

Differing mechanisms of cold-induced changes in capillary supply in m. tibialis anterior of rats and hamsters

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Summary

The physiological, metabolic and anatomical adaptations of skeletal muscle to chronic cold exposure were investigated in Wistar rats (*Rattus norvegicus*), a species that defends core temperature, and Syrian hamsters (*Mesocricetus auratus*), which may adopt a lower set point under unfavourable conditions. Animals were exposed to a simulated onset of winter in an environmental chamber, progressively shortening photoperiod and reducing temperature from 12h:12h L:D and 22 °C to 1h:23h L:D and 5 °C over 4 weeks. The animals were left at 4 °C for a further 4 weeks to complete the process of cold-acclimation. M. tibialis anterior from control (euthermic) and cold-acclimated animals of similar mass showed a significant hyperactivity-induced hypertrophy in the rat, but a small disuse atrophy in the hamster. Little evidence was found for interconversion among fibre types in skeletal muscle on cold-acclimation, and only modest differences were seen in activity of oxidative or glycolytic enzymes in either species. However, adjustments in Type II fibre size paralleled the muscle hypertrophy in rat and atrophy in hamster. Cold-induced angiogenesis was present in the rat, averaging a 28 % increase in capillary-to-fibre ratio (C:F) but, as this was balanced by fibre

hypertrophy across the whole muscle, there was no change in capillary density (CD). In contrast, the C:F was similar in both groups of hamsters, whereas CD rose by 33 % in line with fibre atrophy. Within distinct regions of the m. tibialis anterior, there was a correlation between angiogenesis and fibre size in rats, in which oxygen diffusion distance increased, but not in hamsters, in which there was a reduced oxygen diffusion distance. Consequently, the change in C:F was greatest (39 %) in the glycolytic cortex region of the m. tibialis anterior in rats. We conclude that non-hibernator and hibernator rodents improve peripheral oxygen transport following cold-acclimation by different mechanisms. In rats, an increase in fibre girth was accompanied by a true angiogenesis, while the improved apparent capillary supply in hamsters was due to smaller fibre diameters. These responses are consistent with the strategies of resisting and accommodating, respectively, an annual fall in environmental temperature.

Key words: angiogenesis, capillary, histochemistry, enzyme activity, fibre size, rat, *Rattus norvegicus*, hamster, *Mesocricetus auratus*.

Introduction

The annual onset of cold weather imposes a significant metabolic burden on small animals because of their large surface-area-to-volume ratio and the obligatory thermogenesis required to counter residual heat loss to the environment (Himms-Hagen, 1986). Regulation of core temperature (T_b) in a cold environment involves behavioural and physiological adaptations to reduce heat loss and increase heat production. The typical mammalian response to low environmental temperature is one of avoidance, defending core temperature by increased muscle activity and peripheral vasoconstriction. For example, shivering was the primary source of heat production when rats were initially exposed to low environmental temperature, while subsequent cold-acclimation was associated with a greater involvement of non-shivering thermogenesis, i.e. heat production resulting from tissue

metabolism other than from contractions of skeletal muscle (Griggio, 1982). Although non-shivering thermogenesis is mainly a product of hepatic heat generation, some species also invoke facultative thermogenesis, releasing extra heat from brown adipose tissue (BAT) to enhance the response to cold exposure. Hibernation, the state of winter lethargy associated with a reduction in metabolic rate and core temperature, is an adaptive mechanism which has evolved in some mammalian and avian species to cope with unfavourable conditions. Hibernators regulate their core temperature at a set point differing from that in the state of euthermia (Popovic and Popovic, 1974). They are then able to accommodate low environmental temperatures by virtue of an enhanced capacity for non-shivering thermogenesis and tolerance of a reduced core temperature.

When tissue oxygenation becomes inadequate or oxygen demand rises sufficiently, an increase in vascularity may be required to promote oxygen delivery to cells. This will reduce the distance over which diffusion occurs, increase capillary surface area for exchange and accommodate a greater maximum local blood flow for delivery (Hudlická et al., 1992). In addition to its role in peripheral oxygen transport to the tissues, the capillary supply is also important for the removal of metabolic end-products such as CO₂ and lactic acid, and for the maintenance of local thermal balance. The capillary supply may therefore play an important role in the strategies of both cold avoidance and cold-acclimation. Many approaches have been used to study the vascularisation of muscles in mammals, usually showing that individual muscle fibres are surrounded by a number of capillaries in proportion to their oxidative metabolic capacity (Romanul, 1965).

Exposure to cold is thought to increase the capillary supply to skeletal muscle of rats (Heroux and St Pierre, 1957), guinea pigs (Sillau et al., 1980) and mice (Wickler, 1981), but these data are ambiguous because of the methodologies employed (Egginton, 1990a, 1998). In addition, isolating the relevant stimuli may be difficult, e.g. increased capillarisation at high altitude may reflect hypoxia, cold exposure or a combination of these stimuli. When animals were exposed to cold, an increase in capillarisation of skeletal muscles was observed (Banchero et al., 1985). It was expected that cold plus hypoxia would be more effective in inducing capillarisation than cold alone because of the synergistic effect of increasing metabolic demand by cold and reducing oxygen supply by hypoxia. However, the response depended on muscle fibre composition since cold alone was most effective in increasing capillarisation in soleus muscle (slow oxidative muscle), while cold plus hypoxia was more effective in the gastrocnemius muscle (a mixed muscle) (Banchero, 1982).

The basal metabolic rate of rats is lower than that of hamsters at a common environmental temperature (Himms-Hagen, 1986), so these non-hibernator and hibernator species may respond differently to cold exposure. Given a common stimulus, we reasoned that species with different overwintering strategies would also show differing physiological responses to a period of gradual cooling to overcome the potential limitation in peripheral oxygen transport at low environmental temperatures, the process of cold-acclimation. Such comparisons are difficult to make on the basis of published data because the response evoked by cooling or cold exposure varies among species, and depends on the animal's age and previous thermal history (duration and/or depth of cold exposure), leading to conflicting reports in the literature on the effects of cold exposure. An experimental protocol was therefore adopted that followed gradual alterations in both temperature and photoperiod in order to mimic seasonal fluctuations (summer/winter transition) rather than acute exposure to low temperatures, as used previously, and exposed non-hibernator and hibernator rodents to the same conditions. As far as we are aware, this is the first time such an approach has been adopted. Preliminary data have been presented in abstract form (Deveci and Egginton, 1996, 1998).

Materials and methods

Animals

Animals for this study were used in accordance with the animal welfare regulations of the Animals (Scientific Procedure) Act of 1986. Experiments involved two species, male Wistar rat (an albino strain of *Rattus norvegicus*) and male Syrian hamster (*Mesocricetus auratus*). Both species were divided into two groups of six animals each, euthermic (control), held at the ambient temperature of the animal holding facility, and cold-acclimated (Cold-A), subjected to low environmental temperature. All animals were provided with food pellets and water *ad libitum*, supplemented periodically with fresh vegetable matter.

Cold-acclimation

To minimise any intra-specific allometric (scale-dependent) effects, we compared groups of animals with similar body mass. While this meant starting the 8-week acclimation period with juvenile rats, the changes observed would presumably be accentuated by acclimating older (hence larger and having a reduced intrinsic capacity for non-shivering thermogenesis) animals, and we therefore feel this compromise was justified.

The initial body mass of cold-exposed rats was 60–70 g, and that of hamsters was 130–140 g, with final body masses of approximately 260 and 170 g, respectively. Rearing conditions were an ambient temperature of 21±1 °C and daily photoperiod of 12 h:12 h light:dark (L:D); control animals were maintained under these conditions. Following Home Office guidelines for experimentation, hamsters were housed in individual cages in an environmental chamber whereas rats were kept two or three in a cage. The initial air temperature on transfer was 20 °C, and this was gradually reduced to 5 °C, accompanied by a photoperiod reduced by 1.5 h each week from an initial setting of 8 h:16 h L:D to 1 h per day by week 4. After 4 weeks and up to 8 weeks, photoperiod was held at 1 h:23 h L:D, and a temperature of 5±1 °C was maintained.

Histochemistry

At the end of the cold-acclimation period, the m. tibialis anterior (TA) was quickly removed from one side of animals under anaesthesia and weighed, and slices from the proximal one-third were frozen in isopentane cooled in liquid nitrogen for subsequent histological processing. Serial 10 µm cryostat sections were stained to demonstrate myofibrillar ATPase (for fibre typing), succinic dehydrogenase (for oxidative capacity) and alkaline phosphatase (for capillary endothelium) activities. We did not distinguish between Type IIb and Type II_{d/x/c} fibres (see Fig. 1), whose oxidative capacity is between those of Types IIa and IIb (Schiaffino et al., 1990). Thus, the oxidative capacity of hamster TA may have been slightly underestimated by fibre typing relative to oxidative enzyme activity. Capillary supply was evaluated as the numerical capillary-to-fibre ratio (C:F) or capillary density (CD; mm⁻²), and muscle composition was evaluated as relative fibre type density and mean cross-sectional area, using an unbiased sampling protocol (Egginton, 1990a,b).

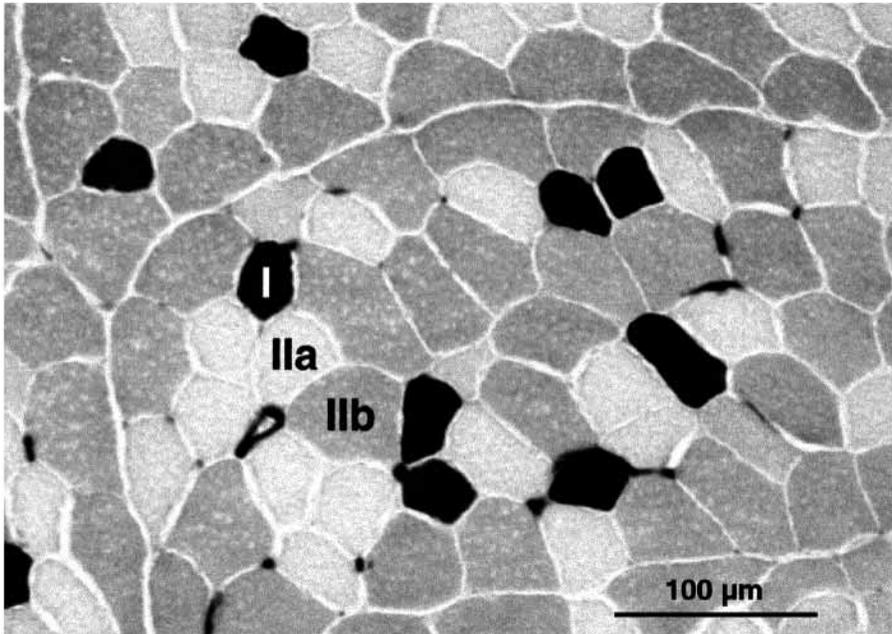


Fig. 1. A representative photomicrograph of ATPase staining from the tibialis anterior core region of a control rat; 10 μm cryostat section. The characterisation of fibre types on the basis of staining intensity is illustrated for Type I, IIa and IIb fibres. Scale bar, 100 μm.

Morphometric analyses

A square-lattice counting frame was superimposed on the image of muscle sections by means of a microscope drawing arm. For each section, the frame was systematically oriented on the same circumscribed regions, relative to the major axis and the section boundaries, using a sample area of 0.194 mm² (Fig. 2). This is different from most other studies, but was used because random samples of tissue will increase data variance and tend to obscure any localised response of specific muscle regions. Capillaries and fibres were counted in three fields in the TA cortex and two fields in the core, and averaged to give a single value for each muscle region. The muscle cross-sectional area occupied by the three main fibre types was quantified using standard stereological point-counting methodology (Egginton, 1990a). Type I or Type IIa fibres from fast or mixed muscles are approximately half the area of Type IIb fibres in adult rats (Egginton, 1990b) and, consequently, their total oxidative capacity is overestimated if only numerical frequency is quantified. In addition, muscle tension development is proportional to fibre cross-sectional area (FCSA), so a quantitative estimate of their relative areas may be a better index of functional capacity.

The relative area (A_A , areal density) of individual fibre types was estimated by standard point-counting techniques, the number fraction (N_N , numerical density) was estimated by dividing the number of fibres for each individual fibre type by the total number of fibres uniquely contained within the counting frame, and the mean FCSA of individual fibre types was estimated as the product of areal density and sample area divided by the appropriate fibre number (Egginton, 1990b).

The distance over which oxygen is transported is one of the most important limiting factors in the supply of oxygen from a capillary to the tissues, accentuating the resistance to oxygen flux existing at the capillary/fibre interface (Egginton, 1990a).

We estimated diffusion distance (D) according to the formula of Rakusan and Poupa (1963) as: $D=500/\sqrt{CD}$, where CD is capillary density. We also used a formula recommended by Snyder (1987) for hexagonal capillary arrays which estimates the maximum (ninety-fifth percentile) oxygen diffusion distance as $R_{max}=[(0.326+0.312)/C:F] \sqrt{FCSA}$ and the mean oxygen diffusion distance as $R_{ave}=[(0.140+0.231)/C:F] \sqrt{FCSA}$. Clearly, such formulae are sensitive to both cold-induced angiogenesis and changes in fibre size since CD will vary reciprocally with $FCSA$.

Enzyme activity

Muscles contralateral to those obtained for histochemical analysis were quickly removed, frozen in liquid nitrogen and stored at -80 °C until analysis. Samples were homogenised in a 30-fold excess (w/v) of ice-cold buffer (2 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ EDTA, 50 mmol l⁻¹ imidazole, pH 7.4 at 20 °C). Measurement of enzyme activity was performed at 25±0.1 °C under optimal conditions of pH and substrate/cofactor concentrations, using substrate deletion as a control for background activity, monitored by following the oxidation of the pyridine nucleotide NADH at 340 nm (using a millimolar extinction coefficient, E_{mM} , of 6.22) or the reduction of

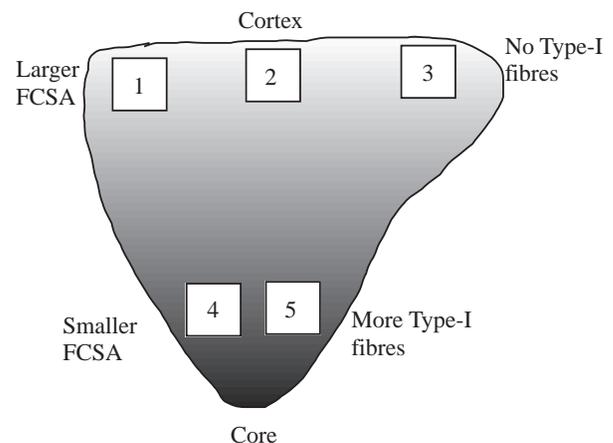


Fig. 2. Schematic cross section of tibialis anterior muscle. The numbers in squares show the relative position of the fields counted. In the cortex, fibre size is large and fibres are mainly glycolytic, whereas in the core region fibre size is smaller and fibres are more oxidative. FCSA, fibre cross-sectional area.

Table 1. Muscle mass and metabolic characteristics in euthermic and cold-acclimated rats and hamsters

	Rat		Hamster	
	Euthermic	Cold-acclimated	Euthermic	Cold-acclimated
Body mass (g)	256±3.7	263±2.1	167±3.5	168±5.6
Tibialis anterior (mg)	408±17.8	467±18.3*	137±3.8	129±6.8
Enzyme activity ($\mu\text{mol min}^{-1} \text{g}^{-1}$ wet mass)				
PFK	82.5±9.6	65.6±5.6	67.4±4.4	60.8±4.3
LDH	445±15.8	528±18.4**	202±30.5	215±18.8
CS	30.0±2.1	25.8±1.0	37.2±2.3	39.9±3.8
HAD	2.05±0.45	1.97±0.25	3.70±0.63	2.30±0.37
Activity ratio				
LDH/PFK	5.76±0.62	8.46±0.97*	3.11±0.56	3.53±0.22
PFK/CS	2.81±0.41	2.58±0.28	1.83±0.12	1.56±0.10
HAD/CS	0.07±0.01	0.08±0.01	0.10±0.01	0.06±0.01*

Values are means \pm S.E.M., $N=6$ animals per group.

* $P<0.05$, ** $P<0.01$ compared with the corresponding euthermic group.

PFK, phosphofructokinase; LDH, lactate dehydrogenase; CS, citrate synthase; HAD, 3-hydroxyacyl-CoA dehydrogenase.

5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 412 nm ($E_{mM}=13.6$). The results are expressed as micromoles of product formed per minute per gram wet mass. The anaerobic capacity of muscle was evaluated by assaying phosphofructokinase (PFK) and lactate dehydrogenase (LDH) activities, the aerobic capacity by the activity of citrate synthase (CS) as a marker for the citric acid cycle and 3-hydroxyacyl-CoA dehydrogenase (HAD), which is a marker of fatty acid catabolism (β -oxidation).

Phosphofructokinase (EC 2.4.1.1)

PFK activity was determined in the presence of 1.5 mmol l^{-1} NADH, 7 mmol l^{-1} MgCl_2 , 2 mmol l^{-1} AMP, 2 units ml^{-1} α -glycerophosphate dehydrogenase, 0.5 units ml^{-1} aldolase and 75 mmol l^{-1} triethanolamine-HCl buffer, pH 8.2 at 25°C . The reaction was initiated by addition of ATP and D-fructose 6-phosphate to the medium, and PFK activity was determined at 340 nm.

Lactate dehydrogenase (EC 1.1.1.27)

LDH was assayed as pyruvate reductase in a medium consisting of 1.5 mmol l^{-1} NADH, 50 mmol l^{-1} imidazole-HCl buffer, pH 7.4 at 25°C . The reaction was initiated by addition of 3 mmol l^{-1} sodium pyruvate, and LDH activity was measured at 340 nm. The pyruvate concentration that gave the highest LDH activity was determined for each tissue.

Citrate synthase (EC 4.1.3.7)

The assay medium consisted of 2.5 mmol l^{-1} DTNB, 5.0 mmol l^{-1} oxaloacetic acid (OAA), 3.0 mmol l^{-1} S-acetyl CoA, 50 mmol l^{-1} triethanolamine-HCl buffer, pH 8.0 at 25°C . CS activity was determined at 412 nm after the addition of 0.3 mmol l^{-1} acetyl coenzyme A (sodium salt), following subtraction of background deacylase activity from activity in the presence of OAA.

3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)

HAD activity was determined in the presence of 1.5 mmol l^{-1} NADH, 1 mmol l^{-1} EDTA and 50 mmol l^{-1} imidazole buffer, pH 7.4 at 25°C ; the reaction was initiated with 2.0 mmol l^{-1} acetoacetyl coenzyme A (sodium salt), and followed at 340 nm.

Statistical evaluation

All results are given as means \pm S.E.M. Analysis of variance (ANOVA) was used to determine statistical significance (taken to be $P<0.05$), using Fisher's PLSD test for multiple comparisons.

Results

Muscle mass

All animals tolerated cold exposure well, with no individual excluded from analysis because of poor condition. Using weight-matched controls, there was clear evidence of muscle hypertrophy in Cold-A rats after 8 weeks (muscle mass increasing from 1.6 to 1.8% of body mass, $P<0.05$), and a modest (6%) atrophy in Cold-A hamsters (not significant, Table 1).

Enzyme activity

There was a clear inter-specific difference in metabolic characteristics, with the fast muscle in rats having a significantly higher glycolytic (PFK, LDH) and lower oxidative (CS, HAD) enzyme activity than that of hamsters. This relationship was unaffected by cold-acclimation (Table 1), which in both species led to a modest (10–20%, not significant) reduction in glycolytic capacity as indicated by PFK activity. LDH activity was significantly elevated in Cold-A rats, leading to a greater LDH/PFK ratio, which indicates an increased terminal anaerobic to glycolytic metabolic activity.

There was little change in the potential tricarboxylic acid cycle flux, as indicated by CS activity in both species, but a 40% reduction in HAD activity in hamster TA on cold-acclimation (not significant). Consequently, there was little difference in the balance between glycolytic and oxidative metabolic capacity (PFK/CS) in either species (Table 1).

Fibre type composition

The compositions of muscles in euthermic rats and hamsters were distinct (Table 2), but the relative distributions of fibre types among regions within the TA were quite similar for both species (Fig. 3A,B). The TA was composed mainly of Type IIb (fast glycolytic, FG) fibres, with a smaller proportion of Type IIa (fast oxidative glycolytic, FOG) and a few Type I (slow oxidative, SO) fibres. The numerical density (N_N) of fibre types showed modest alterations between euthermic and Cold-A groups in either species, although the modest inter-specific differences were reduced further on cold-acclimation, nor were there any significant differences in the areal density of fibre types (Table 2). The muscles showed no evidence of oedema or necrosis, with the interstitium occupying approximately 5% of muscle cross section in all groups. Fibre cross-sectional area (FCSA) was the parameter most affected by cold-acclimation, showing a general hypertrophy in rat muscles and atrophy in hamster muscles compared with euthermic groups. Mean FCSA increased significantly in the Cold-A rat from 1980 ± 112 to $2512 \pm 210 \mu\text{m}^2$ ($P < 0.05$, see Fig. 5A), while it declined in Cold-A hamsters from 2346 ± 88 to $2035 \pm 108 \mu\text{m}^2$ ($P < 0.05$, see Fig. 5B). Differences in mean FCSA were due primarily to the response of fast muscle fibres (mainly Type IIb) in both species (Fig. 3A,B).

There was a similar fibre type distribution in the core and cortex regions of TA muscle in both species, and cold-acclimation had no effect on this distribution in the rat (Fig. 3A) for either numerical or areal fibre type proportion. However, Type I fibre numerical density was significantly reduced in the core region of TA of Cold-A hamsters (Fig. 3B). In euthermic animals, there were regional differences in FCSA within the same muscle. Type IIb fibres were almost double the size of Type IIa and I fibres in both species, while Type IIb fibres in the TA cortex were larger than those in the core region of both rat and hamster ($P < 0.01$). The same was true for Type IIa fibres in rat (Fig. 3A), but not in hamster (Fig. 3B). These regional differences were maintained in Cold-A rats, while the differences between regions disappeared for Type II fibres in hamster TA as a result of cold-acclimation. Following cold-acclimation, there was a significantly increased FCSA of Type IIb fibres in the core and cortex regions of TA muscle in the Cold-A compared with the euthermic rat, while hypertrophy of Type IIa fibres occurred only in the cortex regions (Fig. 3A). In contrast, there was a general atrophy in hamster TA induced by cold-acclimation, seen as a reduction in mean FCSA that was significant in the cortex region for both Type IIa and IIb fibres (Fig. 3B). The influence of these changes on oxidative capacity is reflected in the succinic

Table 2. *Fibre type composition and fibre cross-sectional area in tibialis anterior of euthermic and cold-acclimated rats and hamsters*

	Rat	Hamster
Numerical density, N_N (%)		
Type-I		
Euthermic	5±0.8	10±1.0
Cold-acclimated	7±1.4	8±0.7
Type-IIa		
Euthermic	29±1.1	23±1.6
Cold-acclimated	28±1.0	24±1.3*
Type-IIb		
Euthermic	66±1.5	67±1.6
Cold-acclimated	65±2.1	68±1.9
FCSA (μm^2)		
Type-I		
Euthermic	1111±96	1562±120
Cold-acclimated	1322±173	1353±101
Type-IIa		
Euthermic	1242±60	1587±47
Cold-acclimated	1540±127*	1324±77**
Type-IIb		
Euthermic	2338±124	2691±105
Cold-acclimated	2970±214**	2372±138
Areal density, A_A (%)		
Type-I		
Euthermic	3±0.3	7±0.7
Cold-acclimated	4±0.7	5±0.8
Type-IIa		
Euthermic	17±0.6	17±0.8
Cold-acclimated	17±0.6	17±1.0
Type-IIb		
Euthermic	75±1.1	72±1.7
Cold-acclimated	73±1.3	75±1.6
Interstitium		
Euthermic	5±0.4	4±0.4
Cold-acclimated	6±0.4	3±0.3

Values are means ± S.E.M., $N=6$ animals per group.

* $P < 0.05$, ** $P < 0.01$ compared with the corresponding euthermic group.

FCSA, fibre cross-sectional area.

dehydrogenase staining, and on capillary supply by alkaline phosphatase staining, of muscle sections (Fig. 4).

Capillary supply

Consistent with the lower aerobic capacity of rat muscles compared with those of hamsters was a reduced capillary supply (Fig. 5A,B). The capillary-to-fibre ratio (C:F) showed a significant (28%) increase in Cold-A rats, although there was no change in CD since the cold-induced angiogenesis was balanced by fibre hypertrophy (Table 3), with a positive relationship between the extent of capillary growth and FCSA among regions (Fig. 5A). In contrast, the C:F was similar in

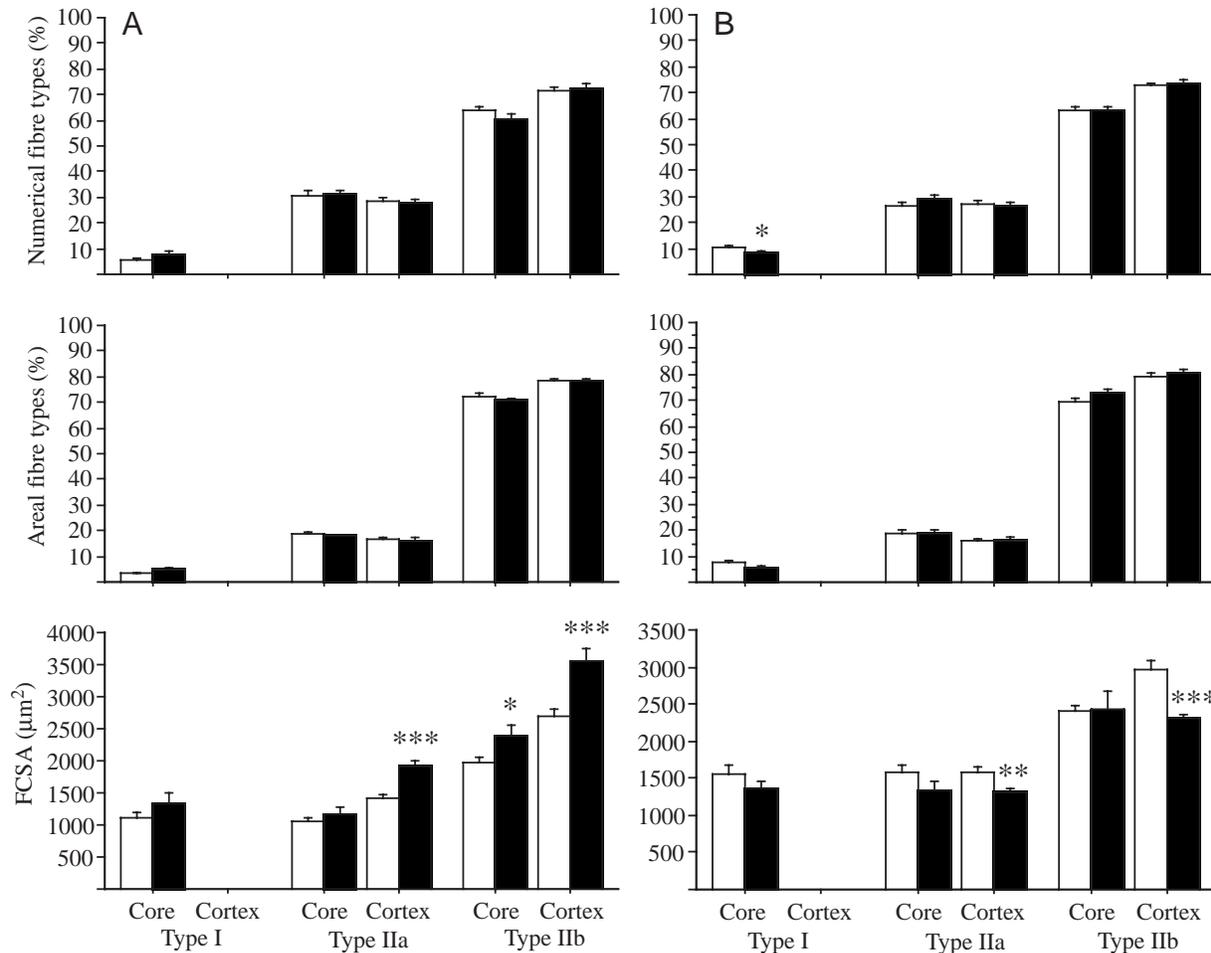


Fig. 3. (A) Rat tibialis anterior (TA) regional fibre type distribution and mean fibre cross-sectional area (FCSA) in euthermic (open columns) and cold-acclimated (filled columns) groups. (B) Regional fibre type distribution and FCSA in hamster TA of euthermic (open columns) and cold-acclimated (filled columns) groups. $N=6$ animals per group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with the corresponding euthermic group. Values are means + S.E.M.

both groups of hamster whereas the CD rose by 33% following cold-acclimation (Table 3).

There was a clear regional differentiation between TA cortex and core in response to cold-acclimation in the rat, with a 39% greater C:F in the cortex but only a 20% increase in the core ($P<0.001$, Fig. 5A). In the hamster, there were, as seen in the average for whole TA muscle, no significant alterations in C:F in either region. However, there was a significant increase in CD in both regions with reciprocal muscle fibre atrophy, with the greatest change again being found in the cortex ($P<0.01$, Fig. 5B).

The estimated intramuscular diffusion distances (D) were similar for euthermic and Cold-A rats: $D=19.8\pm 1.3\ \mu\text{m}$ compared with $19.3\pm 0.9\ \mu\text{m}$ (not significant), $R_{\text{max}}=25.5\pm 1.3\ \mu\text{m}$ compared with $25.6\pm 1.4\ \mu\text{m}$ (not significant), $R_{\text{ave}}=14.3\pm 0.9\ \mu\text{m}$ compared with $13.9\pm 0.8\ \mu\text{m}$ (not significant) for euthermic compared with Cold-A, respectively. In contrast, values were significantly smaller in Cold-A hamster TA: $D=16.8\pm 0.5\ \mu\text{m}$ compared with $14.7\pm 0.4\ \mu\text{m}$ ($P<0.01$), $R_{\text{max}}=22.9\pm 0.6\ \mu\text{m}$

($P<0.05$) and $R_{\text{ave}}=12.0\pm 0.3\ \mu\text{m}$ compared with $11.1\pm 0.3\ \mu\text{m}$ ($P<0.05$). Regional differences were also evident, with diffusion distance lower in the core than the cortex in both species, reflecting both the smaller FCSA and the higher CD.

Table 3. The effect of cold acclimation on capillary supply in tibialis anterior muscle

	Rat	Hamster
C:F		
Euthermic	1.34±0.08	2.13±0.07
Cold-A	1.72±0.08**	2.21±0.08
CD (mm ⁻²)		
Euthermic	715±75	919±52
Cold-A	735±70	1204±68*

C:F, capillary-to-fibre ratio; CD, capillary density.

Values are means ± S.E.M., $N=6$ animals per group.

* $P<0.01$, ** $P<0.001$ compared with the corresponding euthermic group.

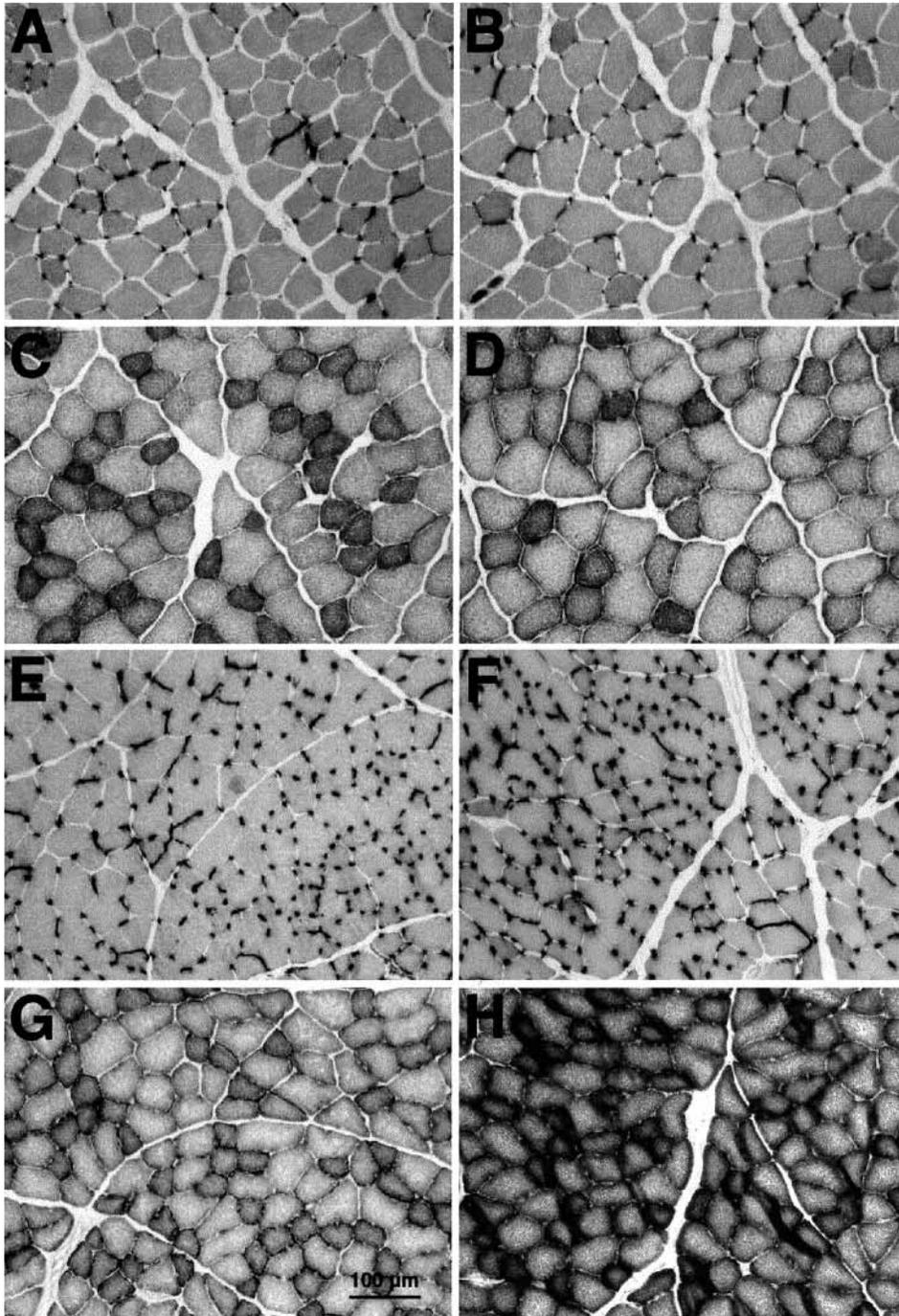


Fig. 4. Photomicrographs showing alkaline phosphatase (A,B,E,F) and succinate dehydrogenase (C,D,G,H) staining from tibialis anterior cortex of euthermic (left-hand column) and cold-acclimated (right-hand column) rat (A–D) and hamster (E–H). Scale bar, 100 μm .

of O_2 and fuels by increasing the carrying capacity of blood, capillary supply, mitochondrial quantity and/or regulation, and the enzyme activity of the tissues (Banchemo, 1982; Buser et al., 1982). Maintenance of core temperature in a cold environment is achieved by an increased oxidative metabolism, primarily in brown adipose tissue (BAT) and skeletal muscle (Jansky and Hart, 1968; Foster and Frydman, 1979). Until recently, heat production by non-shivering thermogenesis in both non-hibernator and hibernator mammals was thought to originate principally through activation of a mitochondrial uncoupling protein (UCP1) specific to BAT (Trayhurn and Nicholls, 1986). These small proteins reside in the inner membrane of the mitochondria, where they dissipate the proton gradient linked to oxidation of metabolic fuels, thus generating heat. However, skeletal muscle is an important contributor to standard metabolic rate because of the large percentage of body mass that it represents (Rolfe and Brand, 1996) and is becoming recognised as an important source of non-shivering thermogenesis following the discovery of muscle-specific UCP2 and UCP3 isoforms (Boss et al., 1997). Metabolic efficiency may be

While these differences among regions were more evident in the rat, cold-acclimation did not alter calculated diffusion distance. However, all measures were reduced in both regions of the TA in Cold-A hamsters (Fig. 6).

Discussion

Mammals subjected to prolonged cold exposure usually show an increased metabolic heat production through both shivering and non-shivering thermogenesis (Himms-Hagen, 1976). An increased metabolic rate can be supported by increased delivery

improved upon cold exposure as a result of altered enzyme activity, fibre type composition, fibre size or intramuscular diffusion distance.

Enzyme activity

There are few metabolic data available that compare hibernator with non-hibernator animals under similar experimental conditions. During hibernation of ground squirrels, LDH activity increased and CS activity decreased in plantaris muscle (Steffen et al., 1991). In contrast, we looked at changes occurring during the process of cold-acclimation

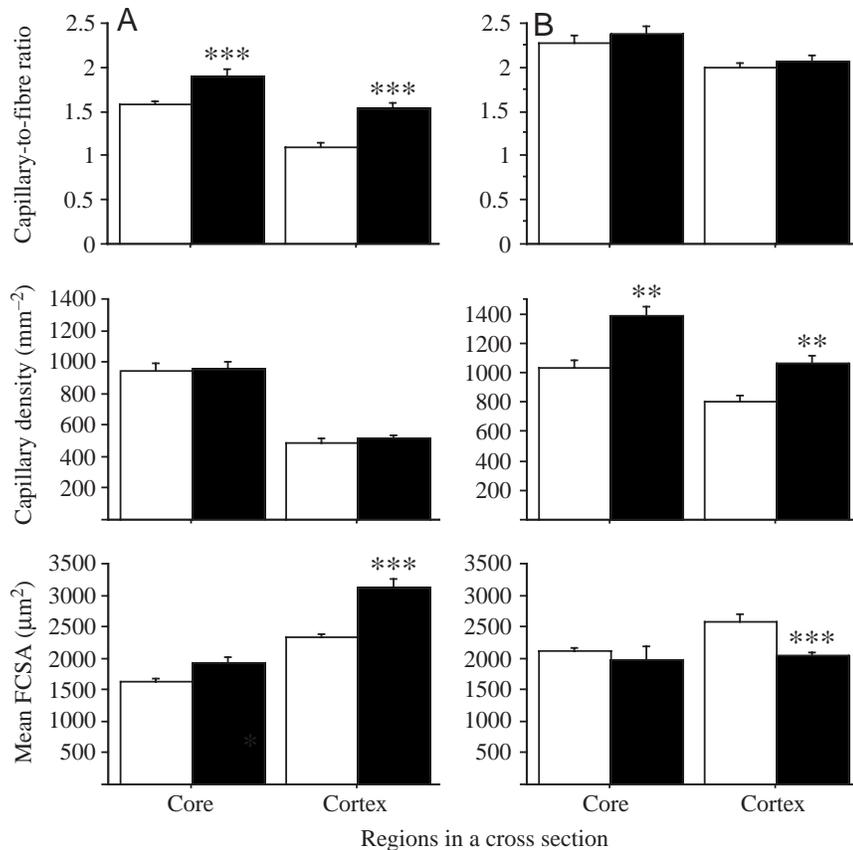


Fig. 5. (A) Rat tibialis anterior (TA) regional differences in capillary-to-fibre ratio (C:F), capillary density (CD) and mean fibre cross-sectional area (FCSA) in core and cortex regions of euthermic (open columns) and cold-acclimated (filled columns) groups. (B) Regional differences in C:F, CD and FCSA in core and cortex regions of euthermic and cold-acclimated hamster TA. $N=6$ animals per group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared with the corresponding euthermic group. Values are means + S.E.M.

that precedes hibernation and found no significant alteration in activities of the oxidative marker enzymes CS and HAD in either species. Similarly, although higher mitochondria volume density and higher cristae surface density have been reported in Cold-A rat muscles (Buser et al., 1982), the effective mitochondrial mass, as indicated by the content of mitochondrial protein and of cytochrome oxidase activity per gram of muscle, was not altered (Behrens and Himms-Hagen, 1977). In addition, an increase in the maximal rate of muscle oxygen consumption upon cold-acclimation and exercise in guinea pigs revealed no increase in mitochondrial volume, suggesting no change in oxidative capacity (Hoppeler et al., 1995).

To combat the cold, rats may stimulate anaerobic metabolism in skeletal muscle as a result of shivering thermogenesis. Blood lactate levels have been shown to increase in Cold-A guinea pigs (Turner et al., 1995), so an increase in LDH activity may allow increased gluconeogenesis. However, the somewhat lower activity of the flux-limiting enzyme PFK in Cold-A rats strongly suggests that shivering thermogenesis was not fuelled by glycolysis in the present study. Depression of PFK activity may be a result of the change in intracellular adenylate charge ([ATP]/[ADP] ratio) since the enzyme is inhibited by high levels of ATP and H^+ , which prevents excessive formation of lactate and a precipitous drop in blood pH. PFK inhibition also occurs by accumulation of citrate, an indication that biosynthetic precursors are abundant and additional glucose need not be

degraded for this purpose (Newsholme and Leech, 1983). The increased LDH activity in rat fast muscles may therefore result from hyperkinesia and greater fast glycolytic (Type IIb) fibre activity during cold-acclimation. In contrast, hamsters show little change in either LDH or PFK activities and are therefore unlikely to rely on shivering thermogenesis.

Enzyme ratio alterations typically paralleled changes in absolute enzyme activities in both species during cold-acclimation, suggesting a broad maintenance of metabolic pattern. The PKF/CS activity ratio, indicating relative glycolytic rather than oxidative capacity, suggests that the former was greater in rats than in hamsters and that this was not

significantly affected by cold-acclimation. Interestingly, there was a clear reduction in fast skeletal muscle HAD/CS activity ratio during cold-acclimation of hamsters, indicating a reduced reliance on lipid metabolism during cold-acclimation. In contrast, significantly larger numbers of fat droplets were observed in both diaphragm and soleus muscle in rat, indicating a shift to lipids as a source of energy in oxidative muscles of Cold-A animals (Buser et al., 1982). The lack of change in HAD activity in our study of fast skeletal muscle would argue against sparing of fatty acids as a substrate for non-shivering heat production *via* UCP in more glycolytic muscle. This is consistent with elevated UCP2 mRNA levels in BAT of both cold-exposed and hibernating ground squirrels (Boyer et al., 1998). Mobilisation of lipid depots to BAT, of which the hamster has a greater relative mass than the rat (D. Deveci and S. Egginton, unpublished data), rather than muscle would be a more efficient strategy for non-shivering thermogenesis in hamsters.

Fibre type composition

Histochemical staining of rodent skeletal muscle identifies large differences in the metabolic profiles of three major fibre types. Importantly, Type IIa fibres possess high activities of both glycolytic and oxidative enzymes and may be more oxidative than Type I fibres (Saltin and Gollnick, 1983). In the TA muscle, two different regions can be distinguished, a predominantly glycolytic cortex and a mainly oxidative core. In rat, 33% of fibres are Type IIa in the TA core compared

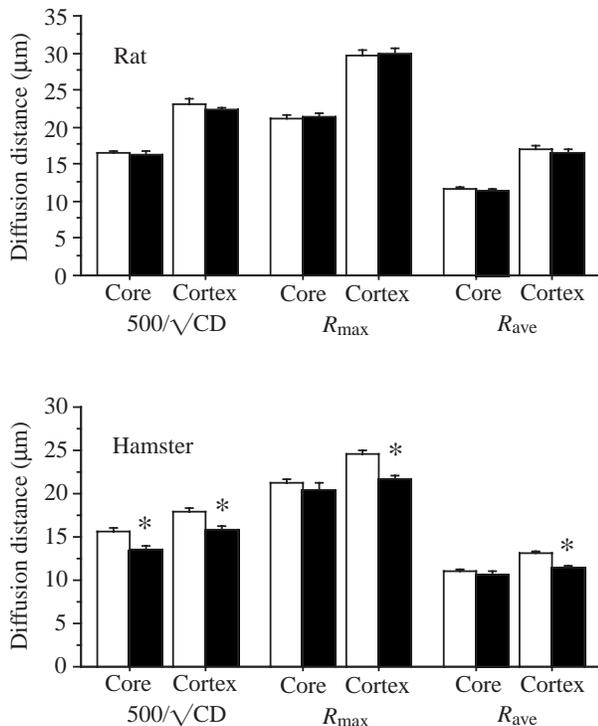


Fig. 6. Estimated regional diffusion distance in tibialis anterior (TA) muscle of euthermic and cold-acclimated rats and hamsters. $500/\sqrt{CD}$, diffusion distance (see Rakusan and Poupa, 1963); R_{max} , maximum diffusion distance; R_{ave} , average diffusion distance (see Snyder, 1987). $N=6$ animals per group. Values are means + S.E.M.

with 22% in the slow oxidative soleus, while in hamster the values are 26% and 30% respectively.

Increased muscle activity can induce fibre type transformation from fast glycolytic to fast oxidative phenotypes (Andersen and Henriksson, 1977). An additional stimulus for muscle fibre transformation in a tissue-specific manner is hormone levels, for example with slow muscle being more sensitive than fast muscle to thyroid status (Izumo et al., 1986). Thyroid hormone (T_3) activity and basal metabolic rate were increased in cold-exposed rats (Storm et al., 1981) and hamsters (Sigurdson and Himms-Hagen, 1988). Why, then, despite observing behavioural changes in muscle activity (i.e. shivering thermogenesis or hypokinesia), was there little evidence of fibre transformation in the TA? It may be that the sensitivity of fibre transformation varies with hormonal status. For example, stimulation and hyperthyroidism had antagonistic effects on myosin expression in rat fast-twitch muscle (Kirschbaum et al., 1990), while high resistance training decreased Type IIb myosin heavy chain (MHC) content in the medial gastrocnemius muscle, but again this transformation was blunted by T_3 administration (Caiozzo et al., 1995). Fibre composition expressed as relative number and area was qualitatively similar in the present study since cold-acclimation did not affect the size difference among fibre types. Moreover, cold-induced hypertrophy and atrophy did not alter these indices in the TA cortex of rat and hamster,

respectively, suggesting an adaptive change in muscle mass that is tightly regulated to preserve the original muscle composition or relative fibre size.

Fibre size

In spite of little or no fibre transformation, the predominantly glycolytic TA of conspicuously active rats showed a significant hypertrophy in response to lowered temperature and light levels. This may be the result of a combination of factors including cold-induced hyperthyroidism, increased metabolic and sympathetic activity, shivering and/or an age-dependent response. Muscle hypertrophy reflects an increase in protein content resulting from both greater synthesis and reduced degradation rates, while the inverse is true of atrophied muscle (Goldberg et al., 1975). However, in the present study, rat fast muscle showed hypertrophy under conditions where the animals were allowed to undertake a physiological adaptation to gradually reduced temperature, rather than the reported pathological atrophy in response to a sudden exposure to low temperatures (typically 5–6 °C in other studies) and exposure to an appropriate photoperiod (usually a constant 12 h:12 h L:D). This hypertrophy was primarily due to an increased FCSA of Type II fibres in the cortex and core regions and may be stimulated by the anabolic effect of cold-induced hyperthyroidism, although thyroid hormone has catabolic effects at higher doses (Tata et al., 1963), and preventing transformation from fast to slow fibre types. The lack of Type I fibre response suggests either that slow fibre malleability in response to cold-induced stimuli was less than that of fast fibres or that induction of hypertrophy depended on the original fibre size.

Whole muscle and/or muscle fibre atrophy has been reported in most studies of cold exposure in the rat (Heroux and St Pierre, 1957; Snyder et al., 1992; Suzuki et al., 1997), in guinea pig (Sillau et al., 1980; Aquin et al., 1980) and in pigeon (Mathieu-Costello et al., 1998). However, these studies used larger rats than we did (body mass 200–300 g, compared with our initial mass of 60 g), so there may be a compounding allometric response. Cold exposure in early life may be less disruptive of growth because the inherent potential for non-shivering thermogenesis provides some defence against low environmental temperature. In contrast, adult animals will adopt a resistance to the adverse effects of cold exposure rather than adaptations that permit continuance of normal anabolism, leading to retardation of growth. It may be that the progressive cold exposure in our experiment produced more continuous shivering than the acute cold exposure used previously, leading to hypertrophy of the TA in cold-acclimated rats. This may parallel the condition induced by short trains of tetanic contractions when the response of fibre metabolism was dissociated from that of capillary supply (Egginton and Hudlická, 2000). The muscle regions with larger fibres were the most superficial, so they were also likely to be subjected to a larger thermal gradient and to be stimulated most by shivering activity. Our experimental procedure increased the time the nocturnal rats were awake, but stimulated the onset of

hibernation in the hamster (previous observations have indicated that this occurs between 10 and 12 weeks with our protocol). The corresponding increase in activity in the rat could therefore also have contributed to muscle hypertrophy.

In contrast, the mean FCSA of hamster TA was significantly lower after cold-acclimation, mainly due to atrophy of Type II fibres. Hamsters were observed to have a reduced level of motor activity and thus showed modest atrophy, in agreement with other studies on hibernator animals (Wickler et al., 1991; Steffen et al., 1991). It is well known that hypokinesia, as seen during the hibernation period, causes muscular atrophy (Fitts et al., 1986). Disuse atrophy during cold-acclimation was evident, particularly during the last 4 weeks at low temperatures and short photoperiod, when the animals spent most of the time sleeping in their nest. Short photoperiod and cold together are more effective as a stimulus for hibernation than either alone (Hoffman, 1968). Shivering is an asynchronous stimulus, resulting in isometric exercise of both flexor and extensor muscles, and may have contributed to the hypertrophy of rat TA. The hamster, in contrast, has a very large non-shivering thermogenic capacity and the gradually reduced photoperiod and temperature may not cause shivering but prepare the animal for hibernation. One reason for hibernation is to avoid adverse environmental conditions, such as scarcity of food, and hence hypometabolism usually occurs in parallel with lower feeding rates (Hoffman, 1968). Thus, atrophy of hamster muscle may additionally be due to inadequate nutrition during the preparation for hibernation. Decreasing FCSA has been demonstrated in other hibernators, such as the ground squirrel (Musacchia et al., 1989), and this atrophy was accompanied by decreased protein synthesis during hibernation. However, in the present study, animals were presumably able to improve their oxygen-carrying capacity (e.g. haematocrit) (Deveci et al., 2001a) and substrate extraction (capillary supply) before atrophy became established, whereas in other studies acute cold exposure would have allowed inadequate time for compensatory changes to be made. The response to a sudden increase in metabolic activity, due to either physical activity (rat) or metabolic thermogenesis (hamster), therefore involved reducing oxygen diffusion distances (i.e. increasing CD or reducing FCSA).

Capillary supply

In the thermoneutral zone (ambient temperature $T_a=27-29^\circ\text{C}$), rat and hamster basal metabolic rates are similar, although in response to progressively lowering environmental temperature the metabolic rate of hamsters increased much more than that of rats at the same temperature (Himms-Hagen, 1986). Mortola (1991) reported that the resting rate of oxygen consumption of adult hamsters was 44% higher than that of rats, presumably supporting non-shivering thermogenesis. Our data also support the hypothesis that oxygen demand is matched with oxygen supply: hamsters had a higher blood oxygen-carrying capacity than rats (Deveci et al., 2001a), but also a higher oxidative enzyme activity and an overall higher capillarity in the TA.

Although aerobic marker enzymes and fibre type composition changed little during cold-acclimation, Cold-A animals may better exploit this capacity. Under physiological conditions, increased muscle capillarity may be induced by increased blood flow *via* its effects on shear stress and wall tension (Hudlická et al., 1992), although a metabolic stimulus for angiogenesis is compelling (Adair et al., 1990).

In a survey of previous studies on capillary growth (angiogenesis), Hudlická et al. (1992) concluded that, in addition to the widely reported influence of cytokines and growth factors, capillary growth may also be elicited by mechanical factors associated with increases in fibre girth. Fibre cross-sectional area (FCSA) data are useful for distinguishing between an apparent increase in capillarisation (CD), as a result of fibre atrophy, and true angiogenesis. Regional analysis showed that there was a good correlation between the site of angiogenesis and FCSA within the TA muscle of rats. In the cortex region, where mean FCSA was largest ($>2250\ \mu\text{m}^2$), the relative change in C:F was approximately double that of the core region (FCSA $1600\ \mu\text{m}^2$).

Heroux and St Pierre (1957) first reported apparent capillary growth in rats following cold exposure, although their results were ambiguous because of the method used to identify capillaries and the interpretation of results on the basis of CD (Egginton, 1998). Similar conclusions followed more detailed studies subsequently performed on guinea pigs by Sillau et al. (1980) and Banchemo et al. (1985). Likewise, angiogenesis has not been observed in the rat (Snyder et al., 1992; Suzuki et al., 1997) or pigeon (Mathieu-Costello et al., 1998) upon cold-acclimation to a T_a of 5°C . These researchers all reported an increase in CD, but this was due entirely to muscle fibre atrophy since the C:F did not change. In addition to the possible allometric component, these authors exposed the rats to a sudden drop in temperature, whereas we gradually lowered temperature and reduced the duration of the light phase of the photoperiod, simulating natural conditions (summer/winter transition). In both cases, a cold-induced increase in basal metabolic rate will increase muscle oxygen demand. In the present study, this was offset by increasing oxygen supply after stimulating angiogenesis, whereas in the other cited examples rats were subjected to a 'cold shock' rather than a natural adaptation and increased oxygen demand was offset by reducing oxygen diffusion distance as a result of muscle fibre atrophy.

In general, the TA showed a different response both within muscle (cortex and core) and between species. In Cold-A rats, the TA cortex and core C:F increased significantly by approximately 40% in the former and by half that in the latter, but CD showed no significant change. In contrast, Cold-A hamster CD increased significantly in both cortex and core (by approximately 33%), but the C:F did not change. In hamster, the increased CD was due to a significant decrease in FCSA rather than to capillary growth, whereas the increased C:F in rat was due to the new capillary growth which paralleled an increase in FCSA. It is reasonable to assume that these new

capillaries compensate at least partially for the increase in diffusion distance that occurs as the capillaries are pushed apart by fibre hypertrophy. The lack of angiogenic stimuli in hamster may result from two important adaptive responses, the absence of significant shivering in hamster muscles and the reduction in FCSA/increased CD which reduced oxygen diffusion distances sufficiently to prevent an adequate metabolic error signal developing.

The greatest change in capillarisation was seen in the TA cortex region of Cold-A rat, induced by cold-induced shivering, which also showed the greatest degree of fibre hypertrophy. The data from the TA cortex support our hypothesis that cold-induced shivering may be considered as a parallel to chronic low-intensity exercise. Muscle stimulation using a regime that activated glycolytic, but not oxidative, fibres (very low voltage and short trains of tetanic contractions) caused capillary growth in the glycolytic cortex of the TA muscle but not in the oxidative core, without any increase in the activity of cytochrome oxidase (Egginton and Hudlická, 2000). Moreover, the first appearance of an increased C:F has been shown to occur in the vicinity of glycolytic fibres of stimulated muscles (Hudlická et al., 1982). Collectively, these data suggest that larger fibres are more responsive to stimulation. It has been assumed that glycolytic fibres are subjected to hypoxia during activity and that this stimulates angiogenesis. Although direct measurements of oxygen tension in the glycolytic region of rabbit TA using oxygen electrodes did not demonstrate any changes that could explain capillary growth as a result of increased muscle activity (Hudlická and Schroeder, 1978; Kanabus et al., 1980), more recently we looked at regional differences within muscles and observed that chronic systemic hypoxia induced angiogenesis where muscle fibre size was largest (Deveci et al., 2001b). The angiogenic potential therefore scaled directly with oxygen diffusion and, hence, should be low in the TA core where the higher CD produces shorter diffusion distances and the fibres are unlikely to become hypoxic.

In hamster TA cortex and core regions, there were no significant changes in C:F, but CD responded to cold to the same extent (by approximately 33%) as fibre atrophy. Clearly, an alternative angiogenic signal to activity or FCSA is operative, presumably hormonal, in origin. For example, hyperthyroidism may stimulate new capillary growth, possibly *via* a mitochondrial pathway since a high-affinity mitochondrial receptor for T₃ has been shown to regulate the synthesis and activity of enzymes involved in oxidative phosphorylation (Roodyn et al., 1965; Sterling et al., 1978). It is a striking example of an integrated physiological adaptive response that elevating T₃ levels, leading to higher basal metabolic rate and some of the secondary morphological changes that appear in the cold such as improved capillary supply, may aid substrate delivery to, and removal of metabolic end-products from, active muscle.

Concluding remarks

Small rodents are limited in their ability to increase

insulation when faced with low environmental temperatures and thus rely on changes in their basal metabolic rate. This may ultimately take the form of either a hypermetabolic (rat) or hypometabolic (hamster) strategy to survive prolonged exposure to cold. The species-specific responses reported in the present study may be explained as follows. Shivering thermogenesis as part of a cold-avoidance strategy would tend to increase capillarisation in fast muscles of rats. In contrast, the ability of hamsters to tolerate a lower core temperature by increased BAT activity under the same conditions would not be expected to increase capillarisation in skeletal muscle. However, altered photoperiod and gradual cold-acclimation may also invoke hormonal changes that would counter the progressive lowering of activity and also increase capillarisation in rat, but in hamster the increased capillary density was due to cold-induced atrophy of muscle fibre cross-sectional area. In addition, within distinct regions of the TA, there was a correlation between angiogenesis and fibre size in the rat, in which oxygen diffusion increased, but not in the hamster, in which there was a reduced oxygen diffusion distance.

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