Markers of oxidative stress in the skeletal muscle of patients on haemodialysis

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Abstract

Background. Increased oxidative stress may play a role in morbidity and mortality of patients with renal failure. Most studies have examined serum markers of oxidation, but it is unclear whether oxidative stress is involved in skeletal muscle atrophy.

Methods. This study examined markers of oxidative stress in the skeletal muscle of 10 haemodialysed patients and 10 control subjects. Biopsies from the quadriceps femoris were analysed for reduced and oxidized glutathione, protein thiols, malonaldehyde and heat shock proteins (HSP27, HSP60 and HSP70), superoxide dismutase and catalase activities. A novel microdialysis procedure was used to examine hydroxyl radical activity in the interstitial fluid of the tibialis anterior.

Results. Patients had muscle atrophy with a reduced diameter of both type I and II fibres (by 15 and 20%, respectively). Muscle microdialysates contained 2,3- and 2,5-dihydroxybenzoates formed from salicylate indicating hydroxyl radical activity, with no differences between patients and control subjects. Muscle protein thiol and oxidized glutathione contents were unchanged in patients, but malonaldehyde content was reduced. In contrast, total muscle glutathione and heat shock protein contents were increased. Muscle superoxide dismutase activity was unchanged, but catalase activity was reduced in patients.

Conclusions. The muscle of patients undergoing haemodialysis undergoes some adaptive responses in total glutathione content, heat shock protein content and catalase activity that are potentially related to chronic oxidative stress. However, there is no evidence of gross oxidation, nor any clear relationship between oxidative stress and muscle fibre atrophy, arguing against a direct role of oxidants in the degenerative processes.

Keywords: free radicals; glutathione; heat shock proteins; microdialysis; reactive oxygen species

Introduction

Patients with end-stage renal disease (ESRD) undergoing haemodialysis (HD) have limited exercise capacity that contributes to poor quality of life and high mortality. Several potential causes have been proposed including anaemia, uraemic myopathy and detraining. Muscle of patients with ESRD on HD shows decreased oxygen diffusing capacity [1], fibre atrophy [2] and reduced oxidative ATP synthesis [2,3] which may reflect at least a component of intrinsic mitochondrial dysfunction. It has been suggested that HD may compound the effect of renal failure on muscle, and increased oxidative stress has been implicated [3,4] although a comprehensive study of the interplay between atrophy and changes in reactive oxygen species (ROS) generation and markers of oxidative stress is lacking. Furthermore, oxidative stress mechanisms have been proposed to play a role in the possibly related situations of disuse atrophy, age-related muscle atrophy and cancer cachexia [5].

Studies of serum markers of the reactions of free radicals and ROS in HD have shown conflicting results. Daschner et al. [6] reported elevated serum malonaldehyde (MDA), a marker of lipid peroxidation, one kind of ROS-mediated damage, in patients with ESRD pre-HD that normalized following dialysis. They also reported normal serum antioxidant levels, unchanged by HD [6]. Other workers have reported abnormal serum markers of oxidative stress in patients [7] exacerbated by HD [7,8], but interpretation is complicated by potential confounding factors such as fluid shifts, heparin use and anaemia [9], and the effects of altered renal clearance of markers [10]. Nevertheless, there is a consensus that ROS are produced at the dialysis membrane, which leaves open the question of whether this can influence muscle. Thus, the general
difficulty of interpreting extracellular ROS measurements and the physical separation between the dialysis membrane and the circulation on the one hand, and the intracellular milieu of the muscle on the other, means that establishing the possible role of ROS in muscle, in patients with ESRD on HD will require direct measurements in muscle. In this article, we report results using two techniques: muscle biopsy and microdialysis.

The potential role of oxidative stress in the muscle dysfunction in patients with chronic renal failure undergoing HD does not appear to have been defined. Skeletal muscle cells generate a number of ROS and this increases during different stresses such as contraction [11]. Skeletal muscle has a substantial capacity to adapt following increased ROS exposure, including increases in the activities of endogenous antioxidant defence enzymes such as glutathione peroxidase (which catalyses the reduction of lipid hydroxyperoxides), superoxide dismutase (SOD) (which catalyses the reduction of superoxide anions to hydrogen peroxide) and catalase [12,13] (which catalyses the breakdown of hydrogen peroxide to oxygen and water). Heat shock proteins (HSPs) also protect cells against oxidative stress and specific HSPs are increased in muscle following a variety of stresses including exercise [12,13]. We have recently demonstrated that the increased production of ROS during exercise may be the signal for activation of a rapid adaptive response in the activity of protective enzymes such as superoxide dismutase and catalase, and an increase in a range of HSPs [12,13]. This would be expected to help protect the tissue against subsequent exposure to pathological increases in ROS.

Here we investigate whether skeletal muscle of patients with ESRD on HD shows evidence of increased generation of free radicals or ROS that could be linked to muscle dysfunction. In addition to analysis of conventional markers of oxidative stress, we used a novel microdialysis procedure to examine hydroxyl radical activity in the interstitial fluid of human skeletal muscle. We hypothesized that patients would show muscle atrophy and that within the muscle tissue there would be increased hydroxyl radical activity, increased MDA content and adaptive increases in defences against oxidative stress.

### Subjects and methods

#### Subjects

We studied 10 patients with ESRD undergoing regular HD, mean age 49 (range 37–60, SD 8) years, mean dialysis duration 6 (range 1–26) years and 10 sex-matched control subjects on no medication, mean age 35 (range 30–49, SD 6) years. The patients’ clinical characteristics are given in Table 1. Smoking, diabetes, infections or acute inflammatory conditions were exclusion criteria. Patients had haemoglobin levels between 11.5–13.5 g/dl. Patients received erythropoietin and intravenous iron, and standard heparin anticoagulation. Each patient received 4 h of HD on three occasions each week, using polyacrylonitrile dialysing membranes and a single-pass dialysate delivery system, a Hospal Crystal 2800 plate membrane and glucose-free, and bicarbonate dialysate containing 139 mM sodium, 2.0 mM potassium, 1.75 mM calcium, 0.5 mM magnesium and 109.5 mM chloride. Dialysate flow rate was 500 ml/min and blood flow >300 ml/min. Patients had primary arteriovenous fistulae (radiocephalic or brachiocephalic) and used two needles for each dialysis session. Ethical approval for the study was granted by the Liverpool Research Ethics Committee and all subjects gave informed consent.

#### Muscle biopsy and sample handling

A muscle sample was taken by percutaneous needle biopsy from the lateral portion of the quadriceps femoris, using local anaesthetic to skin and subcutaneous fascia. Biopsies from patients with ESRD on HD were taken on a dialysis-free day.
analysed per patient, in 1–3 muscle sections. The assessor was blinded and 50–100 fibres were counted in serial transverse sections stained for myosin ATPase activity at pH 9.4, and cooled in liquid nitrogen for histological analysis. In 6 patients with ESRD on HD, microdialysis samples were collected for 2 h prior to HD, throughout the 4 h HD procedure and for 2 h after HD. Samples were stored at −70°C prior to analysis for 2,3- and 2,5-dihydroxybenzoic acid (2,3- and 2,5-DHB) by HPLC with electrochemical detection [14].

Microdialysis study of interstitial hydroxyl radical activity

Microdialysis studies of skeletal muscle were undertaken in five patients with ESRD on HD and five healthy volunteers. Sterile microdialysis probes (CMA60; CMA, Sweden) with a 30 mm long dialysis membrane, 0.6 mm diameter and molecular cut-off of 20 kDa were inserted into the tibialis anterior muscle using a stainless steel split introducing needle. The probe was perfused at 0.5 mM sodium salicylate at 0.3 µl/min using a perfusion pump (CMA106; CMA, Sweden). The probes were allowed to stabilize for 30 min and perfusate was collected hourly for 8 h. For patients with ESRD on HD, microdialysis samples were collected for 2 h prior to HD, throughout the 4 h HD procedure and for 2 h after HD. Samples were stored at −70°C prior to analysis for 2,3- and 2,5-dihydroxybenzoic acid (2,3- and 2,5-DHB) by HPLC with electrochemical detection [14].

Biochemical analysis

Muscle antioxidant defence enzymes. The homogenate in phosphate buffer was centrifuged at 14 000 g and 4°C for 10 min and the supernatant analysed [13] for catalase activity by following the decomposition of hydrogen peroxide at 240 nm, for total SOD activity using the SOD-525 kit (R & D Systems Europe, Oxon, UK) and for protein content using the bicinchoninic acid assay kit (Sigma Chemical Co. Dorset, UK).

Muscle markers of ROS activity. The homogenate in sulphosalicylic acid was centrifuged and the supernatant analysed for total and oxidized glutathione content by an enzymatic recycling assay. The pellet was washed and resuspended in 0.5 M Tris–HCl buffer, pH 7.6 and the protein thiol content measured [15], the protein content in an aliquot digested in 0.66 M KOH being determined by the Lowry method. The saline homogenate was assayed for MDA by HPLC [16].

Muscle HSP content. After centrifugation of the homogenate in SDS, the HSP content of the supernatant was determined by SDS-PAGE and western blotting [12,13] and the total protein content measured using the bicinchoninic acid assay kit.

Results

Muscle fibre types and areas

There was no difference in the proportion of type I to type II fibres in patients with ESRD on HD compared with control subjects, but there was a significant decrease in the mean diameter of both fibre types (Table 2). Type I fibre and type II fibre diameter was reduced by 15 and 20%, respectively, which corresponds (assuming no decrease in total fibre number) to an overall 31±10% reduction in muscle area.

Muscle antioxidant defence enzymes

No significant difference in SOD activity was detected (Figure 1A), but in contrast, the activity of catalase was significantly reduced in patients with ESRD on HD (Figure 1B).

Muscle markers of ROS activity

There was a striking and significant increase in the muscle content of total glutathione in patients with ESRD on HD (Table 2). Type I fibre and type II fibre diameters were measured by image analysis system (Seescan plc, UK). The assessor was blinded and 50–100 fibres were analysed per patient, in 1–3 muscle sections.

Table 2. Fibre type diameters in muscle biopsies from control and patients with ESRD on HD

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Patients</th>
<th>% change in patient</th>
<th>P</th>
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<tbody>
<tr>
<td>Number of type I fibres (% total)</td>
<td>52 ± 6</td>
<td>51 ± 5</td>
<td>−4 ± 16%</td>
<td>1.0</td>
</tr>
<tr>
<td>Type I diameter (µm)</td>
<td>75 ± 5</td>
<td>64 ± 2</td>
<td>−15 ± 7%</td>
<td>0.04</td>
</tr>
<tr>
<td>Type II diameter (µm)</td>
<td>74 ± 5</td>
<td>59 ± 4</td>
<td>−20 ± 8%</td>
<td>0.03</td>
</tr>
<tr>
<td>Type I fibre area (% total area)</td>
<td>54 ± 5</td>
<td>55 ± 5</td>
<td>2 ± 12%</td>
<td>0.9</td>
</tr>
</tbody>
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P is the statistical significance of the difference between patients with ESRD on HD and control subjects.
ESRD on HD (Figure 2A), but no significant difference in the absolute amount of oxidized glutathione (Figure 2B); thus oxidized glutathione formed 7% of the total glutathione pool in controls but only 2% in patients with ESRD on HD. There was no significant difference in the protein thiol content (Figure 2C), but MDA content in patients with ESRD on HD was significantly reduced in comparison with controls (Figure 2D).

**Muscle HSP content**

Muscle HSP27 content from patients with ESRD on HD was significantly elevated compared with control (Figure 3A). There appeared to be a tendency of HSP60 and HSP70 to be increased, but this effect was not significant (Figure 3B and C).

**Hydroxyl radical activity in muscle interstitial fluid**

In the microdialysates from muscles of both control subjects and patients with ESRD on HD, 2,3- and 2,5-DHB were detected and the time-courses are shown in Figure 4. Significant inter-subject variability was observed, but there were no significant differences between the levels in control and patients with ESRD on HD either prior to, during or immediately following HD in the patients.

**Discussion**

Skeletal muscle of the patients with ESRD on HD showed atrophy with a significant decrease in the mean diameter of both fibre types (Table 2). There was no significant change in the proportions of type I and II fibres. Previous studies have proposed a role for ROS
generation and/or handling as part of the cause of HD-mediated muscle loss [2]. HD has been, controversially, associated with serum markers of increased ROS activity [6,7,17], but few studies have examined a role for increased ROS production in muscle. The present findings indicate significant structural and biochemical changes in the muscle of patients with ESRD on HD, although there was no evidence (at the time of sampling) of any major increase in ROS activity in muscle.

One potential limitation of the current study was the 14-year difference in mean age between the patients with ESRD on HD and the control group. However, changes such as those reported in this study are not compatible with reported changes in muscle with increasing age, and no age-dependence of biochemical data was evident in patients with ESRD on HD and control subjects (data not shown in detail).

This study appears to be the first to use the novel microdialysis technique to examine directly the extracellular activity of hydroxyl radicals in humans. Microdialysis, when used in conjunction with detector molecules such as salicylate, cytochrome c or spin trapping agents, has been shown to be a useful tool in the detection of free radical species in animals in vivo [11,12,14]. The results presented here show that 2,3-DHB formation could be detected in microdialysates from human muscle, in agreement with animal studies that indicate a detectable activity of hydroxyl radicals in muscle interstitial fluids [11,14]. In rodent studies 2,3-DHB formation from salicylate is increased by contractile activity [14] and by reperfusion of ischaemic muscle tissue [11]. There was no significant change in 2,3-DHB or 2,5-DHB concentrations in microdialysates from patients with ESRD on HD compared with control subjects either prior to, during or following HD. Data from our laboratory

![Fig. 3. Muscle content of HSP27 (A), HSP60 (B) and HSP70 (C) in control subjects (●) and patients with ESRD on HD (●). Representative western blots are shown in panel D. Lanes 1–3: patients; lanes 4–6: control subjects. Data are presented as arbitrary units derived from densitometry readings. *P < 0.05 compared with control muscles. The increase in HSP27 is 2.2 ± 0.9 -fold in patients with ESRD on HD compared with control subjects (P = 0.04), that in HSP60 is 2.4 ± 0.8 -fold (P = 0.06) and that in HSP70 is 1.6 ± 0.4 -fold (P = 0.09). HSP27 and HSP60 are strongly correlated in patients with ESRD on HD (R = 0.82, P = 0.001).](image-url)

![Fig. 4. Concentration of 2,3-dihydroxybenzoic acid (A) and 2,5-dihydroxybenzoic acid (B) in microdialysates prior to, during and following HD in patients with ESRD on HD (■) and for an equivalent collection time in healthy control subjects (●). The time period during which HD occurred in patients is shown.](image-url)
using transgenic mice, heterozygotic for mitochondrial SOD (MnSOD) suggest that the extracellular hydroxyl radical activity in muscle interstitial fluid depends on mitochondrial generation of hydrogen peroxide [11]. The lack of difference in 2,3- and 2,5-DHB between control subjects and patients with ESRD on HD therefore suggests that muscle mitochondrial superoxide and hydrogen peroxide generation are not grossly abnormal in the patients. Of course the results showed significant variability (Figure 4), and so it remains possible that there are more subtle differences.

Surprisingly, a decreased MDA content was found in the muscle of patients with ESRD on HD, suggesting that no gross lipid peroxidation was occurring. This was in contrast to the increased MDA reported in the serum of patients with ESRD on HD in some studies [18] and a recent report of an increased MDA in skeletal muscle of patients with ESRD on HD [4]. The study by Lim et al. [4] used a very broad range of patient ages (from <40 years, with a substantial percentage of the HD cohort (32%) between 61 and 80 years and a further 20% in excess of 81 years. In this instance, it is likely that there is an age-related effect of studying >50% of elderly patients with ESRD on HD. Glutathione is an endogenous ROS scavenger which protects against oxidative injury, and the increase in the reduced glutathione content of muscle seen in patients with ESRD on HD represents a large increase in reducing power. Similar adaptive increases in the total glutathione content of other tissues have been reported in response to oxidative stress [19] and in erythrocytes in patients with ESRD on HD [20]. Protein thiols in blood have been shown to be reduced in patients with ESRD and to increase following HD [7], but we found no changes in the protein thiol or oxidized glutathione content of muscle from patients with ESRD on HD (Figure 2). This lack of an increase in oxidized glutathione and lack of change in protein thiols together with the reduction in tissue MDA and lack of change in hydroxyl radical activity indicate that no gross oxidation was occurring in the muscle of patients with ESRD on HD.

The SOD and catalase data are also compatible with a lack of ongoing oxidative stress in the muscle of patients with ESRD on HD. We found a decrease in muscle catalase activity in comparison with control muscles, and it is conceivable that this may be a response to the large increase in muscle glutathione content such that less catalase is required for detoxification of hydrogen peroxide. In contrast, no change in total SOD activity was seen (Figure 1), despite the induction of SOD activity that occurs following oxidative stress to muscle during contractile activity [12].

HSPs are intracellular chaperones synthesized in response to a variety of stresses, including oxidative stress in skeletal muscle [21], which are cytoprotective by binding to destabilised cellular proteins, stabilizing them until conditions become more favourable, or by facilitating rapid adaptation of the cell. The present study shows a significant increase in the content of HSP27. We and others have reported that a single bout of exercise causes an adaptive increase in HSPs in rodents and humans which appears to be in response to an increase in the generation of ROS by muscle [12,13]. Changes in muscle content of HSP27 appear particularly relevant since experimental manipulation of cellular HSP27 content has been associated with changes in cellular glutathione [22]. The data reported here are in accord with this, although we found no direct relationship between HSP27 and glutathione contents.

A potential speculative explanation for the increase in muscle HSP content, decreased MDA and increased total glutathione seen in patients with ESRD on HD is that the muscle of patients is exposed to chronic oxidative stress but has successfully adapted to this stress with little evidence of ongoing ROS generation. Thus, the present data may not be incompatible with that in some studies of serum markers of oxidative stress [6,7].

Data from the microdialysis studies also suggest that the biochemical adaptations which had occurred in these patients do not predispose their muscles to greater oxidative stress during HD.

Are the changes in muscle biochemistry reported here a non-specific result of muscle atrophy rather than the disease process? Muscle atrophy is a prominent feature of patients with ESRD on HD [2,23] and is partially reversible by exercise training, although it does not appear that the changes in skeletal muscle of patients with ESRD on HD are entirely due to disuse atrophy [23]. Oxidative stress has been reported to be a feature of muscle atrophy of various causes [24,25]. In accord with our data, decreased catalase activity has been reported in one study of disuse atrophy [25] although this has not been universally observed [24]. Since some studies reported an increase in cytosolic Cu, ZnSOD activity [25], with no change [24] in mitochondrial MnSOD activity in atrophic muscle, it has been suggested that some of the changes in disuse atrophy are due to an increase in non-mitochondrial generation of superoxide anions [24]. However, we found no evidence for this in patients with ESRD on HD. Although the study sample size was relatively small, data from microdialysis studies provide no evidence of increased ROS generation during HD in any individual patient studied or in the group of patients, although the between-patient variability was substantial. Although we did not formally study activity levels, it may be possible that some, but not all of the changes observed were due to decreased activity although there was little evidence of ongoing increased ROS as a mechanism by which the atrophy was occurring.

Studies of HSP in atrophied muscle have usually reported a decrease in HSP70 content that is associated with muscle weight loss and a slowing of protein synthesis [26]. In contrast, we see no significant change in HSP70. In previous studies there have been reports of a proportionately greater atrophy of type II fibers [23] in patients with ESRD on HD and this would
result in an overall increase in HSP70 content since HSP70 levels are greater in type I compared with type II fibres. However, we did not observe any significant difference in the fraction of type I fibers, although the mean diameters of both types of fibres were decreased, consistent with general muscle atrophy. Thus, overall the changes reported here do not appear to reflect non-specific changes in atrophic muscle or changes in fiber type proportions.

In summary, notwithstanding the small size and limitations of this study (which include a lack of any formal activity- or age-matching) our data do not indicate that the muscle atrophy observed in patients with ESRD on HD is primarily related to increased ROS generation. More research is required to further understand the functional effects of the changes in muscle biochemistry in patients with ESRD on HD reported in this study.

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Conflict of interest statement. None declared.

References


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