Fibre type specificity of haem oxygenase-1 induction in rat skeletal muscle

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Abstract The expression of the inducible haem oxygenase (HO-1) gene was examined in different skeletal muscles. Rats were treated with haemin and a time course of HO-1 mRNA expression was determined in soleus and extensor digitorum longus (EDL) muscles. Fibre type composition and tissue myoglobin content were also measured. We found that HO-1 mRNA expression markedly increased in soleus (type I fibres) muscle but was only slightly affected in EDL (type II fibres). HO-1 expression directly correlated with both percentage of red fibres and tissue myoglobin. These data demonstrate that HO-1 gene expression follows a fibre type-specific pattern which might indicate an important role for this protein in the maintenance of skeletal muscle function.

Key words: Heme oxygenase; Skeletal muscle; Fiber type; Nitric oxide; Carbon monoxide; Ischemia

1. Introduction

Haem oxygenase is a ubiquitous microsomal enzyme that catalyses the breakdown of haem to carbon monoxide (CO) and biliverdin, the latter being converted to bilirubin by the cytosolic biliverdin reductase [1]. The inducible isoform of haem oxygenase (HO-1 or HSP32) is susceptible to up-regulation in different tissues by a diversity of stress-related agents and conditions, including ultraviolet radiations [2], hypoxia [3], ischaemia-reperfusion [4], nitric oxide (NO) donors [5–9], peroxynitrite [7,10], as well as its own substrate haemin [1] and various haemoglobins [11,12]. Data from our own and other laboratories have demonstrated that both haem oxygenase-derived CO and bilirubin act as important effector molecules in the control of vessel contractility [13,14] and in protection against oxidant-mediated cytotoxicity [10,15], respectively. Recently, Essig and co-workers reported that HO-1 mRNA increases in rat skeletal muscle following exercise [16] and we have shown that cultured skeletal muscle cells have the ability to up-regulate the expression of HO-1 when appropriately stimulated by haemin and NO donors [8]. However, only limited information is available on the possible physiological role of HO-1 induction in skeletal muscle.

Three main types of skeletal muscle fibres have been identified and are generally referred to as type I (slow-twitch oxidative), IIA (fast-twitch oxidative) and IIB (fast-twitch glycolytic) fibres [17]. Traditionally, they can also be distinguished as red (type I and IIA) and white (type IIB) fibres. The numerous biochemical and ultrastructural differences between the various fibre types determine distinct metabolic and physiological functions in a given muscle. For instance, type I fibres have a low contraction speed and low glycolytic capacity, and are characterised by a higher content in mitochondria and myoglobin compared to type IIB fibres. In addition, type I fibres are well supplied with capillaries and are adapted for continuous work output with slow development of fatigue, whereas type IIB fibres rely mainly on stored glycogen and have low resistance to fatigue. Another peculiar feature of skeletal muscle tissue is the expression of important regulatory proteins which also appear to be confined to specific fibre types. In fact, recent studies have revealed that rat skeletal muscle contains neuronal NO synthase and that NO synthase protein and activity correlate strongly with type II fibre composition [18]. Furthermore, heat shock proteins (HSPs), which function as molecular chaperones and are highly induced in stress conditions, are expressed predominantly in type I fibres [19,20]. These findings prompted us to investigate whether treatment of rats with haemin results in skeletal muscle HO-1 (HSP32) induction and whether HO-1 gene expression follows a fibre type-specific pattern.

2. Materials and methods

2.1. Materials

Male Sprague-Dawley rats were bred within our institution and used in all studies. Haemin was obtained from Porphyrin Products Inc. (Logan, UT, USA). Stock solutions of haemin were prepared by dissolving the compound in 0.02 M NaOH and then adjusting the pH to 7.4 by addition of 0.01 M phosphate buffer. [α-^{32}P]dCTP was obtained from Amersham International (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise specified.

2.2. Animal treatment

Rats (400–500 g) were injected intraperitoneally either with haemin solution (10 mg/kg) or with vehicle (control group). Animals were returned to their cages and were allowed free access to drink and food. For HO-1 gene expression studies, animals were killed at various time points (2, 4, 6 and 24 h) following haemin or vehicle injection. Soleus and extensor digitorum longus (EDL) skeletal muscles were removed and immediately stored in liquid nitrogen until RNA extraction. A second group of haemin-treated animals was used to measure tissue myoglobin content, whereas the determination of fibre type composition was performed in skeletal muscles of untreated rats.

2.3. RNA extraction and Northern blot analysis

Freshly removed rat soleus and EDL muscle tissues were snap-frozen in liquid nitrogen and ground in a mortar and pestle. Total RNA was extracted by phenol-chloroform using the method described by Chomczynski and Sacchi [21]. Total RNA (10 μg/lane) was run on a 1.3% denaturing agarose gel containing 2.2 M formaldehyde and

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Abbreviations: HO-1, haem oxygenase-1; NO, nitric oxide; CO, carbon monoxide; EDL, extensor digitorum longus muscle; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSPs, heat shock proteins

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transferred onto a nylon membrane, according to the method of Tyrrell and Basu Modak [22]. The membrane was hybridised using [α-^{32}P]dCTP-labelled cDNA probes to the rat HO-1 gene (the 88–971 nucleotide residue fragment of the rat haem oxygenase gene) [23] and rat GAPDH gene. The hybridised membrane was exposed to radiographic film and bands quantified by densitometric analysis.

2.4. Fibre type determination

Soleus and EDL rat muscle specimens were mounted on small cork discs and snap-frozen in melting isopentane. Frozen sections (10 mm thick) were cut by means of a cryostat at −20°C and then allowed to dry at room temperature for 4 h before staining. Sections were mounted on slides and stained for ATPase activity at pH 4.5, 4.8 and 9.4, and NAD diaphorase activity using standard methods as previously described [24,25]. Slides were viewed using a conventional microscope (Olympus BX 40F, Olympus Optical Co., Tokyo, Japan) at a magnification of 40–200×. Five areas were selected at random from each section and all fibres within the field of view were counted. Three separate groups of fibres were clearly identifiable, corresponding to type I, type IIA and type IIB fibres.

2.5. Myoglobin measurements

Myoglobin content in soleus and EDL muscles was determined using a previously described technique [26]. Briefly, 200 mg of freshly extracted muscle was ground in a mortar under liquid nitrogen and suspended in 2 ml phosphate buffer (20 mM K_2HPO_4; pH 6.6). Samples were homogenised using a glass-Teflon homogeniser and then centrifuged at 28 000×*g* for 50 min. The supernatant was transferred to a capped tube and CO gas was slowly bubbled through it (25 ml/min) for 10 min. The absorbance at 538 and 568 nm of each sample was measured against a blank containing phosphate buffer. The concentration of myoglobin was calculated from the difference between the two readings and expressed as mg/g wet tissue.

2.6. Statistical analysis

Data were analysed by one-way analysis of variance followed by Bonferroni *t*-test. Differences were considered significant at $P < 0.05$.

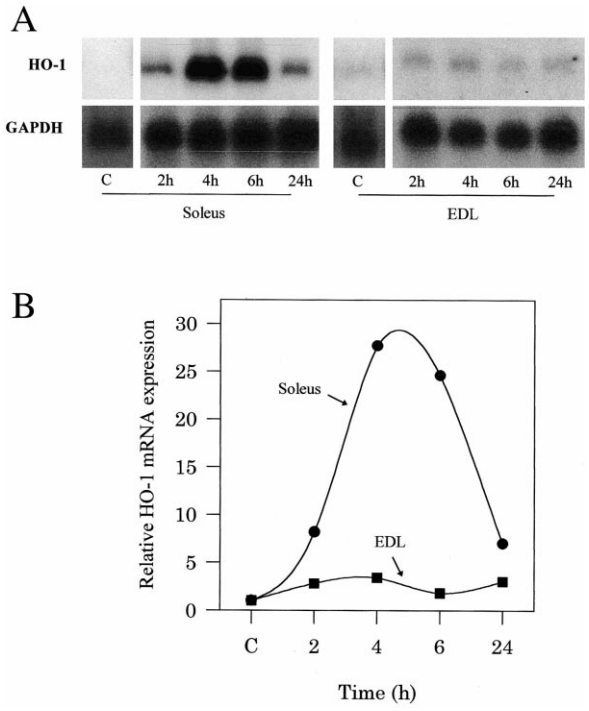

Fig. 1. A: Time course of HO-1 mRNA expression in rat muscles following haemin treatment. Rats were injected intraperitoneally with haemin (10 mg/kg) and total RNA was extracted from soleus and EDL muscles at the times indicated after injection. Control animals were injected with an equivalent volume of vehicle and harvested after 6 h (lane C). Northern blot analysis was performed for HO-1 and GAPDH genes as described in Section 2. B: Densitometric determination of relative HO-1 mRNA expression in soleus and EDL muscles following treatment of animals with haemin.

3. Results

HO-1 mRNA expression following haemin treatment was markedly different in soleus and EDL muscles (Fig. 1A). A significant increase in HO-1 gene expression was found in soleus, with maximal induction observed at 4 h after haemin treatment (Fig. 1B). At 24 h, HO-1 expression had decreased but was still greater than control. In contrast, haemin treatment produced only a slight response in EDL muscle and HO-1 mRNA levels were found to be elevated at 4 h after haemin treatment but the increase was much less pronounced than in soleus (Fig. 1B).

The fibre type composition of EDL and soleus muscles was determined by staining for pH-sensitive ATPase and NAD diaphorase activities and values from the two methods were averaged. As shown in Table 1, soleus was confirmed to be composed predominantly of type I fibre (91%) with much less type IIA fibres (7%), making it a primarily red fibre muscle (type I+type IIA = 98%). Type IIB (white) fibres in soleus muscle represented only 1.5%. In contrast, EDL muscle was composed mainly of type IIB (white) fibres (54%). Red fibres in EDL muscle represented 46.5% of the total and were primarily of type IIA (41%) with little type I (5.5%). Measurements of tissue myoglobin content revealed that the tissue composed primarily of red (type I and IIA) fibres, the soleus muscle, had the highest concentration (Table 1). From a mean of six samples, soleus contained 5.01 ± 0.78 mg/g wet tissue of myoglobin, whereas EDL contained only 2.14 ± 0.25 mg/g wet tissue. Interestingly, there was a direct correlation between HO-1 mRNA expression and both percentage of red fibres

Table 1
Fibre type composition and tissue myoglobin content of rat soleus and EDL muscles

Muscle	Staining method	Fibre type (%)			Myoglobin (mg/g wet tissue)	HO-1 mRNA (relative units)
		Type I	Type IIA	Type IIB		
Soleus	NAD diaphorase	89 ± 3.4	9 ± 1.8	2 ± 1.2	–	–
	ATPase	94 ± 3.7	5 ± 1.6	1 ± 1.1	–	–
	Average	91 ± 3.5	7 ± 1.7	1.5 ± 1.1	5.01 ± 0.78	27
EDL	NAD diaphorase	4 ± 1.7	37 ± 3.6	59 ± 2.6	–	–
	ATPase	7 ± 2.0	45 ± 2.4	48 ± 1.8	–	–
	Average	5.5 ± 1.9	41 ± 3.0	54 ± 2.2	2.14 ± 0.25	2

Fibre type composition was determined by NAD diaphorase and ATPase staining techniques (see Section 2). The relative increase in muscle HO-1 mRNA expression after treatment of animals with 10 mg/kg haemin (4 h) is also indicated. Values are expressed as mean ± S.E.M. of six independent experiments. Data shown for the ATPase staining method are the mean of values obtained at pH 9.4, 4.8 and 4.5.

and myoglobin concentration in skeletal muscles (Table 1). As shown, a more pronounced HO-1 mRNA expression was observed in skeletal muscles with a higher proportion of red fibres and a greater myoglobin content.

4. Discussion

In recent years, several studies have been investigating the biological role of haem oxygenase enzymes in different tissues. However, little is known about the regulation of these enzymes in skeletal muscle. Here, we report that a marked increase in the expression of the inducible isoform of haem oxygenase (HO-1) occurs in skeletal muscle in vivo after treatment of rats with haemin. This finding is not surprising since haem is a potent inducer of HO-1 in all tissues examined so far [1], and we have recently shown that in vitro exposure of skeletal muscle cells to haemin is associated with a time-dependent increase in HO-1 gene, protein expression and activity [8]. In addition, up-regulation of HO-1 mRNA has been detected in rat skeletal muscle tissue following exercise [16]. The novelty of the present study is, however, represented by the result showing that HO-1 induction in skeletal muscle follows a fibre type-specific pattern. We found that HO-1 is expressed predominantly in soleus muscle, which contains a high percentage of red fibres (type I and IIA), whereas HO-1 mRNA in EDL (type IIB) was only slightly affected. It is also interesting to note that HO-1 mRNA levels directly correlated with the amount of tissue myoglobin in the given muscles.

The fibre type specificity in the expression of certain genes could be an intrinsic characteristic of skeletal muscle tissue. Indeed, previous studies have shown that the stress protein HSP60 is expressed more in red (type I) than in white (type IIB) muscle [19]. Constitutive HSP72 protein levels are also high in rat soleus muscle and their content is proportional to the percentage of type I fibres in muscles of mixed fibre type [20]. In addition, the levels of inducible HSP72 continuously accumulate in the soleus following exercise, while they rise only transiently in EDL [27]. Notably, numerous studies have shown that soleus muscle and, in general, muscles rich in type I fibres are more resistant to oxidative injury mediated by ischaemia-reperfusion than either EDL or other muscles composed primarily of type IIB fibres [28–30]. This increased resistance may be partly explained by the differential expression of intracellular defensive systems, such as the above-mentioned HSPs, which act as molecular chaperones for the correct folding of native and damaged proteins. According to our data, HO-1 could also play a crucial role in the maintenance of soleus muscle functions for multiple reasons. Firstly, increased bilirubin production following HO-1 induction would provide an efficient and potent antioxidant system against free radical-mediated damage [10,31]. Indeed, experimental evidence from our laboratory supports a direct involvement of HO-1-derived bilirubin in the amelioration of post-ischaemic function in cardiac muscle (Clark et al., unpublished observation). Secondly, HO-1-derived CO appears to modulate vascular tone during stress conditions [13,14] and, in the case of soleus muscle (fibre I), it may have an important role in regulating microcirculatory functions [9]. In this context, it is tempting to speculate that CO and NO may act as signalling molecules following fibre type-specific patterns as skeletal muscle contains a neuronal-type NO synthase restricted to the sarcolemma of type II fibres [18]. Finally, our findings that HO-1 up-regulation is confined to red fibres and directly correlates with high myoglobin content might have an important biological significance. In fact, the haem moiety of myoglobin is a suitable substrate for haem oxygenase as it has been reported that myoglobin is degraded in vivo under conditions of increased haem oxygenase activity [32].

Thus, our study is the first to demonstrate that HO-1 induction follows a fibre type-specific pattern suggesting an important role for haem oxygenase in the catabolism of haem-dependent proteins and its contribution in the maintenance of skeletal muscle functions.

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