Xanthine oxidase in human skeletal muscle following eccentric exercise: a role in inflammation

Ylva Hellsten, Ulrik Frandsen, Niels Ørthenblad*, Bertil Sjødin† and Erik A. Richter

*Copenhagen Muscle Research Centre, August Krogh Institute, DK-2100 Copenhagen, Denmark, *Department of Physical Education, Odense University, Odense, Denmark and †Institute for Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden*

1. The present study tested the hypothesis that the level of xanthine oxidase is elevated in injured human skeletal muscle in association with inflammatory events. Seven male subjects performed five bouts of strenuous one-legged eccentric exercise. Muscle biopsies from both the exercised and the control leg, together with venous blood samples, were obtained prior to exercise and at 45 min, 24, 48 and 96 h after exercise. The time courses of xanthine oxidase immunoreactivity and indicators of muscle damage and inflammation were examined.

2. The number of xanthine oxidase structures observed by immunohistological methods in the exercised muscle was up to eightfold higher than control from day 1 to day 4 after exercise ($P < 0.05$). The increase was attributed to an enhanced expression of xanthine oxidase in microvascular endothelial cells and an invasion of leucocytes containing xanthine oxidase.

3. The concentration of plasma interleukin-6 was significantly higher 90 min after exercise than before exercise ($P < 0.05$) and remained higher than pre-exercise levels throughout the 4 days. On day 4 the plasma creatine kinase activity was approximately 150-fold higher ($P < 0.05$) than resting levels.

4. Despite the increase in xanthine oxidase in the muscle there were no detectable changes in the levels of muscle malondialdehyde or in plasma antioxidant capacity up to 4 days post-exercise.

5. It is concluded that eccentric exercise leads to an increased level of xanthine oxidase in human muscle and that the increase is associated with secondary inflammatory processes. The increase in xanthine oxidase in the muscle occurs mainly in microvascular endothelial cells, but occurs also via infiltrating leucocytes containing xanthine oxidase. A role for leucocytes in xanthine oxidase induction in endothelium is proposed.

It is well known that inflammatory events involve the generation of free radicals via NADPH oxidase and myeloperoxidase in immunoparticipating cells for the purpose of bacterial defence and phagocytosis. More recent evidence suggests that superoxide radicals ($O_2^{\cdot-}$) are also of importance in neutrophil attraction and neutrophil adherence to endothelium (Petrone, English, Wong & McCord, 1980; Suzuki, Inauen, Kvietys, Grisham & Meininger, 1989; Yoshida, Granger, Anderson, Rothlein, Lane & Kvietys, 1992). A role for superoxide radicals in neutrophil adherence has been indicated during anoxic conditions by inhibition of neutrophil adhesion to cultured endothelial cells by superoxide dismutase (Yoshida *et al.* 1992). One of the suggested sources of superoxide radicals during inflammatory events is the enzyme xanthine oxidase, which in skeletal muscle, as well as in most other tissues, is localized in the vascular walls (Jarasch, Grund, Bruder, Heid, Keenan & Franke, 1981; Hellsten-Westing, 1993). In healthy tissue xanthine oxidase mainly exists in a dehydrogenase form not capable of producing superoxide radicals, but the enzyme may be modulated to its superoxide-generating oxidase form via oxidation of critical sulfhydryl groups (Della Corte & Stirpe, 1972) or via limited proteolysis (Della Corte & Stirpe, 1968). The initiation of these modulating mechanisms has not been fully elucidated. In addition to the effect of superoxide radicals generated by xanthine oxidase on neutrophils it has been demonstrated that activated neutrophils, upon binding to cultured endothelial cells, induce an enhanced expression of xanthine oxidase (Wakabayashi, Fujita, Morita, Kawaguchi & Murota, 1995), suggesting a two-directional interaction between xanthine oxidase and neutrophils.

It has been proposed that xanthine oxidase contributes significantly to cellular damage during a number of conditions such as reperfusion injury (Grisham, Hernandez & Granger, 1986; Terada, Dormish, Shanley, Leff, Anderson

& Repine, 1992), thermal injury (Friedl, Till, Trentz & Ward, 1989), rheumatic (Miesel & Zuber, 1993), renal (Pfeffer, Hueckstead & Hoidal, 1994) and lung disease (Till, Friedl & Ward, 1991), due to the propensity of xanthine oxidase to generate reactive oxygen species and also due to its interaction with neutrophils. Elevated activity levels of xanthine oxidase in whole-tissue homogenates following reperfusion injury and in various disease conditions have repeatedly been shown and attributed to a protease-induced conversion of xanthine dehydrogenase to xanthine oxidase (Roy & McCord, 1981; Lindsay, Liauw, Romaschin & Walker, 1990; Smith, Carden & Korthuis, 1991). In these studies the actual sites for the increases in xanthine oxidase have not been assessed.

Little information exists regarding xanthine oxidase in human tissues undergoing inflammation. In a recent study we observed an increased level of xanthine oxidase in the muscle of subjects that had performed several days of strenuous exercise (Hellsten, Hansson, Johnson, Frandsen & Sjödin, 1996). Due to the nature of the study it was, however, not possible to determine a time course of xanthine oxidase induction or whether the increase occurred in association with inflammation. One approach to elucidate whether xanthine oxidase may increase in human muscle in association with inflammatory processes is to use eccentric exercise as an experimental model. Skeletal muscle subjected to exercise with an eccentric component is well known to suffer ultrastructural damage, probably in part as a consequence of mechanical stress (Fridén, Sjöström & Ekblom, 1981). Subsequent to this initial injury there appears to be a secondary phase of damage, which has been attributed to inflammatory processes. The secondary phase, which generally occurs 24–96 h after the exercise, is associated with a large release of muscle enzymes, oedema, increased muscle soreness, increased levels of cytokines (Evans *et al.* 1986) and infiltration of leucocytes (Jones, Newham, Round & Tollfree, 1986).

In the present study the induction of xanthine oxidase in muscle undergoing inflammation was examined in subjects after eccentric one-legged knee extensor exercise. The time course and distribution of induction of xanthine oxidase in the muscle was assessed in conjunction with indicators of muscle damage and inflammatory processes.

METHODS

Subjects

Seven sedentary young men aged 20–28 years participated in the study. Their mean body mass was 81 kg (range, 65–98 kg) and height, 184 cm (range, 175–193 cm). They were all healthy and without a history of any medical conditions, including clotting disorders, and they had not been involved in any regular training programmes within the last 3 years. All subjects were informed of the risks and discomforts associated with the experiment and that they were free to withdraw from the project at any time, before giving their informed consent to participate. Subjects were provided with an honorarium according to an hourly rate for student employees. The study conformed to the code of ethics of the World Medical Association (Declaration of Helsinki) and was approved by the local ethics committee. Students were covered by state medical insurance and, in addition, by the same insurance as that covering hospitalized patients, in case of complications.

Experimental design

The subjects were instructed to maintain their normal diet, to avoid alcohol, smoking and drugs during the 2 days prior to the experiment and to fast for 12 h prior to the exercise. The subjects reported to the laboratory in the morning and were placed in a room especially intended for invasive procedures. After 30 min rest a Teflon catheter was placed in a forearm vein. Basal blood samples were obtained from the catheter, and two muscle biopsies were obtained from the vastus lateralis muscle of the control thigh using a Bergstrøm needle with suction. The skin overlying the sampling site was anaesthetized and incisions were made through the subcutaneous tissue and fascia. All biopsy procedures were performed by a medical doctor with 10 years of experience with this procedure. For the one-legged exercise, subjects were placed in a chair with a rod connecting the foot with the pedal arm of a bicycle ergometer. The bicycle ergometer was placed behind the subjects and driven by an engine at a constant velocity of 30 r.p.m., thereby moving the lower leg forwards and backwards. The subjects performed maximal eccentric exercise with the quadriceps muscle by resisting a backward motion of the rod. When the lower leg was moved forward the subjects were instructed to relax all muscle groups. The movement of the lower leg was performed from an angle around the knee of 160 to 90 deg. The hip joint was fixed during the exercise, but in order to maximally stress the quadriceps muscle the hip joint angle was altered every session to be at either 90 or 130 deg, alternately, by changing the angle of the back rest. The eccentric exercise was performed in five sessions each lasting 5 min with 4 min rest between sessions. After an initial warm-up session of submaximal eccentric contractions the subjects were instructed to perform as hard as they could during the second, third, fourth and fifth work sessions. Work rate, oxygen consumption and heart rate were monitored during the second and fifth exercise period. At the end of the fifth exercise session, at 15, 45 and 90 min and at 24, 48 and 96 h, post-exercise blood samples were drawn from the forearm vein. Biopsies were obtained from the vastus lateralis muscle in the control leg prior to, 45 min and 48 h post-exercise, and in the exercised leg at 45 min, 24, 48 and 96 h post-exercise.

Sample preparation and storage

Biopsy samples were divided into two parts, one of which was mounted in an embedding medium and frozen in isopentane cooled in liquid nitrogen for subsequent immunohistochemical analysis. The other part was immediately frozen in liquid nitrogen for malondialdehyde determination. The biopsy material was stored at −80 °C until analysed.

Blood samples for interleukin-1β (IL-1β), IL-6, creatine kinase, xanthine oxidase, hypoxanthine and antioxidant capacity analysis were placed on ice in plastic tubes containing heparin. All samples were centrifuged at 4000 r.p.m. for 5 min, within 10 min of sample collection. The plasma samples were stored at −80 °C until analysed.

Oxygen uptake and heart rate

Oxygen uptake and heart rate were determined with a cardiopulmonary exercise-testing system (CPX; Medical Graphics Corporation, St Paul, MN, USA) and with a Polar Vantage XL heart rate monitor, respectively.

Maximal voluntary isometric contraction force

The maximum voluntary isometric contraction force (MVC) of the quadriceps muscle was measured with the knee joint at 90 deg. Bilateral MVC recordings were obtained before exercise and at 90 min, 24, 48 and 96 h post-exercise.

Muscle soreness

Muscle soreness was rated subjectively by palpation of the proximal, middle and distal areas of the vastus lateralis muscle, using a rating scale that ranged from 0 (no soreness) to 4 arbitrary units (a.u.) (extreme soreness). Soreness was evaluated pre-exercise and at 45 min, 24, 48 and 96 h post-exercise. Palpation and recording was always performed by the same person.

Immunohistochemistry

The distribution of xanthine oxidase-immunoreactive cells was determined immunohistochemically with the use of a monoclonal antibody X1-7, specific to xanthine oxidase (Hellsten-Westing, 1993). The distribution of leucocytes was assessed with a monoclonal antibody specific to the leucocyte C3bi receptor (CD11b; Dako, Glostrup, Denmark; code no. M741), and endothelium was identified with the endothelial cell-specific monoclonal antibody 7E9 (Koho *et al.* 1984). Frozen muscle biopsies cut at 8 μm were fixed in −20 °C acetone for 30 s followed by 2% formaldehyde for 2 min. The sections were then carefully rinsed in 0·01 M Tris-buffered saline (TBS) and incubated for 30 min with TBS containing 1% bovine serum albumin. After rinsing in 0·01 M TBS the sections were incubated with the primary X1-7 (2·4 μg ml^{-1}) or the C3bi antibody for 60 min. After rinsing off the excess antibody with 0·01 M TBS, biotinylated goat-anti-mouse immunoglobulin (Dako, code no. E433) diluted 1 : 400 in 0·01 M TBS was applied for 45 min. After rinsing off the excess secondary antibody with 0·01 M TBS, avidin biotinylated alkaline phophatase (ABComplex/AP; Dako, code no. K376) was applied for 30 min. Levamisol was added to block the activity of endogenous alkaline phosphatase. The staining was visualized by the addition of New Fuchsin chromogen (Dako, code no. K 698) for 5 min. Immunostaining was also performed with fluorescein isothiocyanate (FITC) conjugated antibodies and Texas Red. The sections were mounted in glycerine. Specificity of the staining for the antibodies used was assessed by staining without primary antibody and staining without secondary antibody. The number of xanthine oxidase-immunoreactive cells were counted on biopsy sections with a minimum of 200 muscle cells. Connective tissue areas with large numbers of leucocytes were not included in the quantification as such areas were not present or similar in size in all biopsy samples. Immunoreactive cells were examined and photographed under bright-field illumination in a photomicroscope (Nikon Microphot-FXA, Nikon Corporations, Tokyo). Nomarski optics were used to facilitate identification of the structures.

Muscle lipid peroxidation

Muscle lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) from muscle lipid peroxides in the thiobarbituric acid (MDA–TBA) assay (Draper & Hadley, 1990), fractionated on HPLC according to the method described by Wong (1987). This assay of muscle lipid peroxides involves hydrolysis in dilute H_3PO_4 at 100 °C, complexation of MDA with TBA, fractionation on HPLC and spectrophotometric quantification of the MDA–TBA adduct at 532 nm. The method was modified as follows. About 30 mg of muscle tissue was homogenized with twenty strokes on a Potter–Elverheim homogenizer in 550 μl 5% water trichloroacetic acid (TCA) and 2·4 mM methanol butylated hydroxytoluene (BHT). The homogenate was then heated for 30 min before centrifugation at 1000 *g* for 10 min to obtain a clear aliquot. A 200 μl volume of the aliquot was mixed with a 200 μl saturated solution of thiobarbituric acid (TBA) reagent and 600 μl 1% H_3PO_4 and heated for a further 30 min, neutralized with methanol NaOH, centrifuged at 10000 *g* for 3 min and injected onto the HPLC column to separate the MDA–TBA adduct from interfering chromogens. Separation was performed with a Merck Lichrosphere 100 RP-18/5 column (250 mm × 4 mm). Separation was achieved with a flow of 0·5 ml min^{-1}. Detection was at 532 nm. The composition of standard solution A (12, 15 mM) was 30 μl TEP (1,1,3,3-tetraethoxypropane) in 9·70 ml water. The composition of standard solution B (50 μM) was 40 μl solution A in 9·60 ml water. With an injection of 50 μl of a 1·0 nM standard solution, 0·05 pmol MDA was analysed per injection. The detection limit was 0·05 μM.

Plasma cytokines

Plasma levels of IL-1β and IL-6 were assessed in duplicate samples with high sensitivity ELISA kits, Quantikine IL-1b and Quantikine HS IL-6, respectively (R&D Systems Europe Ltd, Oxon, UK).

Plasma xanthine oxidase activity

Plasma xanthine oxidase activity was determined with chemiluminescence as described by Corbisier, Houbion & Remacle (1987).

Plasma creatine kinase activity

Plasma creatine kinase activity was assessed at 30 °C in duplicate samples using a kit (Boehringer Mannheim GmbH, Germany).

Plasma hypoxanthine and uric acid concentration

Plasma hypoxanthine and uric acid concentrations were determined by reverse-phase HPLC according to the methods described by Tullson, Whitlock & Terjung (1990). Separation was achieved by a 30 min gradient elution using a Hibar Lichrosphere 100 CH-18/2 (Merck) column (250 mm × 4 mm). The linear gradient programme for buffers A and B of the mobile phase was as follows: 0 min, 100% A; 0·1–4 min, 75% A; 4·1–17 min, 0% A; 17·1–22 min, 0–100% A; 22·1–30 min, 100% A, where the composition of buffer A was 150 mM ammonium phosphate (pH 5·80). Buffer B contained 150 mM ammonium phosphate with 20% methanol and 2% acetonitrile added as organic modifiers (pH 5·45). Separation was achieved at room temperature (20–23 °C) with a flow of 0·8 ml min^{-1}. Detection was at 254 nm, with peak identification by comparison of retention times with commercially obtained compounds.

Plasma antioxidant capacity

The antioxidant capacity was assessed using the enhanced chemiluminescent method described by Whitehead, Thorpe & Maxwell (1992). Briefly, a chemiluminescent reaction was created by mixing the chemiluminescent substrate luminol with an oxidant (perborate) in the presence of an enhancer (paraiodophenol) and the reaction catalysed by the enzyme horseradish peroxidase. This mixture provides a stable light emission for several minutes that is susceptible to quenching by antioxidant solutions for a time period that is proportional to their concentration. The total antioxidant capacity (TAOC) of plasma can then be derived by relating the period of quenching it produces to a known concentration of uric acid, as uric acid is responsible for the majority of the plasma antioxidant capacity.

Statistics

All values are reported as means and the standard error of the mean (± S.E.M.).

The number of xanthine oxidase and leucocyte C3bi receptor-immunoreactive cells found per muscle fibre, muscle malondialdehyde concentration and the MVC recordings were analysed using a two-way analysis of variance with repeated measures. The

plasma concentrations of IL-1β, IL-6, creatine kinase, uric acid, hypoxanthine and the plasma antioxidant capacity were analysed using a one-way analysis of variance with repeated measures. When significant exercise effects were found, differences were computed to compare pre- *vs.* post-exercise values, using Fisher's least significant difference test. Differences are reported at $P < 0{\cdot}05$.

RESULTS

Mean contraction force, oxygen uptake and heart rate

The mean force generated during the eccentric exercise decreased significantly from 523 ± 33 N during the second exercise bout to 295 ± 33 N during the fifth exercise bout, corresponding to 88 ± 8 and 52 ± 7% of the isometric MVC, respectively. Oxygen uptake during the experiment increased from 1·04 ± 0·07 l min^{-1} during the second exercise to 1·35 ± 0·1 l min^{-1} during the fifth exercise bout ($P < 0{\cdot}05$). Mean heart rate during the experiment increased from 118 ± 4 beats min^{-1} during the second exercise bout to 140 ± 4 beats min^{-1} during the fifth exercise bout ($P < 0{\cdot}05$).

Maximal voluntary isometric contraction force

Isometric MVC of the exercising leg decreased from a pre-exercise level of 651 ± 61 to 302 ± 30 N at 90 min after the exercise and remained lower than pre-exercise and control levels throughout the 4 days (Fig. 1). Isometric MVC of the non-exercising leg was similar before and after the exercise.

Xanthine oxidase and leucocyte immunoreactivity

The number of xanthine oxidase-immunoreactive cells per muscle fibre increased ($P < 0{\cdot}05$) from a pre-exercise value of 0·07 ± 0·03 to 0·37 ± 0·07 at 24 h post-exercise in the exercised leg and the level was still elevated (0·57 ± 0·07; $P < 0{\cdot}05$) at 96 h post-exercise (Figs 2*B* and 3). There was no significant difference in the number of xanthine oxidase-immunoreactive cells per muscle fibre in biopsies obtained from the control leg prior to the exercise bout compared with biopsies from the control leg at 45 min and 48 h post-exercise (Fig. 3).

The number of C3bi receptor-immunoreactive cells per muscle fibre increased from a pre-exercise value of 0·02 ± 0·01 to 0·20 ± 0·03 at 24 h post-exercise ($P < 0{\cdot}05$) and remained elevated throughout the 96 h (Fig. 4).

In the exercised leg the xanthine oxidase immunoreactivity post-exercise was mainly expressed in capillary endothelial cells but was also evident in cells other than endothelial cells, as verified by staining with an endothelial cell-specific antibody in serial sections (Fig. 2*A* and *B*). In several cases, as illustrated in Fig. 2*B* and *C*, xanthine oxidase immunoreactivity was also observed in the same location as C3bi receptor-immunoreactive leucocytes in serial sections, indicating the existence of xanthine oxidase either in the actual leucocyte or in the adjacent tissue. Expression of xanthine oxidase in the actual leucocytes was indicated in connective tissue areas in between the muscle cells, in which a large number of C3bi receptor-immunoreactive leucocytes had accumulated and where the xanthine oxidase antibody showed staining of similar structures (Fig. 2*D* and *E*). Areas of a biopsy section where xanthine oxidase was greatly expressed generally also contained a relatively large number of leucocytes, but the number of xanthine oxidase-immunoreactive structures was almost 2-fold greater than the number of C3bi receptor-immunoreactive structures. Smooth muscle cells of larger vessels displayed xanthine oxidase immunoreactivity in biopsy specimens obtained both pre- and post-exercise. It was not possible, however, to systematically assess changes in xanthine oxidase immunoreactivity in the smooth muscle cells as only some of the biopsy samples contained larger vessels. Muscle cells and connective tissue showed no xanthine oxidase immunoreactivity either before or any time after exercise. No immunoreactivity was observed in the absence of primary or secondary antibodies.

Plasma IL-1β and IL-6

The plasma level of IL-1β was 0·49 ± 0·13 pg ml^{-1} before exercise ($n = 5$) and remained unaltered throughout the 96 h measured. The level of IL-6 increased from a resting value

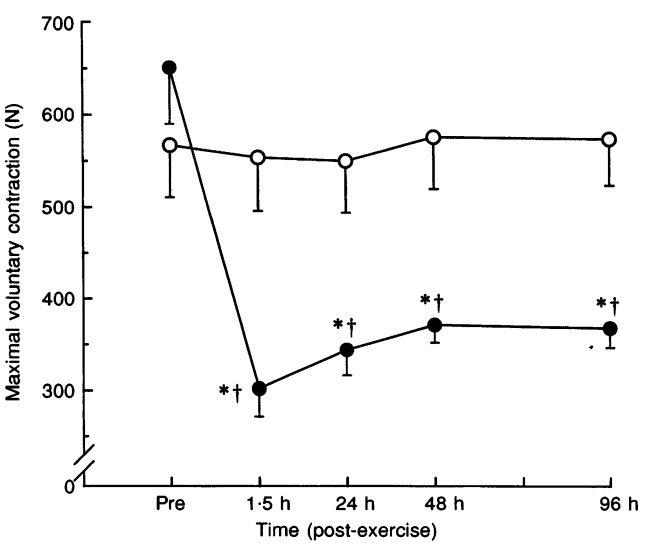

Figure 1. Maximum voluntary isometric contraction force

Maximal voluntary knee extensor contraction (MVC) with control leg (○) and experimental leg (●) before and after repeated bouts of one-legged eccentric exercise. Values are means ± S.E.M. ($n = 7$). * Significant difference from control leg. † Significant difference from pre-exercise value.

of $2{\cdot}40 \pm 0{\cdot}63$ pg ml^{-1} to a value of $4{\cdot}50 \pm 1{\cdot}28$ pg ml^{-1} 90 min after exercise ($n = 7$; $P < 0{\cdot}05$; Fig. 5).

Muscle malondialdehyde

Prior to exercise the mean malondialdehyde level in muscle was $9{\cdot}87 \pm 0{\cdot}90$ μmol kg^{-1} wet weight. The muscle malondialdehyde level did not change ($P > 0{\cdot}05$) after exercise and was $10{\cdot}99 \pm 1{\cdot}16$ at 45 min post-exercise, $10{\cdot}05 \pm 1{\cdot}83$ at 24 h post-exercise and $9{\cdot}61 \pm 0{\cdot}80$ μmol kg^{-1} wet weight 96 h post-exercise. There were no significant differences between the experimental and the control legs ($P > 0{\cdot}05$).

Creatine kinase

Plasma creatine kinase activity increased significantly from a pre-exercise value of 90 ± 25 U l^{-1} to a peak level of $13\,345 \pm 3110$ U l^{-1} 96 h post-exercise ($P < 0{\cdot}05$; Fig. 6).

Plasma hypoxanthine concentration

During the eccentric exercise the plasma concentration of hypoxanthine increased significantly from a pre-exercise value of $0{\cdot}8 \pm 0{\cdot}2$ to $6{\cdot}6 \pm 1{\cdot}5$ μmol l^{-1} at the end of the fifth exercise period ($P < 0{\cdot}05$). At 90 min post-exercise the plasma hypoxanthine concentration was not different from the pre-exercise value.

Plasma antioxidant capacity and uric acid concentration

The plasma antioxidant capacity was $38{\cdot}6 \pm 2{\cdot}3$ TAOC prior to exercise, $39{\cdot}0 \pm 2{\cdot}4$ at the end of the fifth exercise bout, $39{\cdot}7 \pm 2{\cdot}3$ at 45 min post-exercise and $38{\cdot}6 \pm 3{\cdot}5$ TAOC 48 h after exercise ($P > 0{\cdot}05$). Plasma uric acid levels were 341 ± 28 μmol l^{-1} prior to exercise, 295 ± 19 at the end of the fifth exercise bout, 307 ± 19 45 min post-exercise and

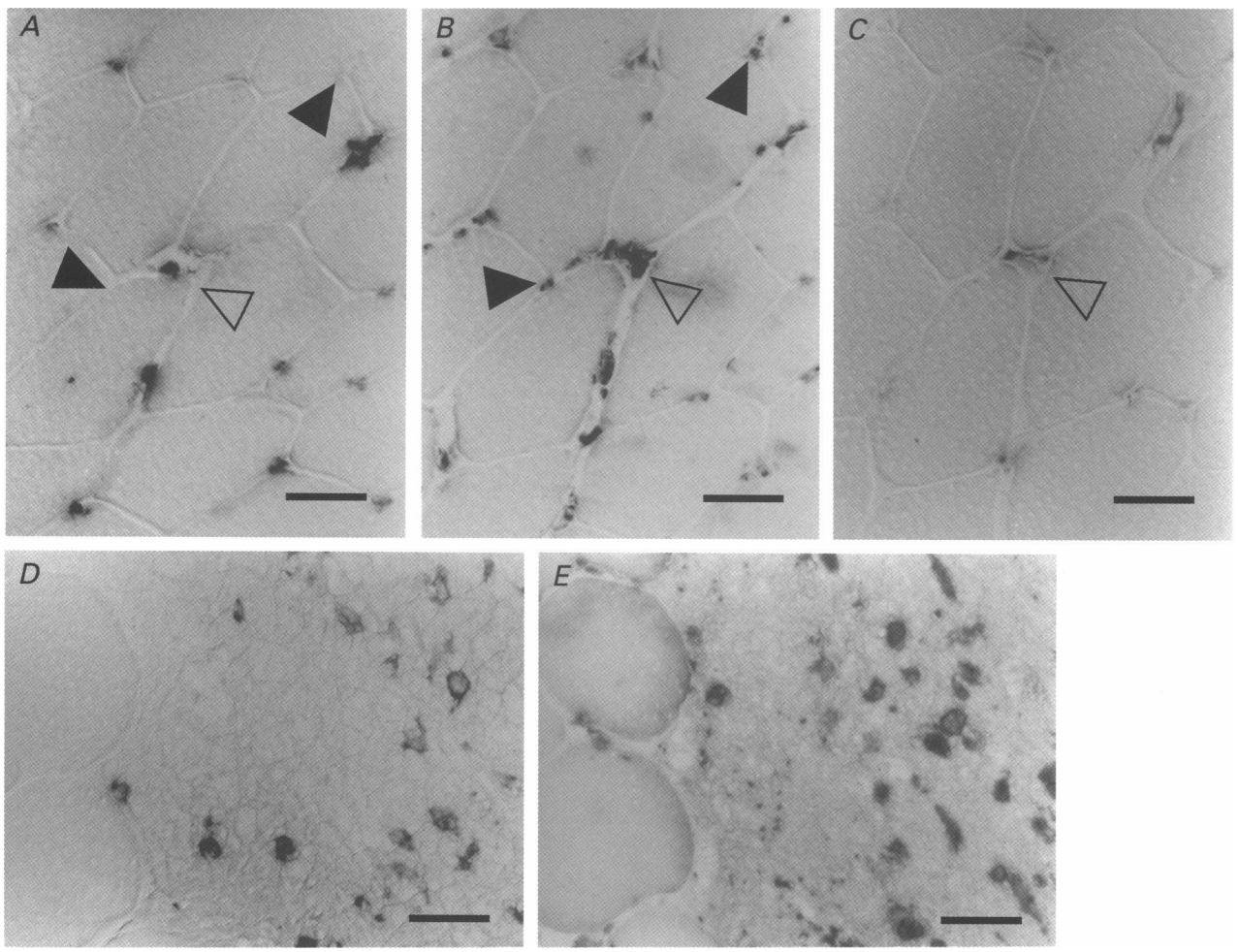

Figure 2. Micrographs of immunohistochemically stained human muscle sections

Serial sections of biopsy sample obtained from the experimental leg 48 h after eccentric exercise showing: *A*, microvascular endothelium (antibody 7E9); *B*, xanthine oxidase-immunoreactive structures (antibody X1-7); *C*, C3bi receptor-immunoreactive cells (antibody Dako M741). Open arrowheads in *A*, *B* and *C* indicate an area displaying immunoreactivity with all three antibodies. Filled arrowheads in *A* and *B* indicate cells that show xanthine oxidase immunoreactivity but not immunoreactivity with the endothelial cell-specific antibody. Micrographs *D* and *E* are serial sections of muscle biopsy sample obtained 48 h after exercise showing an area with connective tissue between muscle fibres with a high number of C3bi receptor-immunoreactive cells (*D*) and xanthine oxidase-immunoreactive cells (*E*). The scale bar is 40 μm.

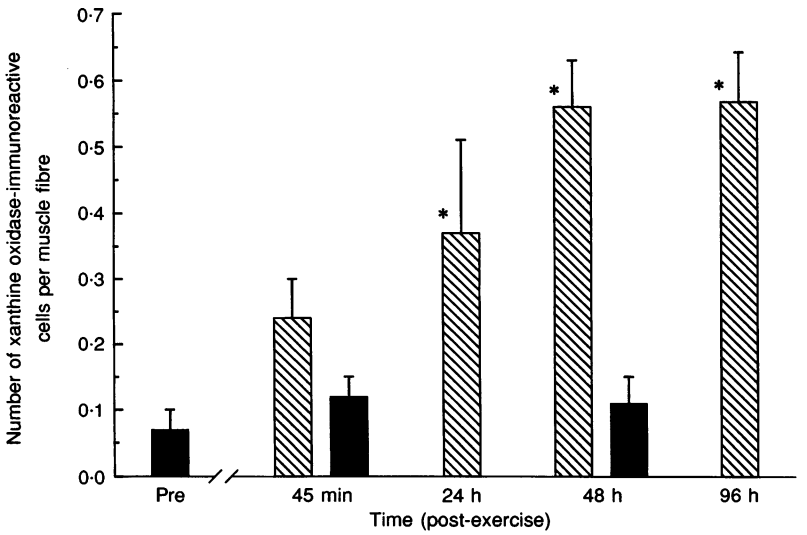

Figure 3. Xanthine oxidase immunoreactivity
Number of xanthine oxidase-immunoreactive cells per muscle fibre in muscle biopsy samples obtained from the control (■) and the experimental (▨) leg before and after one-legged eccentric knee-extensor exercise. Values are means ± S.E.M. ($n = 7$). * Significant difference from control value.

306 ± 26 μmol l^{-1} 48 h post-exercise ($P > 0{\cdot}05$). As uric acid is the major antioxidant in plasma, the lack of change in uric acid after exercise is in accordance with the lack of alteration in antioxidant capacity.

Muscle soreness

There was no soreness immediately after the eccentric exercise. Within the first 24 h following the eccentric exercise all subjects experienced soreness (1·3 ± 0·1 a.u.) in the quadriceps muscle of the eccentrically exercised leg. The soreness became more pronounced, reaching a maximum (1·6 ± 0·2 a.u.) at 48 h post-exercise. There was no soreness in the non-exercised control leg.

DISCUSSION

The present study demonstrates for the first time that human muscle undergoing inflammatory processes expresses an increased xanthine oxidase immunoreactivity. The elevated expression occurred mainly in the endothelial cells of microvessels but also in leucocytes present in the muscle. This restricted localization suggests that during exercise-induced inflammatory processes in the muscle, xanthine oxidase could cause injury primarily to microvessels and to tissues in the near vicinity.

The eccentric exercise regime led to muscle injury apparent as a sustained attenuation in maximal voluntary isometric force by the exercised leg. The injury was succeeded by an elevation in plasma IL-6 indicating the onset of inflammation within hours after exercise. From 24 to 96 h an augmentation in muscle damage and the inflammatory response was apparent with large increases in plasma creatine kinase activity, muscle soreness, invading leucocytes and a maintained elevation in plasma IL-6 levels. Based on the delay in the increase in muscle xanthine oxidase and the appearance of inflammatory markers it is suggested that the increase in xanthine oxidase in the microvascular endothelium and in leucocytes in muscle occurs in association with inflammatory events subsequent to exercise-induced muscle damage.

Animal studies investigating the effect of ischaemia and reperfusion on tissue damage and the level of xanthine oxidase have demonstrated an increase in tissue xanthine

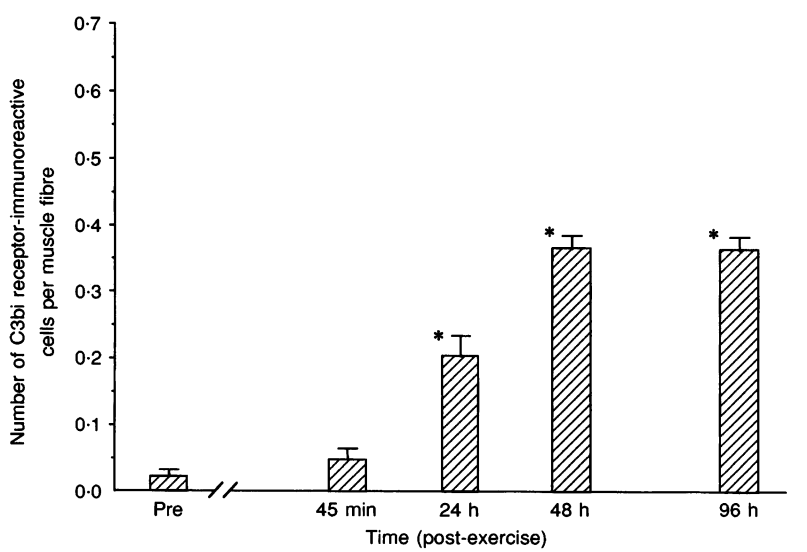

Figure 4. C3bi receptor immunoreactivity
Number of C3bi receptor-immunoreactive cells per muscle fibre in muscle biopsy samples obtained before and after one-legged eccentric knee-extensor exercise. Values are means ± S.E.M. ($n = 7$). * Significant difference from pre-exercise value.

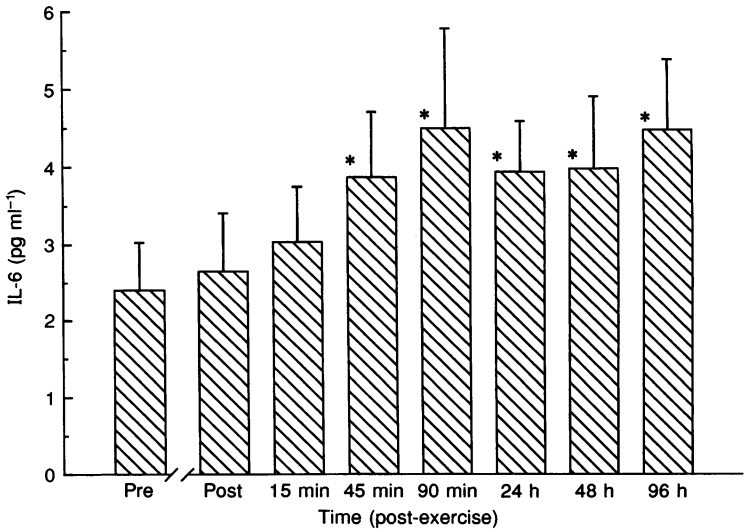

Figure 5. Plasma concentration of IL-6 before and after one-legged eccentric knee-extensor exercise

Values are means ± S.E.M. ($n = 7$). Post, immediately before the end of the last exercise session. * Significant difference from pre-exercise value.

oxidase activity in ischaemic tissue as determined from measurements in whole-tissue homogenates (Roy & McCord, 1983; Grisham *et al.* 1986; Lindsay *et al.* 1990). The present study illustrates that the location of an increase in xanthine oxidase in tissue undergoing inflammation is restricted to endothelial cells and that some leucocytes contain xanthine oxidase and thereby contribute to the increase in xanthine oxidase in muscle. Figure 2*D* and *E* demonstrates xanthine oxidase immunoreactivity in cells identified as leucocytes present in the connective tissue of a muscle biopsy obtained after eccentric exercise. As an additional verification of the fact that xanthine oxidase may be expressed in leucocytes, we have observed xanthine oxidase immunoreactivity in C3bi receptor-immunoreactive leucocytes adhering to the vascular walls of arterioles in human muscle subjected to ischaemia followed by reperfusion. There are several cell layers of leucocytes in such vessels and the localization is therefore more clearly distinguished from endothelium (U. Frandsen & Y. Hellsten, unpublished observation). The contribution to tissue xanthine oxidase activity levels by leucocytes has to a large extent been neglected; however, Grum, Gross, Mody & Sitrin (1990) demonstrated that isolated murine peritoneal leucocytes contain xanthine oxidase activity. These authors proposed that leucocytes may synthesize xanthine oxidase and may represent a source of tissue xanthine oxidase. It is possible that xanthine oxidase is expressed in leucocytes upon activation or infiltration into the muscle. IL-1, IL-6, tumour necrosis factor-α, interferon-α and histamine have all been documented as inducing an enhanced level of xanthine oxidase in a variety of tissues (Ghezzi, Bianchi, Mantovani, Spreafico & Salmona, 1984; Terao *et al.* 1992) and cultured cells (Falciani, Ghezzi, Terao, Cazzaniga & Garattini, 1992). As the eccentric exercise induced a cytokine response it is plausible that any of these immunomodulators could have induced an expression of xanthine oxidase in the leucocytes. The apparent close association between xanthine oxidase and leucocytes observed in the current investigation, as well as in previous animal studies (Phan, Gannon, Varani, Ryan & Ward, 1989; Terada *et al.* 1992; Wakabayashi *et al.* 1995) suggest that the appearance of xanthine oxidase in tissue undergoing inflammation may be partly related to leucocyte function.

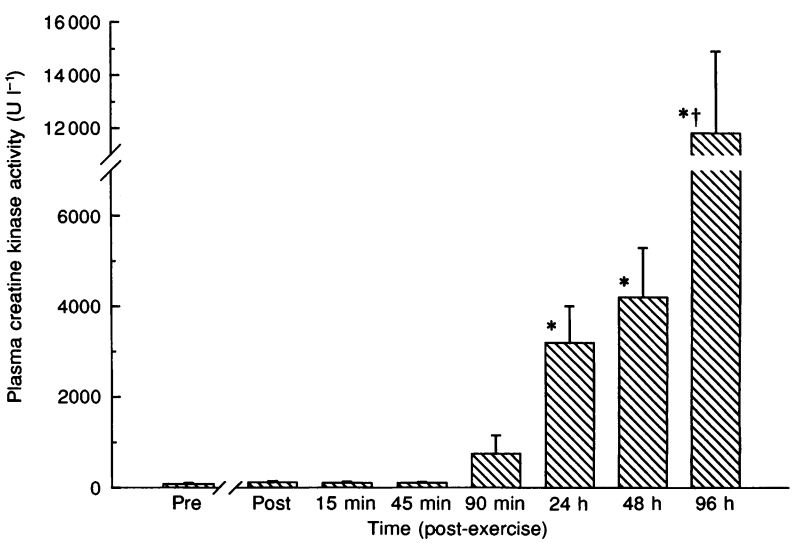

Figure 6. Plasma creatine kinase activity before and after one-legged eccentric knee-extensor exercise

Values are means ± S.E.M. ($n = 7$). Post, immediately before the end of the last exercise session. * Significant difference from pre-exercise value. † Significant difference from 24 h and 48 h value.

The mechanism behind the observed increase in xanthine oxidase in endothelial cells can be speculated upon. As leucocytes were present in the exercised muscle at the times when the level of endothelial xanthine oxidase was also elevated, a possibility could be that the enhanced endothelial xanthine oxidase level was a result of leucocyte adhesion. It has been observed that activated leucocytes induce an increase in the level of xanthine oxidase in cultured endothelial cells (Phan *et al.* 1989; Wakabayashi *et al.* 1995). The increase in xanthine oxidase, which occurs within minutes of leucocyte exposure, appears to be dependent on adhesion of the leucocytes to the endothelial cells, while blocking with antibodies against CD11a and CD18 receptors attenuates the increase in xanthine oxidase (Wakabayashi *et al.* 1995). Figures 3 and 4 illustrate that the time course for the increase in xanthine oxidase immunoreactivity was similar to that of the leucocytes.

An alternative mechanism for the increase in endothelial xanthine oxidase is an induction by IL-6. When added to cell cultures for 24 h IL-6 has been shown to induce an elevated expression and activity of xanthine oxidase in renal epithelial cells (Pfeffer *et al.* 1994). Cultured endothelial cells have similarly been shown to respond to cytokine exposure by an increased activity of xanthine oxidase (Friedl *et al.* 1989). The present increase in plasma IL-6 soon after exercise is assumed to originate from inflammatory cells in the muscle tissue. Therefore, an elevation in the local IL-6 level could have increased the expression of xanthine oxidase in the endothelium. According to Pfeffer and co-workers (Pfeffer *et al.* 1994) the cytokine-stimulated increase in cellular xanthine oxidase activity requires new protein synthesis, which could explain the observed time delay between the rise in plasma levels of IL-6 and endothelial xanthine oxidase content. The increase in IL-6 following strenuous eccentric exercise is probably initiated by mechanically induced cellular damage in the muscle due to the high forces developed, since concentric exercise does not appear to result in the same cytokine response as eccentric exercise at the same relative workload (Bruunsgaard, Halkjær-Kristensen, Johansen, Galbo & Pedersen, 1995). It is not known whether the endothelium in muscle may also be mechanically injured by eccentric exercise and whether damage to endothelial cells could initiate inflammatory processes as proposed by Jones & Round (1994). Considering the present findings of increased xanthine oxidase in the muscle microvasculature after eccentric exercise, endothelial cell injury would appear to be a plausible initiating event in eccentric exercise-induced inflammation.

A third possibility for an increase in tissue xanthine oxidase in damaged and inflamed tissue is the binding of circulating xanthine oxidase to endothelium (Tan, Yokoyama, Dickens, Cash, Freeman & Parks, 1993). Xanthine oxidase has been found in high amounts in the bloodstream following ischaemia–reperfusion (Yokoyama *et al.* 1990) and in patients suffering from disease conditions such as pulmonary dysfunction (Grum, Ragsdale, Ketai & Simon, 1987). As xanthine oxidase may attach to the membrane of endothelial cells via proteoglycans (Adachi, Fukushima, Usami & Hirano, 1993; Tan *et al.* 1993), circulating xanthine oxidase could theoretically bind to endothelial cells at the sites of the tissue injury. To examine this possibility we analysed xanthine oxidase activity in the plasma from our subjects. No detectable levels of xanthine oxidase were found in the blood samples obtained before or after the eccentric exercise suggesting that binding of circulating xanthine oxidase was not the mechanism behind the present increase in endothelial xanthine oxidase immunoreactivity.

Due to the propensity of xanthine oxidase to generate reactive oxygen species the enzyme has been considered to be a potential cause of muscle damage during exercise (Sjödin, Hellsten-Westing & Apple, 1990; Jackson, 1994). Although there is a theoretical possibility that xanthine oxidase may indeed generate harmful oxygen species during strenuous exercise, there is to date no direct evidence for such an event. The present data show that exercise may increase the level of xanthine oxidase in human muscle in association with inflammation, but indicate also that despite the severity of the exercise, the increase occurs only in specific locations, mainly at the site of microvessels. This suggests that during exercise-induced inflammatory processes in the muscle, xanthine oxidase could cause injury to microvessels and the tissue surrounding these. This possibility is consistent with findings of ultrastructural changes at the site of capillaries in human muscle following long-distance running (Crenshaw, Fridén, Hargens, Lang & Thornell, 1993). The restricted localization of xanthine oxidase in the muscle after exercise, and thus the limited tissue mass that could be subjected to a radical attack via xanthine oxidase, could explain the lack of increase in malondialdehyde levels, measured in whole-muscle homogenates, as well as the lack of change in plasma antioxidant capacity. Measurements of whole-muscle malondialdehyde and plasma antioxidant capacity may not be sufficiently sensitive or specific to detect limited radical formation (Gutteridge & Halliwell, 1990). It should again be stressed, however, that the sites in the muscle where the increase in xanthine oxidase occurred must be considered highly susceptible to free-radical damage. Also, muscle protein leakage after exercise, as indicated by plasma creatine kinase activity, was exceptionally high considering that exercise was confined to the quadriceps muscle of one leg.

There was only a modest rise in the plasma level of hypoxanthine, the substrate for the xanthine oxidase reaction, and the increase occurred acutely in response to the exercise, whereas after 24 h, when the xanthine oxidase concentration in the muscle was highest, hypoxanthine in plasma was back to pre-exercise levels. This suggests that

substrate availability could not have elevated the activity level of xanthine oxidase, although as plasma contains approximately 1 μmol l^{-1} at rest the xanthine oxidase reaction could proceed. We were unable to supplement our immunohistochemical data on xanthine oxidase with measurements of xanthine oxidase activity, as the levels were so low that the changes could not be assessed reliably, even with two highly sensitive methods available to us. The reason for the low whole-muscle homogenate xanthine oxidase activity is illustrated by the limited localization sites.

The present data show that the expression of xanthine oxidase is increased in endothelial cells in human muscle after eccentric exercise and indicate that infiltrating leucocytes may contain xanthine oxidase. The data furthermore suggest that xanthine oxidase is not involved in the initial exercise-induced muscle damage but that xanthine oxidase may contribute to the generation of reactive oxygen species during secondary inflammatory events. The evident association between xanthine oxidase and leucocytes observed in the current investigation, as well as in previous *in vitro* studies, suggests that the leucocytes may be involved in the induction of xanthine oxidase in the endothelium. It appears, however, that more serious muscle inflammation than can be elicited by hard eccentric exercise, or possibly an impaired muscle antioxidant defence system, is required for the markers of radical damage used here to be detectable in humans.

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Author's email address

Y. Hellsten: Ylva.Hellsten@pop.aki.ku.dk

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