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HORMONAL INFLUENCE ON DIURNAL GLYCOGEN RHYTHMS IN RAT SKELETAL MUSCLES

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SUMMARY

1. Normal, diabetic, and sympathectomized rats were killed at intervals during a 12 h light (6 a.m. to 6 p.m.)/12 h dark cycle to determine (1) the patterns of diurnal glycogen rhythms in skeletal muscle fibre types, and (2) if the absence of insulin or the catecholamines epinephrine and norepinephrine abolished these rhythms.

2. Normal rat muscles lost and restored 40–60 % of their glycogen stores during one 24-h cycle. Glycogen losses were linear between 6.30 a.m. (acrophase time for all muscles) and 8.30 a.m. and between 8.30 a.m. and nadir points (4.30 p.m. to 8.30 p.m.), but the rates of loss were non-uniform between these time frames and among fibre types. All muscles had similar linear restoration rates.

3. Glycogen stores in most muscles from diabetic and sympathectomized rats exhibited changes similar to those seen in normal animals.

4. Skeletal muscle fibre types have notable and non-uniform diurnal glycogen rhythms that should be considered in the design of metabolic and exercise experiments. These rhythms do not appear to be regulated, either differentially or *in toto*, either by insulin or by the catecholamines epinephrine and norepinephrine.

INTRODUCTION

There have been several reports documenting the existence of diurnal rhythms in skeletal muscle glycogen stores (Conlee, Rennie & Winder, 1976; Garwaite, Morgan & Meyer, 1979), but the mechanisms responsible for regulating these daily changes in muscular glycogen content have not been identified. Insulin and the catecholamines epinephrine and norepinephrine are important blood-borne hormonal regulators of carbohydrate metabolism in muscular tissues, but their possible roles in diurnal glycogen changes have not been closely examined. The purpose of the present study was (1) to characterize the patterns of diurnal glycogen fluctuations in different types of rat skeletal muscle fibres, (2) to test the hypothesis that these daily glycogen

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rhythms would be abolished in both diabetic and sympathectomized animals, and (3) to determine whether or not glycogen rhythms in individual skeletal muscle fibre types are differentially affected by the diabetic state or by sympathectomy.

METHODS

Experimental animals

Male and female Sprague-Dawley rats with initial body weights of 180–200 g were used in these studies. They were segregated by sex and housed in temperature controlled rooms (23 ± 1 °C) regulated to provide alternating 12-h periods of light (6 a.m. to 6 p.m.) and darkness with food (commercial pellets) and water available *ad libitum*.

Experimental designs

The first study examined the magnitudes and patterns of diurnal muscular glycogen changes in normal rats. Male animals were killed at 2-h intervals during one 24-h period, beginning at 6.30 a.m., and muscle samples were removed and analysed for glycogen content.

The second study evaluated the impact of altered hormonal status on diurnal muscular glycogen changes. Female animals were randomly divided into three groups: normal (N), diabetic (D), and sympathectomized (S). Animals in the N group received no special treatment. Animals in the D group received a single injection of streptozotocin (STZ), which is toxic to pancreatic β-cells (Junod *et al.* 1967) and produces symptoms characteristic of uncontrolled diabetes mellitus (Junod *et al.* 1967; Chang & Schneider, 1971; Ianuzzo & Armstrong, 1976). Animals in the S group were treated with 6-hydroxydopamine (60HD), which produces selective degeneration of post-ganglionic adrenergic nerve terminals (Thoenen & Tranzer, 1968). In addition, adrenal medullas were surgically removed bilaterally because 60HD does not affect their catecholamine content (Weinshilboum & Axelrod, 1971). Animals in the second study were terminated at either 7 a.m. or 7 p.m., 2–4 weeks after hormonal modifications were initiated, and muscle samples were removed and analysed for glycogen content.

Hormonal modifications

Animals in the D group were rendered diabetic by a single injection of STZ (80 mg·kg body wt^{-1}) dissolved (40 mg·ml^{-1}) in 50 mM-citrate buffer, pH 4·5 (Junod *et al.* 1967). Injections were administered under ether anaesthesia via the lingual vein, and the animals were killed 3–4 weeks later.

Initially each animal in the S group was subjected to a bilateral adrenodemedullectomy (ADMX) under pentobarbital anaesthesia (40 mg·kg body wt^{-1}). Two days following surgery, each animal received 2 injections (6 h apart) of 60HD (34 mg·kg body wt^{-1}) under ether anaesthesia. The 60HD was dissolved in 0·5 ml of a 0·9 % saline-0·1 % ascorbic acid solution just prior to its injection into the lingual vein (Gollnick, 1973). One week later each animal received two final injections of 60HD as above. Animals in the S group were sacrificed 5–21 days following these final injections.

Sacrifice procedures

In both studies, animals were killed by exsanguination under sodium pentobarbital anaesthesia (50 mg·kg body wt^{-1}, i.p.). Samples of the deep axial (red) portion of the lateral head of gastrocnemius muscle (RG), the peripheral (white) portion of the medial head of gastrocnemius muscle (WG), and the soleus muscle (SOL) were excised for glycogen determinations. These muscle sites were chosen because of their dominant compositions of fast-twitch oxidative glycolytic (FOG), fast-twitch glycolytic (FG), and slow-twitch oxidative (SO) fibres, respectively (Saubert, Armstrong, Shepherd & Gollnick, 1973).

In the second study, diabetes was confirmed by removing and analysing blood samples for glucose content, and sympathectomy was verified by removing and analysing heart and adrenal tissues for catecholamine contents. All samples except blood were frozen in liquid nitrogen immediately following their excision and then stored at −85 °C until analysis.

Tissue analyses

For determination of blood glucose concentration a sample of whole blood was immediately deproteinized with 3 M-perchloric acid and centrifuged for 15 min at 4 °C at 5000 ***g***. The supernatant fluid was then neutralized with 2 M-potassium bicarbonate and the sample centrifuged as above. The perchlorate-free extract was removed and frozen at −20 °C until analysis for glucose units using the fluorometric procedures described below for tissue glycogen determination. A glucose standard was used, and results were expressed in mM.

Heart samples and adrenal glands were analysed for catecholamine concentrations using the procedures described by Jacobowitz, Cooper & Barner (1967), except that the tissues were first homogenized in 750 μl of 0·01 N-HCl (minus tissue sample water volume, assumed to be 70 %) before extraction with 5 ml butanol. Norepinephrine was used as the standard for heart samples, and a norepinephrine–epinephrine mixture (20 %–80 %) was used as the standard for adrenal glands. Recovery averaged 84·7 % using these procedures, and values were corrected to 100 %. Results were expressed as nmol and μmol catecholamines·g tissue wet wt^{-1} for heart and adrenal tissues, respectively.

Tissue samples were analysed for glycogen content using either the anthrone technique described by Seifter, Dayton, Novic & Muntwyler (1950) or the fluorometric procedures described by Lowry & Passonneau (1972). Results were expressed as μmol glucosyl units·g tissue wet wt^{-1}.

Statistics

In study one, differences among means within and among tissues were tested using analysis of variance, and the Student-Newman-Keul's test was used to locate the sites of significant differences. Glycogen losses and gains were independently regressed against time using least square analyses, and analysis of variance was used to determine if the calculated functions were linear and if the calculated slopes were significantly different from zero. A correlation coefficient was calculated for each

regression equation and tested for significance using an appropriate statistical tab (Zar, 1974). Significant differences between slopes were determined using the Student's t test as described by Zar (1974).

In study two, the influences of hormonal status and time of killing on each variable were evaluated using an analysis of variance for a 3×2 (group×time) factorial design, and the Student-Newman-Keul's test was used to locate sites of significant ($P < 0{\cdot}05$) differences.

RESULTS

Study One

Significant ($P < 0{\cdot}025$ or less) diurnal changes of 43–59 % occurred in the glycogen concentrations of all muscles examined (Table 1). In all three muscles peak values were observed at 6.30 a.m., and the greatest rates of glycogen loss occurred between 6.30 a.m. and 8.30 a.m., though the rate of loss in SOL was noticeably less than those in RG and WG (Table 1). Between 8.30 a.m. and nadir points (4.30 p.m. in RG and SOL and 8.30 p.m. in WG) glycogen concentration decreased as a linear function of time ($P < 0{\cdot}05$ or less) in each muscle, but the rate of loss in WG was significantly less ($P < 0{\cdot}05$) than those in RG and SOL (Table 1). Between nadir points and 6.30 a.m.

Table 1. *Muscular glycogen acrophase, nadir, loss rate, and restoration rate values for male animals*

Tissue	Glycogen concentration		Linear loss and restoration rates		
	Acrophase	Nadir	6·30 a.m. – 8·30 a.m.	8·30 a.m. – Nadir	Nadir – 6·30 a.m.
WG	45·46 ± 3·47	26·63 ± 3·69	−6·38	−0·54 (−0·86)	1·53 (0·84)
RG	39·44 ± 8·30	16·90 ± 0·87	−6·79	−1·16 (−0·98)	1·29 (0·91)
SOL	30·61 ± 8·72	15·79 ± 2·28	−2·95	−1·05 (−0·99)	1·07 (0·89)

Glycogen values are expressed as mean ± S.E. in μmol glucose units·g muscle wet wt^{-1}; $N = 3$ for acrophase means; $N = 5$ for nadir means. Rate values are given in μmol glucose units·g muscle wet wt^{-1}·h^{-1}. Statistical differences for concentrations and rates are given in Results. Values in parentheses are correlation coefficients, and all are statistically significant ($P < 0{\cdot}05$ or less).

WG = white gastrocnemius; RG = red gastrocnemius; SOL = soleus.

the glycogen levels in all three muscles were restored as linear functions of time ($P < 0{\cdot}05$) and the rates of restoration were not significantly different from each other (Table 1). At each sampling time the mean value for WG was higher than those for RG and SOL, but this difference was significant ($P < 0{\cdot}05$) only between 12·30 p.m. and 10·30 p.m.

Study Two

D animals experienced little or no body weight gain (Table 2) and demonstrated excessive fluid intake and excretion. Compared to N and S animals their blood gluco

Table 2. *Body weight, blood glucose, catecholamine, and glycogen values for normal, diabetic, and sympathectomized female animals*

Group	Normal		Diabetic		Sympathectomized	
	7 a.m.	7 p.m.	7 a.m.	7 p.m.	7 a.m.	7 p.m.
Body weight (g)	244 ± 4[a]		184 ± 6		267 ± 5	
N	19		17		16	
Catecholamines						
Heart (nmol·g^{-1})	7·42 ± 0·38		7·67 ± 0·43		1·21 ± 0·19	
N	19		17		14	
Adrenals (μmol·g^{-1})	3·478 ± 0·147		3·814 ± 0·199		0·011 ± 0·002	
N	18		16		11	
Blood glucose (mM)	6·25 ± 0·08	6·64 ± 0·18[b]	35·26 ± 1·32	35·60 ± 1·36	5·73 ± 0·17	6·62 ± 0·09[b]
N	10	8	9	8	7	6
Glycogen (μmol·g^{-1})						
Liver	332·87 ± 22·47	121·86 ± 15·14[b]	162·20 ± 15·00	114·52 ± 12·57	462·98 ± 30·08	203·53 ± 18·45[b]
N	10	9	9	8	8	6
Heart	21·63 ± 1·31	18·34 ± 1·57	66·87 ± 4·61	63·36 ± 3·17	25·38 ± 1·81	21·11 ± 0·90
N	9	9	8	8	8	6
WG	32·38 ± 1·57	34·89 ± 1·72	48·39 ± 2·97	38·34 ± 1·91[b]	41·68 ± 2·10	33·85 ± 3·22[b]
N	9	9	9	8	8	5
RG	30·46 ± 2·34	21·11 ± 1·36[b]	31·12 ± 2·25	25·17 ± 0·94[b]	38·05 ± 1·35	29·06 ± 1·12[b]
N	10	8	9	8	7	6
SOL	36·14 ± 2·64	29·75 ± 2·07[b]	25·03 ± 1·93	22·25 ± 1·03	49·93 ± 1·32	38·58 ± 1·28[b]
N	9	8	9	8	8	6

[a] Values are expressed as mean ± S.E.
[b] Value significantly different ($P < 0·05$) from 7 a.m. value.

levels were 6-fold higher, their heart glycogen concentrations were 3-fold higher, and their liver glycogen stores were 50 % lower (Table 2). All of these changes are characteristic of the diabetic state.

Within each animal group, heart and adrenal catecholamine concentrations did not differ between 7 a.m. and 7 p.m., so the values for these two time points were pooled to produce a single group mean for each tissue. Treatment with 60HD reduced the mean heart catecholamine concentration of S animals to 16 % of the mean heart values for N and D animals, which did not differ from each other (Table 2). Individual catecholamine values (not shown) for S animal hearts ranged from 2 % to 46 % of the N group mean and were directly related to the number of days between the final 60HD injections and sacrifice for each animal. ADMX reduced the mean adrenal catecholamine content of S animals to 0·3 % of N and D group means (Table 2). The heart and adrenal catecholamine data suggest that S animals had minimally functioning sympathetic systems.

In agreement with the report of Conlee *et al.* (1976), no consistent differences were observed in the diurnal muscular glycogen rhythms of N female rats compared to those of the male rats of study one. WG samples from D and S animals demonstrated significant diurnal glycogen rhythms, but the WG of N animals did not. Peak WG values for all three groups were significantly different from each other. The glycogen content of RG in all three groups underwent significant diurnal changes, and peak S group values were significantly higher than those of N and D animals. SOL samples from S and N animals demonstrated a diurnal glycogen rhythm and at both time points there were significant differences between S, N, and D group means (Table 2).

DISCUSSION

Soon after this project was initiated, Conlee *et al.* (1976) published their report on diurnal variations in the glycogen content of rat liver and skeletal muscles. We continued with our investigations because we routinely use a different strain of rat and sample different muscles, and because these authors did not detail or discuss similarities and/or differences in the patterns they observed among muscles and fibre types.

In study one, all three muscles lost approximately 50 % of their glycogen stores during the light phase of the 24-h cycle, and the absolute values and diurnal patterns were quite similar to those of equivalent tissues reported by Conlee *et al.* (1976) when adjusted for the different timing of the light–dark cycle. The existence of these rhythms and their magnitude impose constraints on several types of experiments. First, studies using glycogen depletion patterns as an index of fibre recruitment and utilization should be uniformly timed from day to day to avoid misinterpretation of results. Likewise, experiments examining the influence of dietary manipulations on muscular fuel depots should be conducted at similar times each day. In addition, because there is a good correlation between time to exhaustion and initial muscular glycogen content during prolonged submaximal physical activity (Bergström, Hermansen, Hultman & Saltin, 1967; Clark & Conlee, 1979), the timing of exercise bouts from day to day should be uniform in experiments where exercise duration is an important measure.

The pattern of diurnal glycogen depletion and restoration in FOG fibres does not appear to vary from muscle to muscle [comparing the red vastus of Conlee *et al.* (1976) to our RG]. Since this is also true for diurnal glycogen changes in FG fibres from their white vastus and our WG, it suggests that central, rather than local, factors trigger the rhythms observed in each fibre type. The overall similarity in the timing of the glycogen depletion and restoration patterns among the three fibre types also suggests the presence of a central synchronizer.

However, the responses of the three fibre types were not identical, suggesting that the fibre types have different sensitivities to a central stimulus. Both FOG and FG fibres lost glycogen between 6.30 a.m. and 8.30 a.m. at more than twice the rate of SO fibres (Table 1). Thereafter, the rate of glycogen loss from FG fibres was significantly less than those from FOG and SO fibres, which were nearly identical to each other. These observed differences and their importance should be viewed with some caution because of the small number of animals examined at each sampling time (Table 1).

The need for significant muscular glycogen catabolism during the light period is not obvious. At this time the animals are relatively inactive (Bockman, Meyer & Purdy, 1971), so glycogenolysis to fuel muscular activity for either posture or locomotion would be minimal. In addition they have full stomachs (Garwaite *et al.* 1979), replenished liver glycogen stores (unpublished observations; Conlee *et al.* 1976), maintained normal blood glucose and free fatty acid levels (unpublished observations; Conlee *et al.* 1976), and oxygen delivery to muscle in excess of oxygen utilization (Folkow & Halicka, 1968). Clearly, however, the metabolic environment of skeletal muscle is such that glycogen stores are still mobilized in the face of abundant extracellular fuels, but the mechanisms producing this cyclic mobilization, and restoration, of muscular glycogen stores remain unknown.

Unlike in the liver (Shimazu, Matzushita & Ishikawa, 1978), direct autonomic neural links do not appear to be involved. First, the parasympathetic nervous system is not distributed to limb musculature. Second, because all S animal muscles that were examined demonstrated diurnal changes in their glycogen levels, the sympathetic nervous system does not appear to play a direct role. This conclusion must be tempered by the fact that S animals were not completely devoid of catecholamines. While ADMX was almost 100% effective in eliminating the adrenal glands as a source of circulating catecholamines, 60HD only reduced cardiac catecholamine levels by variable amounts, and was most effective when animals were sacrificed soon (5 days) after they received their final injections. With longer periods of time nerve regrowth probably occurred (de Champlin *et al.* 1975), and the increased amount of hormone present, together with supersensitivity to its presence (de Champlin *et al.* 1975), could still exert a controlling influence on glycogen rhythms.

However, data from individual animals tend to minimize this possibility. In animals killed 5 days following the final 60HD injections, heart catecholamine levels were only 2% of those in N animals, while animals sacrificed 21 days following the final 60HD injections had heart catecholamine levels that were 46% of those in N animals. Despite this wide range of catecholamines present, all animals showed similar patterns of diurnal glycogen changes in their tissues, which argues against a dominant role for these hormones in controlling these changes. In addition, the fact that glycogen stores in each muscle sample underwent significant diurnal rhythms

argues against any differential effect of catecholamines on diurnal glycogen rhythms in the three muscle fibre types.

In general, absolute glycogen concentrations in S animal tissues were higher than those of N animals. Elevated liver glycogen values have been observed previously in animals subjected to ADMX (Gollnick, 1973). The reason for these elevated values is not definitely known. Adrenalectomized-chemically sympathectomized rats have elevated basal insulin levels (Ahrén, Järhult & Lundquist, 1981) and increased tissue sensitivity to insulin (Ahrén & Lundquist, 1981). Either one, or both, of these occurrences could facilitate increased tissue glycogen formation and deposition in the S animals.

STZ is commonly used to induce diabetes mellitus in laboratory animals. Rats in the D group exhibited many of the symptoms characteristic of the diabetic state, and their blood glucose values were within the range of those reported by others using this model (Junod *et al.* 1967; Chang & Schneider, 1971).

Significant diurnal glycogen rhythms occurred in the WG and RG of D animals, suggesting that insulin does not play a primary role in regulating diurnal muscular carbohydrate changes. While these animals were severely diabetic, they probably were not completely void of insulin. C. D. Ianuzzo and V. Chen (personal communication, 1981) found that in animals injected with an identical dose of STZ, plasma immunoreactive insulin (IRI) levels were 4 % of those in controls, and similar values have been reported by others using slightly smaller doses of STZ (Junod *et al.* 1967; Chang & Schneider, 1971). It may be argued that, despite this very low insulin level, enough was present to exert control over daily glycogen rhythms. While possible, this appears unlikely. Conlee *et al.* (1976) were unable to observe a relationship between diurnal changes in plasma IRI levels and changes occurring in muscular glycogen stores in normal animals. In addition, the diabetic condition produces major, not minor, shifts in metabolic fuel storage and utilization patterns (Table 2; Randle, 1966; Chang & Schneider, 1971; Ianuzzo & Armstrong, 1976). Since the loss of 96 % of the normal insulin levels has such drastic effects, one would expect similar profound changes in cycles of muscular glycogen stores if insulin were responsible for their regulation. The fact that D animals exhibited, for the most part, normal diurnal changes in muscular glycogen content argues against the role of insulin in controlling such changes.

Since the SOL failed to show diurnal variation in its glycogen stores in the D group, this could be taken as evidence that this muscle, with its dominant composition of SO fibres (Saubert *et al.* 1973), is more sensitive to the absence of insulin than the other muscles. However, the fact that histochemical (Armstrong, Gollnick & Ianuzzo, 1975) and biochemical (Ianuzzo & Armstrong, 1976) studies have demonstrated that SO fibres are least susceptible to the debilitating effects hypoinsulinism has on skeletal muscle fibre types argues against such a possibility.

In conclusion, significant diurnal rhythms occur in locomotory muscle glycogen stores, with peaks occurring at the beginning of the light cycle and troughs occurring 10–14 h later. These rhythms are not confined to one strain of rat and they appear to be similar in muscles with similar fibre type compositions, but the patterns of glycogen loss and restoration differ among fibre types. Neither insulin nor the catecholamines epinephrine and norepinephrine appear to play major roles in regulating diurnal muscular glycogen rhythms, and other synchronizers, probably of a hormonal or neuroendocrine nature, are responsible. In addition, there is no strong

vidence that these hormones exert differential effects on diurnal glycogen changes in the various types of fibres composing skeletal muscle.

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