Skeletal muscle, cytokines, and oxidative stress in end-stage renal disease

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Background. End-stage renal disease (ESRD) is a state of microinflammation, with increased activation of cytokines and augmented oxidative stress. While peripheral blood mononuclear cells are an established source of reactive oxygen species and inflammatory cytokines during hemodialysis (HD), skeletal muscle is also capable of generating these biomolecules.

Methods. Femoral arterio-venous (A-V) balance of interleukin-1 (IL-1), IL-6, IL-10, tumor necrosis factor-α (TNF-α), malondialdehyde (MDA), and carbonyl protein (CP) were measured in 17 ESRD patients and 9 healthy volunteers. ESRD patients were studied before (pre-HD) and during HD. mRNA levels of cytokines, heme oxygenase-1 (HO-1), and suppressors of cytokine signaling-2 (SOCS-2) were quantitated in the skeletal muscle by real-time polymerase chain reaction (PCR).

Results. Arterial concentration of MDA (pmol/mL) was higher pre-HD (325.5 ± 19.6) compared to controls (267.7 ± 14.7), but decreased intradialysis (248.8 ± 16.1) (P < 0.01). Dialysis clearance of MDA was 16.9 ± 3.1 mL/min. CP concentration (nmol/mg protein) in the artery was significantly higher pre-HD (2.29 ± 0.09) than in controls (1.92 ± 0.05), and remained stable during HD (2.23 ± 0.07). Plasma cytokines increased to a variable degree in the artery and vein during HD. A-V balance studies demonstrated that the MDA (17.8%) and CP (5.1%) concentrations increased significantly in the vein intradialysis. Venous concentration of IL-6 was higher than that in the artery during dialysis (16.27 ± 2.42 vs. 11.29 ± 2.17 pg/dL, P < 0.01). mRNA levels of IL-6 (0.028 ± 0.02 vs. 6.69 ± 0.21), HO-1 (0.96 ± 0.01 vs. 5.08 ± 1.11), and SOCS-2 (0.63 ± 0.12 vs. 0.82 ± 0.14) in the muscle increased during HD (P < 0.01). Immunohistochemical studies confirmed the increase in IL-6 protein in the skeletal muscle during HD. The intradialytic increase in IL-1, IL-10, and TNF-α gene expression was not significant.

Conclusion. Skeletal muscle may also contribute to the circulating plasma IL-6 and increased oxidative stress during HD.

Inflammation coexists with increased oxidative stress in end-stage renal disease (ESRD). Overwhelming production of free radicals, and lipid and protein oxidation byproducts could lead to organ damage. Carbonyl protein (CP) is a marker of oxidative damage in cells and tissue proteins [1]. Malondialdehyde (MDA) is a water soluble lipid peroxidation product that is partially excreted by the kidney [2]. Oxidative stress is increased in patients with reduced renal function [3]. It is unknown whether elevated markers of oxidative stress reflect increased generation or reduced metabolic clearance. While some investigators have demonstrated exacerbation of the flux of free radicals with initiation of renal replacement therapy [4], others have observed no change [5] or decrease [6] in indices of oxidative stress during hemodialysis (HD). These discordant results are the reflection of differences in biocompatibility of the dialysis therapy [7], use of disparate markers of oxidative stress [6], and failure to take into account the intradialytic loss of the biomolecules in the dialysate [6].

Oxygen-derived free radicals are produced during mitochondrial respiration in the skeletal muscle and by the activated peripheral blood mononuclear cells (PBMC). Blood membrane interaction during HD promotes secretion of cytokines and also formation to reactive oxygen species (ROS) by the PBMC. Respiratory chain is a powerful source of oxidative stress, with 1% to 4% of oxygen reacting with the respiratory chain incompletely reduced to ROS [8]. Impaired mitochondrial function [9] and decreased antioxidant capacity [10] in ESRD could potentially augment the oxidative stress in ESRD. Oxidants may amplify the inflammatory cascade via activation of the nuclear factor-κB (NF-κB) pathway [11]. Human skeletal muscle has the inherent ability
to express interleukin-6 (IL-6) [12]. IL-6 is produced in response to diverse stimuli and mediates pleiotropic actions, including acute phase response, immune modulation, hematopoiesis, and metabolic regulation. Heme oxygenase-1 (HO-1) is a heat shock protein that is expressed in the skeletal muscle. It plays an important role in the cellular defense against oxidative stress and the detrimental effects of proinflammatory cytokines [13]. The suppressors of cytokine signaling (SOCS) family of proteins play key roles in the negative regulation of cytokine signal transduction [14]. The SOCS family consists of 8 proteins: cytokine-inducible SH2 protein (CIS) and SOCS1-SOCS7. The expressions of SOCS1 to 3 and CIS are induced by cytokine or growth factors, resulting in the inhibition of JAK/STAT-mediated cytokine signaling by a negative feedback loop [15, 16].

We hypothesized that HD induces generation of ROS and release of cytokines from the skeletal muscle. In order to test the hypothesis, we measured the femoral arterio-venous (A-V) balance of MDA, CP, and representative cytokines, IL-1, IL-6, IL-10, and tumor necrosis factor-α (TNF-α) across the leg in 17 ESRD patients and 9 healthy volunteers. We also simultaneously quantified the mRNA levels of the respective cytokines, HO-1 and SOCS-2, in the skeletal muscle. ESRD patients were studied before (pre-HD) and during HD. The changes in markers of oxidative stress were interpreted in the context of removal by dialysis. mRNA levels of IL-6, HO-1, and SOCS-2 in the skeletal muscle increased intradialysis, which was associated with a more pronounced increase in the concentrations of MDA, CP, and IL-6 in the femoral vein than in the artery.

METHODS

Seventeen ESRD patients and 9 healthy volunteers were studied. The study was approved by the Human Research Review Committee at the University of New Mexico. Informed consent was obtained from all the participants. Patients were admitted to the General Clinical Research Center at the University of New Mexico the day before the experiment. All the studies were performed in a postabsorptive state, after overnight fast. The femoral artery and vein were cannulated on the same side, taking all aseptic precautions. In ESRD patients, blood samples were collected from the femoral artery and vein prior to initiation of HD (pre-HD), at midpoint of HD (mid-HD), and during the last 10 minutes of HD (end-HD). Skeletal muscle biopsies were also obtained at the same time points. In controls, muscle biopsy and A-V balance study was performed only once.

Hemodialysis

Only ESRD patients underwent HD. The patients’ standard dialysis prescription was used, except that all the patients received 4 hours of dialysis. Dialysate composition was sodium (Na⁺) 139 mEq/L, bicarbonate (HCO₃⁻) 35 mEq/L, calcium (Ca++) 2.5 mEq/L, magnesium (Mg++) 1 mEq/L, dextrose 200 mg/dL, and potassium (K⁺) (per patient’s need). A new polysulfone membrane (Hemoflow, F70; Fresenius, Walnut Creek, CA, USA) was used. Anticoagulation was not used during dialysis to minimize the risk of bleeding. While the patient’s regular erythropoietin dose was continued, iron infusion was avoided. Representative dialysate samples were collected every 10 minutes in a sterile container. The spent dialysate sample was mixed thoroughly, and a sample was taken and stored at −80°C for future analysis.

Muscle biopsy

Muscle biopsies were taken from the lateral portion of vastus lateralis about 20 cm above the knee using Bergstrom biopsy needle. Approximately 30 to 50 mg of muscle tissue was obtained during each biopsy. Fat and connective tissue were completely removed and the sample immediately frozen in liquid nitrogen and stored at −80°C for future analysis. During repeat biopsies care was taken to obtain samples from sites away from the initial site.

Assay for IL-1, IL-6, IL-10, and TNF-α

Cytokines were measured in plasma samples using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, MN, USA) according to the manufacturer’s directions. Assays were performed in duplicates and the mean of the 2 measurements was used. The inter- and intraassay CV% for each cytokine were <6%.

Measurement of plasma MDA and CP

MDA was measured using ion exclusion and reverse-phase Shodex KC-811 column (Waters, Milford, MA, USA) with detector set at 532 nm [3]. The day to day variation in standard assays of MDA was <7%. Each plasma sample was assayed in duplicate and variation between duplicate samples was <9%.

The hemodialysis clearance of MDA was calculated by the following equation:

\[
\text{MDA clearance} = \frac{\text{volume of dialysate} \times \text{concentration in dialysate}}{\text{area under the curve}}.
\]

Area under the curve was calculated by the trapezoidal rule using the software WinNonLin (version 4.1; Mountain View, CA, USA).

CP was measured as we described earlier [3]. Briefly, duplicate samples were prepared by adding 10 μL of plasma to 1.0 mL of phosphate-buffered saline (PBS) in 13 × 100 mm tube. One of the duplicate sample was treated with 200 μL of 2,4 Di-nitro phenylhydrazine
(DNPH) in 2 N hydrochloric acid (HCl). To the other sample, 200 μL of 2 N HCl was added, which was used as a blank. The mixture was incubated at room temperature for 1 hour, followed by the addition of 1.2 mL of 20% TCA. The samples were then incubated in ice for 10 minutes and centrifuged at 600 g, 40°C for 10 minutes, and the supernatant discarded. The protein pellet was washed 2 times with 3 mL of ethyl alcohol:ethyl acetate (1:1, v/v) and dried under nitrogen gas. The protein pellet was dissolved in 1 mL of 10 mmol/L sodium phosphate (pH 6.8) containing 3% sodium dodecyl sulfate (SDS). Each sample was then scanned from 320 to 410 nm in UV/Vis Spectrophotometer (Beckman Du-640; Beckman, Corona, CA, USA) against a duplicate blank treated with HCl. The concentration of carbonyl groups was calculated from the absorbance spectrum, with the extinction coefficient for aliphatic hydrazone derivatives assayed at 360 nm. The final data are expressed as nmol of carbonyl groups per mg of protein. The CV% for the assay was <6%.

Real-time PCR for IL-1, IL-6, IL-10, TNF-α, HO-1, and SOCS-2

Muscle biopsy material (20 mg) was homogenized in Qiagen’s RNeasy lysis buffer containing 2-mercaptoethanol (Qiagen, Valencia, CA, USA) at room temperature. RNA was isolated and purified using the Qiagen RNeasy mini protocol for isolation of total RNA from muscle, which included Proteinase K digestion and DNase treatment (Qiagen 74104, 19131, and 79254). Using 500 ng of total RNA as template, cDNA was prepared by reverse transcriptase reaction using the ABI high capacity cDNA archive kit (ABI 4322171; Applied Biosystems, Foster City, CA, USA). mRNA expression was assessed by real-time PCR (RT-PCR) method using Applied Biosystems TaqMan® Assays-on-Demand™ Gene Expression Products (Applied Biosystems). Assays-on-Demand™ are a comprehensive collection of predesigned primer and probe sets available “off the shelf” and each assay consists of 2 unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB probe. PCR was performed on the MJ Research Opticon 2 (Bio-Rad, Inc., Waltham, MA, USA) instrument. Negative controls for RT-PCR included reactions containing no template. The amount of target gene in ESRD patients was normalized to an endogenous control (GAPDH) and to the mRNA level in the controls [17]. Data from the RT-PCR experiment can be analyzed for absolute or relative quantification. Absolute quantification determines the input copy number of the transcript of interest, usually by relating the PCR signal to a standard curve. Relative quantification describes the change in expression of gene relative a reference group, such as an untreated control or a sample at time zero in a time-course study. Quantifying the relative changes in gene expression using real-time can be performed using 2-ΔΔCT method [18].

Briefly, data from RT-PCR were imported into an Excel spreadsheet and analyzed using the following equation:

\[ \Delta \Delta C_T = (C_T \text{Target} - C_T \text{GAPDH})_{\text{Time} x} - (C_T \text{Target} - C_T \text{GAPDH})_{\text{Time}0} \]

C_T is the fractional cycle number at which the amplified target reaches a fixed threshold, \( \Delta C_T \) is the difference on threshold cycles for target and reference, \( c_{\text{Time}} \) is the time point of interest, and \( c_{\text{Time}0} \) represents the 1x expression of the target gene normalized to GAPDH.

Immunohistochemical staining for IL-6 protein

We used modified immunohistochemistry method of Zamora et al[19] to detect the IL-6 in the muscle biopsies. Briefly, the sections were rehydrated and incubated with 5% Triton-X (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes and then washed twice with 0.02 mol/L phosphate-buffered saline (PBS). After blocking the sections in 5% goat serum, they were incubated overnight with the primary rabbit IL-6 antibody (R&D Systems) in a 1:20 dilution in 1% sheep serum at 4°C. The following data, sections were washed 5 times with PBS, the sections were incubated with secondary antibody labeled with fluorescein isothiocyanate (FITC; R&D Systems). Sections were mounted, visualized, and photographs (EFD-3; Nikon, Japan) taken after excitation for 30 seconds using the filter for FITC. We did not quantify the fluorescence.

Statistics

Data are given as mean and standard error of mean (SEM). α was set at 0.05. Paired and unpaired t test were used when applicable. Repeated measures of analysis of variance (ANOVA) were used with pre-, mid-, and end-HD as the repeating factors and ESRD versus control as the grouping factor with a post-hoc Tukey test. Simple linear regression analysis was used to identify the association between variables.

RESULTS

The etiology of ESRD was hypertension in 2, diabetic nephropathy in 6, glomerulonephritis in 3, interstitial nephropathy in 2, and unknown in 4. There were 4 diabetics among controls. Hemoglobin concentration was significantly lower in ESRD patients (Table 1). The glycated hemoglobin (HbA1C) levels in ESRD patients and controls subjects with diabetes were 7.4 ± 0.3% and 7.7 ± 0.4%, respectively. ESRD patients were adequately dialedyzed, as evidenced by a urea reduction ratio of 72.0 ± 1.5%. Negative acute phase protein, serum albumin, was higher, and positive acute phase proteins fibrinogen and C-reactive protein (CRP) were lower in controls compared to ESRD patients.
Concentrations of MDA in the artery and vein were significantly elevated in ESRD patients pre-HD compared to controls, but were decreased during HD (P < 0.001) (Table 2). Dialysis clearance of MDA was 16.9 ± 3.1 mL/min. MDA concentration in the vein was higher than that in artery in both ESRD and control subjects (P < 0.01). In ESRD patients (pre-HD and end-HD), the concentration of CP in the artery and vein was higher compared to controls (P < 0.01). While the CP concentration in the artery did not change intradialysis, it increased in the vein (P < 0.01). CP was not detected in the dialysate.

Plasma cytokines in the artery and vein increased to a varying degree during dialysis treatment (Table 3). However, the magnitude of increase in IL-6 intradialysis was significantly higher in the vein than that observed in the artery. mRNA levels of IL-6 (0.028 ± 0.02 vs. 6.69 ± 0.21), HO-1 (0.96 ± 0.01 vs. 5.08 ± 1.11), and SOCS-2 (0.63 ± 0.12 vs. 0.82 ± 0.14) in the muscle increased during HD (P < 0.01) (Fig. 1). The changes in the mRNA levels of IL-1β (0.065 ± 0.019 vs. 0.542 ± 0.328) and TNF-α (0.287 ± 0.62 vs. 0.796 ± 0.351) during dialysis were not significant. IL-10 mRNA was not detectable in the muscle. Immunohistochemical staining for IL-6 protein was performed in multiple 10 μm sections obtained from 2 ESRD patients (pre-HD and end-HD) and 2 controls. IL-6 protein expression in the muscle was increased in samples obtained at the end of HD compared to pre-HD and controls (Fig. 2). Net increase in the IL-6 concentration in the vein, as indicated by the arterio-venous balance studies, correlated positively with IL-6 mRNA (r² = 0.29, P < 0.01). mRNA levels of IL-6 and HO-1 were positively and significantly correlated (r² = 0.41, P < 0.001).

MDA (348.2 ± 32.0 vs. 288.6 ± 24.7 pmol/mL), CP (2.38 ± 0.15 vs. 2.18 ± 0.08 nmol/mg protein), IL-1 (1.15 ± 0.08 vs.1.05 ± 0.07 pg/dL), IL-6 (4.65 ± 0.64 vs. 4.08 ± 0.44 pg/dL), IL-10 (0.87 ± 0.05 vs. 1.07 ± 0.10 pg/dL) in the artery pre-HD among patients with and without diabetes were not significantly different. The changes at mid- or end-HD, or the arterio-venous differences in the plasma concentration of these biomolecules, were comparable between the 2 groups. The mRNA levels were also not significantly different.

**DISCUSSION**

In the present study, we measured the A-V balance of cytokines and markers of oxidative stress, and also genes involved in the regulation of inflammatory response and oxidative stress in ESRD patients and healthy volunteers. The arterial concentration of MDA decreased during HD
because of loss in the dialysate. CP level in the artery did not change significantly during HD because it was not removed by dialysis. A-V balance studies showed that there is a net increase in the concentrations of MDA, CP, and IL-6 in the femoral vein during HD that was accompanied by an increase in the mRNA levels of IL-6, HO-1, and SOCS-2 in the skeletal muscle. These results suggest that skeletal muscle may be an important source of oxidative stress and IL-6 during HD. Femoral arteriovenous difference has been used as an estimate of release of biomolecules from the leg muscle [20, 21].

Cytokines are pleiotropic polypeptides with molecular weight ranging from 10 to 45 kD that are produced by different cells in response to diverse stimuli. Cytokines may be pro- or anti-inflammatory. The production of proinflammatory cytokines is controlled by the regulatory cytokine, IL-10. While IL-1 and TNF-α are classified as proinflammatory cytokines, IL-6 has both pro- and anti-inflammatory properties [22]. In vitro and in vivo data support the hypothesis that cytokines are released by PBMCs intradialysis. We observed that the concentrations of cytokines increased both in the artery and vein during dialysis, indicating increased generation of cytokines intradialysis. The magnitude of increase in the concentration of IL-6 in the femoral vein was significantly higher than that in the artery during HD, possibly due to net release from the leg compartment. This is supported by the increase in gene expression of IL-6 in the skeletal muscle during HD. IL-6 is expressed in the human skeletal muscle [23], and skeletal muscle is capable of releasing this protein [20, 23]. Release of IL-6 is mainly regulated by local stimuli [20], and preliminary studies suggest that it may not be a marker of muscle damage [24, 25]. However,

Fig. 1. Interleukin-6 (IL-6) and hemeoxygenase-1 (HO-1) and suppressors of cytokine signaling-2 (SOCS-2) gene expression in the skeletal muscle were increased during hemodialysis (HD). Relative change in mRNA expression (2-ΔΔCt method). Pre-HD, before HD; mid-HD, midpoint of HD; end-HD, last 10 minutes of HD. ¶End-HD vs. pre-HD, P < 0.05; †Pre-HD vs. mid-HD, P < 0.05; ‡Pre-HD vs. mid-HD, P < 0.01; ∗End-HD vs. pre-HD and mid-HD, P < 0.001.

Fig. 2. Immunohistochemical staining for IL-6 in the muscle. Increased fluorescence was observed in samples obtained at the end of HD compared with pre-HD and controls.
we demonstrated that IL-6 induces expression of genes regulating protein catabolism [26], increases muscle protein breakdown [27], and facilitates acute phase protein synthesis [28] during HD. Investigators have shown that muscle derived IL-6 functions as an exocrine hormone during exercise, exerting an effect on the liver and adipose tissue, thereby maintaining glucose homeostasis [29]. We did not find any significant changes in insulin, glucagon, or glucose levels during HD. While previous investigators have demonstrated that PBMCs are the primary source of cytokines during HD, this study suggests that IL-6 may be released from the skeletal muscle also. Increased plasma IL-6 could augment its own gene expression in the muscle through autocrine mechanism [30]. It is possible that increased cytokine released from PBMC facilitates the IL-6 gene expression in the muscle. Evidence from our laboratory indicates that the cytokine gene expression is not increased locally in the muscle in response to trauma related to biopsy up to 6 hours (unpublished data). While it is true that skeletal myocytes can express IL-6, the contribution from mononuclear cell infiltrates and vascular smooth muscle cells (VSMC) in the biopsy samples cannot be discounted [31, 32]. HD has a variable effect on plasma endothelin-1 (ET-1) concentration [33]. Investigators have demonstrated that ET-1 increases IL-6 mRNA levels in human VSMC [32]. ESRD patients with diabetes are metabolically different from those without diabetes. However, since the same patient is studied before and during HD, the impact of diabetic status on the results of the study probably minimal. There is a bidirectional relationship between cytokines and oxidative stress. Exposure of myotubes to ROS producing agents results in an increase in IL-6 release through the activation of the redox sensitive transcription factor, NF-kB [11]. Oxygen derived free radicals are produced during mitochondrial metabolism by the univalent reduction of O2 to water. Oxidative stress is measured by detection of various modified macromolecules that are generated from the ROS because they themselves have half-lives of just a few seconds. The analysis of oxidative stress during HD is further complicated by a number of factors in vivo, including changes in generation rate, size of the extracellular compartment, intercompartmental flux, and depuration by dialysis. PBMCs are activated by contact with nonbiological surface during HD, leading to NADPH-dependent production of superoxide and other reactive oxygen intermediates [34]. Preliminary evidence indicates that mitochondrial function and energy production via oxidative metabolism is impaired in ESRD [35]. In our study, while the concentration of MDA in the vein was higher than that in the artery in controls (5.4%) and ESRD patients pre-HD (5.0%), the magnitude of increase was more profound intradialysis (17.8%). Despite increased generation, the concentration of MDA decreased by 23% in the artery during dialysis due to loss in the dialysate. Using different techniques, investigators have demonstrated that uremic plasma accumulates oxidants, which are freely dialyzable [6, 36]. The plasma levels of MDA and thiobarbituric acid reactive substances (TBARS) are reported to decrease by 25% to 88% during HD [6, 37]. Similar to our study, Schettler et al [38] observed that while the arterial concentration of TBARS decreased (4%), the venous concentration increased (6%) intradialysis. Because of the larger molecular size, CP is not removed by HD and, hence, the arterial concentration did not decrease during HD. Dissociation between the plasma levels of different markers of oxidative stress has been reported pre- [6] and intradialysis [38] by other researchers also. A-V balance showed that the concentration of CP in the vein increased by 5.1%. While this study does not define the role of blood membrane interaction in increased oxidative stress during HD, these results suggest that skeletal muscle may also be an important source of oxidative stress in ESRD.

We observed that the HO-1 mRNA level increased during HD. Heme oxygenase system is involved in many physiologic as well as pathophysiologic processes, such as cytoprotection, apoptosis, and inflammation. HO is the rate-limiting enzyme in the catabolism of heme. Three isoforms of HO have been identified (HO-1, HO-2, and HO-3). Under physiologic conditions, most cells express low or undetectable levels of HO-1 protein, whereas HO-2 proteins are constitutively expressed. HO-3 awaits further characterization. Cytokines and ROS have been demonstrated to induce HO-1 expression [39], and it is speculated that HO-1 is important in the resolution of acute inflammation [40]. Increase in IL-6 mRNA level during HD was accompanied by increase in SOCS-2 gene expression. IL-6 induces marked increase in expression of CIS, SOCS-1, SOCS-2, and SOCS-3 in tissues, which in turn result in inhibition of signaling of wide range of cytokines [41]. Because SOCS genes are induced by cytokines and the corresponding proteins inhibit further cytokine-induced signaling, SOCS proteins are believed to form part of classic negative feedback loop mechanism.

Loss of lean body mass is an important predictor of mortality and morbidity in ESRD. While malnutrition is touted as a cause of uremic sarcopenia, muscle wasting has been described even in well-nourished patients on hemodialysis, indicating that factors other than malnutrition may contribute to uremic cachexia. Inflammation and oxidative stress coexist in ESRD and may contribute to muscle wasting through mitochondrial dysfunction [42]. The results from this study demonstrate that there is net release of MDA, CP, and IL-6 in to the vein during HD, possibly from skeletal muscle. This is supported by the increase in IL-6, HO-1, and SOCS-2 mRNA levels in the skeletal muscle during HD. Thus, skeletal muscle may contribute to the increase in plasma IL-6 and oxidative stress during HD.
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