Marcaine injection, muscles were excised, homogenized and centrifuged. The supernatant fractions were assayed for glucose 6-phosphate dehydrogenase ($\bullet$) and the homogenate was assayed for β -glucuronidase ($\circ$). Enzyme activity was expressed as nmol/min per mg of supernatant protein (glucose 6-phosphate dehydrogenase) or nmol/min per mg of non-collagen protein (β -glucuronidase).

after Marcaine, after which time phagocytic cells contribute to the increase in enzyme activities. Figs. 1 and 2 show that the secondary increase in the activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from 15–24 h corresponded closely to the increase in β -glucuronidase activity in muscles injected with Marcaine.

Glucose 6-phosphate dehydrogenase in liver has a half-life of about 15 h (Freedland, 1968; Goldberg & St. John, 1976). Although the half-life of glucose 6-phosphate dehydrogenase in regenerating muscle has yet to be determined, the rapid increase in activity

EXPLANATION OF PLATE I

Comparative staining for Nissl substance and glucose 6-phosphate dehydrogenase in control and Marcaine-treated rat anterior tibial muscles

(i) Nissl stain; (ii) histochemical reaction for glucose 6-phosphate dehydrogenase. (a) Control; (b) Marcaine, 8h; (c) Marcaine, 24h. The bar denotes 100 μ m and is the magnification for all Plates.

Table 2. *Transaldolase, transketolase, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase activities in Marcaine-treated and control rat anterior tibial muscles*

Values are nmol/min per mg of supernatant protein and are expressed as means $\pm$ s.e.m. for the numbers of determinations in parentheses. Experimental procedures are described in the text. * $P < 0.01$; † $P < 0.05$ (analysis of variance: transaldolase, $F = 8.31$, $df = 1/11$, $P < 0.01$; transketolase, $F = 8.31$, $df = 2/10$, $P < 0.01$; ribose 5-phosphate isomerase, $f = 44.0$, $df = 1/10$, $P < 0.01$; ribulose 5-phosphate 3-epimerase, $F = 17.08$, $df = 2/11$, $P < 0.01$; F , variance; df , degrees of freedom).

Time after Marcaine injection ...		Activity (nmol/min per mg of supernatant)		
		2h	6h	24h
Transaldolase	Marcaine	27.1 $\pm$ 2.3 (5)*	32.6 $\pm$ 2.4 (4)*	25.4 $\pm$ 1.6 (5)†
	Control	18.2 $\pm$ 0.8 (5)	22.0 $\pm$ 1.1 (4)	21.1 $\pm$ 1.0 (5)
Transketolase	Marcaine	28.3 $\pm$ 3.6 (4)*	31.1 $\pm$ 2.6 (4)*	14.9 $\pm$ 0.6 (5)
	Control	18.3 $\pm$ 1.2 (4)	20.7 $\pm$ 1.6 (4)	16.9 $\pm$ 1.7 (5)
Ribose 5-phosphate	Marcaine	34.2 $\pm$ 4.0 (4)*	57.3 $\pm$ 4.8 (4)*	45.1 $\pm$ 4.0 (5)†
	Control	25.5 $\pm$ 3.0 (4)	33.0 $\pm$ 2.9 (4)	31.7 $\pm$ 3.0 (5)
Ribulose 5-phosphate 3-epimerase	Marcaine	64.2 $\pm$ 7.0 (5)†	67.7 $\pm$ 3.6 (4)*	51.0 $\pm$ 4.9 (5)*
	Control	59.9 $\pm$ 6.6 (5)	49.5 $\pm$ 2.8 (4)	43.8 $\pm$ 4.9 (5)

in response to Marcaine suggests that it is relatively short in regenerating tissue. Short-half-life enzymes are considered to be regulatory enzymes whose activities can fluctuate rapidly in response to environmental changes (Goldberg & St. John, 1976). Thus the rapid induction of glucose 6-phosphate dehydrogenase observed in the present study may represent adaptation to the regenerating state in skeletal muscle.

It should be noted that regulation of glucose 6-phosphate dehydrogenase is complex and the process differs among experimental systems. For example, enhancement of glucose 6-phosphate dehydrogenase activity in liver (Rudack *et al.*, 1971; Garcia & Holten, 1975), uterus (Smith & Barker, 1974) and hepatoma (Selmeci & Weber, 1976), and 6-phosphogluconate dehydrogenase activity in liver (Procsal *et al.*, 1976) and mammary gland (Betts & Mayer, 1977), occur via newly synthesized enzyme protein. On the other hand, increased glucose 6-phosphate dehydrogenase activity may be accomplished without synthesis of enzyme protein, although the increase is blocked by actinomycin D or cycloheximide (Yagil *et al.*, 1974; Hizi & Yagil, 1974). In these situations, enhanced activity apparently results from modification of pre-existing enzyme molecules (Hizi & Yagil, 1974). Post-transcriptional regulation of glucose 6-phosphate dehydrogenase synthesis has been postulated to occur in liver after carbon tetrachloride injury (Watanabe & Taketa, 1973), and in erythrocytes and leukaemic granulocytic cells (Kahn *et al.*, 1976).

The non-oxidative enzymes (Table 1) of the pentose phosphate pathway were not affected to the same extent as were glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Transketolase, transaldolase, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase activities were slightly elevated (1.5, 1.5, 1.3 and 1.1 times

control respectively) 2h after injection of Marcaine. The activities of these enzymes remained elevated at 6h (1.5, 1.5, 1.7 and 1.4 times control respectively). By 24h, transaldolase, ribose 5-phosphate isomerase and ribulose 5-phosphate 3-epimerase were significantly elevated (1.2, 1.4 and 1.2 times control respectively). It is possible that the small increase in the activities of the non-oxidative enzymes is due to the inflammatory cell response. The activities of the non-oxidative enzymes in normal adult muscles are at least 10 times those of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Figs. 1 and 2, Table 2).

Thus elevation of the activities of the oxidative enzymes may represent the major factor contributing to increased flux through the pentose pathway during the initial stages of muscle regeneration. Enhanced activities of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase have been associated with elevated activity of the pentose phosphate pathway (Figs. 1 and 2; cf. Beaconsfield & Carpi, 1964; Rifenberck *et al.*, 1974; Wagner *et al.*, 1977a) and probably reflect accelerated glucose utilization for the production of nucleic acids (Beaconsfield & Reading, 1964) and lipids. In this regard, increased quantities of RNA have been noted in a number of studies on muscle regeneration (Gallucci *et al.*, 1966; Susheela *et al.*, 1968; Carlson, 1970; Neerunjun & Dubowitz, 1974). We conclude that enhancement of the pentose phosphate pathway is important for anabolic processes in the initial stages of skeletal muscle regeneration.

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