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ORIGINAL ARTICLE

Superoxide dismutase type 1 in monocytes of chronic kidney disease patients

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Abstract We analyzed proteomic profiles in monocytes of chronic kidney disease (CKD) patients and healthy control subjects. Two-dimensional electrophoresis (2-DE) and silver staining indicated differences in protein pattern. Among the analyzed proteins, superoxide dismutase type 1 (SOD1), which was identified both by MS/MS mass-spectrometry and immunoblotting, was reduced in kidney disease. We characterized SOD1 protein amount, using quantitative in-cell Western assay and immunostaining of 2-DE gel blots, and SOD1 gene expression, using quantitative real-time polymerase chain reaction (PCR), in 98 chronic hemodialysis (HD) and 211 CKD patients, and 34 control subjects. Furthermore, we showed that different SOD1 protein species exist in human monocytes. SOD1 protein amount was significantly lower in HD (normalized SOD1 protein, 27.2 ± 2.8) compared to CKD patients (34.3 ± 2.8), or control subjects (48.0 ± 8.6; mean ± SEM; $P < 0.05$). Analysis of SOD1 immunostaining showed significantly more SOD1 protein in control subjects compared to patients with CKD or HD ($P < 0.0001$, analysis of main immunoreactive protein spot). SOD1 gene expression was significantly higher in HD (normalized SOD1 gene expression, 17.8 ± 2.3) compared to CKD patients (9.0 ± 0.7), or control subjects (5.5 ± 1.0; $P < 0.0001$). An increased SOD1 gene expression may indicate increased protein degradation in patients with CKD and compensatory increase of SOD1 gene expression. Taken together, we show reduced SOD1 protein amount in monocytes of CKD, most pronounced in HD patients, accompanied by increased SOD1 gene expression.

Keywords 2-DE · Superoxide dismutase · CKD · Hemodialysis · In-cell Western assay · Protein species

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Introduction

Chronic kidney disease (CKD) is associated with a markedly increased morbidity and mortality including stroke, peripheral vascular disease, sudden death, coronary artery disease, congestive heart failure, and infection (Nolan 2005; Tepel et al. 2003). Physiologic and pathophysiologic functions within the cells are mediated by a multitude of proteins. The amount of a certain protein species within a cell depends on its de novo synthesis or import, chemical modifications like protein splicing, exchange between compartments and removal like proteolytic degradation or export (Jungblut et al. 2008). Recent improvements in methods for proteome analysis offer the possibility of identifying disease-associated protein markers to assist in diagnosis or prognosis, and for selecting potential targets for specific therapy (Hoehenwarter et al., 2006a, b). Proteomics technology offers promise to substantially improve

our understanding and treatment of the molecular basis of CKD (Wittmann-Liebold et al. 2006; Knepper 2002; Bumann et al. 2001). Because protein abundance is regulated by both transcriptional and non-transcriptional mechanisms, studies profiling the amount of a protein of interest and its corresponding gene expression have generally demonstrated a limited correlation. Therefore, one gets a limited view of cellular regulation if one looks only at mRNA levels (Wittmann-Liebold et al. 2006; Knepper 2002). Currently, little is known about the relation between the changes of protein amount and gene expression occurring in CKD.

In the present study, we analyzed proteomic profiles in monocytes of patients with CKD and control subjects by two-dimensional electrophoresis (2-DE) and MS/MS mass-spectrometry. Monocytes accumulate in the atherosclerotic plaque and are associated with increased atherogenesis which can frequently be observed in patients with CKD (Watanabe et al. 2006; Quehenberger 2005; Merino et al. 2008). We observed differences in superoxide dismutase type 1 (SOD1) protein amount and quantified them using quantitative in-cell Western assay. SOD1 gene expression was quantified using real-time polymerase chain reaction (PCR).

Materials and methods

Clinical sample

343 adults were enrolled including chronic hemodialysis patients (HD), patients with CKD, and control subjects without kidney disease. HD and CKD patients show impaired kidney function of varying severity. HD patients are patients with life-threatening end stage renal failure with the need of renal replacement therapy. Patients with CKD show impaired renal function with reduced glomerular filtration rate (GFR) and/or pathologic findings in the blood and urine, ultrasound and/or kidney biopsy. Written-informed consent was obtained from each patient, and ethical approval was given by the local ethics committee. 98 patients on chronic HD treatment more than 3 months, 211 patients with CKD, and 34 control subjects without kidney disease were investigated. Exclusion criteria were age less than 18 years or pregnancy. HD patients were routinely dialyzed for 4–5 h three times weekly using biocompatible membranes with no dialyzer reuse. Dialyses were performed using standardized techniques with bicarbonate-based dialysates and controlled ultrafiltration rate. Blood flow rates were 250–300 ml/min, dialysate flow rates were 500 ml/min, and dialysate conductivity was 135 ms. In HD patients, mean ± SEM *Kt*/*V* value, i.e., the amount of plasma cleared of urea divided by the urea distribution volume, was 1.2 ± 0.0. Blood pressure was measured predialysis with a sphygmomanometer after 10 min of recumbency. Phases I and V of the Korotkoff sounds were taken as the systolic and diastolic blood pressure, respectively. Pulse pressure was calculated as the difference between systolic and diastolic blood pressures. Blood samples were taken predialysis. CKD was defined as kidney damage, confirmed by kidney biopsy or markers of damage, or GFR less than 90 ml/min/1.73 m^2 for more than 3 months.

Sample preparation

Human monocytes were obtained from heparinized blood using superparamagnetic polystyrene beads coated with a primary monoclonal antibody specific for the cluster of differentiation 14 (CD14) membrane antigen expressed on human monocytes (Invitrogen, Germany) and washed several times in Hanks balanced salt solution containing (in mmol/l): NaCl 136, KCl 5.40, KH_2PO_4 0.44, Na_2HPO_4 0.34, D-glucose 5.6, $CaCl_2$ 1, *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (HEPES) 10, pH 7.4.

Two-dimensional electrophoresis (2-DE)

Samples analyzed by 2-DE and immunoblottings were obtained from typical patients/subjects for each group which presented consecutively in our nephrology department.

Cells were homogenized in ice-cold lysis buffer [containing 25 mmol/l Tris–HCl, pH 8; 25 mmol/l NaCl, 25 mmol/l NaF, 2 mmol/l ethylenediaminetetraacetic acid, 10 mmol/l β-mercaptoethanol, complete mini protease inhibitor cocktail (Roche Diagnostics, Germany)], homogenized in a Dounce homogenizer on ice, followed by centrifugation at 2500*g* for 5 min at 4°C. Total protein concentration was determined by the Bradford method using bovine serum albumin as a standard.

Preparation buffer was added to a final concentration of 9 mol/l urea, 70 mmol/l dithiothreitol, and 2% ampholytes. After incubation for 30 min and centrifugation for 45 min at 15000*g*, the supernatant was recovered for analysis.

Two-dimensional gel electrophoresis was performed according to standardized technique using 23 cm × 30 cm × 0.1 cm or 16 cm × 15 cm × 0.1 cm gels combining carrier ampholyte isoelectric focusing (IEF) and sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Klose and Kobalz (1995), Scheler et al. (1998), and Diedrich et al. (2007). 65 µg (2-DE gels used for spot analysis of silver-stained spots and for mass-spectrometry), 100 µg (2-DE gels used for statistical analysis of Western blots), or 180 µg (2-DE gel used for Ponceau staining) of total protein was applied to

vertical rod gels [9 mol/l urea, 4% acrylamide, 0.3% piperazine diacrylamide (PDA), 5% glycerol, 0.06% tetramethylethylenediamine (TEMED) and 2% carrier ampholytes (pH 3–11 or 3.5–9.5), 0.02% ammoniumperoxodisulfat (APS)] for isoelectric focusing at 8820 Vh in the first dimension. Protein loading was highly standardized (Diedrich et al. 2007). A variance of protein loading could limit the message of the results. After focusing, the IEF gels were incubated in equilibration buffer, containing 125 mmol/l trisphosphate (pH 6.8), 40% glycerol, 65 mmol/l dithiothreitol, and 3% sodium-dodecyl-sulfate (SDS) for 10 min and subsequently frozen at −80°C.

The second dimension sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared containing 375 mmol/l Tris–HCl buffer (pH 8.8), 15% acrylamide, 0.2% bisacrylamide, 0.1% SDS and 0.03% TEMED, 0.02% APS. After thawing, the equilibrated IEF gels were immediately applied to SDS-PAGE gels. Electrophoresis was performed using a two-step increase of current, starting with 15 min at 65 mA, followed by a run of 6 h at 140 mA, until the front reached the end of the gel. After 2-DE separation, the gels were stained with MS compatible silver.

Image analysis and protein identification by immunoblotting and mass-spectrometry

Protein patterns in the gel were recorded as digitalized images using a high-resolution scanner (GS-710 Calibrated Imaging Densitometer, BioRad, München, Germany). The scanned and high-resolution pseudo-colored gel images were manually analyzed using Odyssey imaging system software (Licor biosciences; Bad Homburg, Germany).

The silver staining intensity of the spots in the selected area was normalized individually, i.e., the staining intensity of individual protein spots was normalized to the total staining intensity of all protein spots in the region of interest. All selected protein spots had clearly defined spot borders and little to no streaking or smearing.

Analysis of protein spots excised from 2-DE gels was performed by in-gel tryptic digestion technique according to Proteome Factory's standardized procedures (www.proteomefactory.com, Berlin, Germany). The desired gel pieces from 2-DE gels were carefully excised and digested with 0.4% w/v trypsin (sequencing grade modified trypsin, Promega, Madison, USA). The peptides were applied to an Agilent 1100 nanoLC system with a trap column online-coupled to an ion-trap mass-spectrometer Esquire3000plus (Bruker-Daltonic, Bremen, Germany). The obtained MS/MS fragment data were searched against NCBInr protein database NCBInr 20081017 (7124886 sequences; 2457960432 residues) using Mascot Search Engine, a search engine that uses mass-spectrometry data to identify proteins from primary sequence databases (www.matrixscience.com). Mascot version 2.1 was used. The following search parameters were applied: type of search: peptide mass fingerprint; enzyme: trypsin; variable modifications: oxidation (M); propionamide (C); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance 0.1%; peptide charge state: 1+, +2; +3; max missed cleavages: 1. We did not use error tolerant search. MS/MS analysis was used to identify protein spot #2 as superoxide dismutase. No MS/MS analysis was performed for the different kidney disease entities. MS/MS analysis performed in different kidney disease stages might be able to find protein modifications including acetylations. The natural SOD1 variants are mostly related to neurological disorders, mainly amyotrophic lateral sclerosis. We did not include patients with clinical apparent ALS, Parkinson's disease or Alzheimer's disease in our study. Protein phenotypes were further examined using iHOP (www.ihop-net.org) and UniProtKB (www.uniprot.org).

To verify the identity of SOD1 in the 2-DE gels by immunoblotting, proteins were transferred to Hybond-ECL nitrocellulose or polyvinylidendifluoride (PVDF) membranes (NEN Life Science Products, Boston or Millipore, Bedford, USA). Membranes were blocked with Odyssey blocking buffer (Licor biosciences) for 24 h at 4°C and incubated with sheep anti-human SOD1 primary antibodies followed by incubation with Alexa Fluor680-allophycocyanin-fluorescence-labeled donkey anti-sheep (MoBiTec, Göttingen, Germany). Imaging was performed using the Odyssey infrared imaging system (Licor biosciences) at 700-nm emission with an excitation wavelength of 680 nm.

To depict the position of immunostained spots, we performed protein blot staining. We used the fluorescent protein stain SYPRO Ruby (BioRad) which exhibits a relatively broad range of excitation and emission wavelengths and stains proteins irreversibly. Imaging was performed using a Typhoon 9400 laser scanner (GE Healthcare, Munich, Germany) at 532-nm excitation and 610-nm emission. We could not completely prevent SYPRO Ruby appearance during measurement of the fluorescent immunostainings and therefore also counterstained blot membranes with Ponceau S (Sigma-Aldrich, Taufkirchen, Germany) and colloidal gold (www.proteomefactory.com) according to standard protocols (Sasse and Gallagher 2008; Berggren et al. 1999). To allow better localization of the proteins in the 2-DE pattern, we compared three different staining techniques. Protein location was manually visualized on the silver-stained gels.

For the quantification of immunoreactive spots, intensities were determined after adjustment of background intensity. SOD1 protein amount was determined in the different patient groups using the abovementioned antibodies, as control we quantified β-actin protein amount

using rabbit anti-human β-actin (Abcam, Cambridge, UK) primary antibodies followed by incubation with IRDye800CW-infrared fluorescent dye-conjugated goat anti-rabbit (Biomol, Hamburg, Germany) secondary antibodies. No difference in β-actin protein amount could be observed between the groups. β-actin values were 30807 $\pm$ 14788 arbitrary units in control subjects ($n = 3$), 32326 $\pm$ 14978 arbitrary units in patients with CKD ($n = 2$) and 36321 $\pm$ 6931 arbitrary units in HD patients ($n = 2$) ($P = 0.96$ by ANOVA).

Immunoblotting

Proteins were separated by 10% sodium-dodecyl-sulfate polyacrylamide gelelectrophoresis (SDS-PAGE) at 150 V for 90 min, and transferred to Hybond-ECL nitrocellulose membranes. Membranes were blocked with Odyssey blocking buffer for 24 h at 4°C and incubated with sheep anti-human SOD1 or rabbit anti-human β-actin (Abcam, Cambridge, UK) primary antibodies followed by incubation with Alexa Fluor680-allophycocyanin-fluorescence-labeled donkey anti-sheep (MoBiTec) or IRDye800CW-infrared fluorescent dye-conjugated goat anti-rabbit (Biomol, Hamburg, Germany) secondary antibodies. Imaging was performed using the Odyssey infrared imaging system (Licor biosciences) at 700- or 800-nm emission with an excitation wavelength of 680 or 780 nm, respectively. These experiments displayed the expected molecular weights of the proteins and confirmed that the antibodies can be used to identify SOD1 and β-actin in human monocytes.

Quantitative in-cell Western assay

For the quantification of SOD1 protein amount, in-cell Western assays of monocytes were performed as recently described by our group (Liu et al. 2006; Krueger et al. 2010). Cells were fixed, permeabilized with Triton X100, blocked, incubated with sheep anti-human SOD1 primary antibodies, then again incubated with Alexa Fluor680-allophycocyanin-fluorescence-labeled donkey anti-sheep secondary antibodies. Imaging was performed at 700-nm emission with an excitation wavelength of 680 nm. Rabbit anti-human β-actin antibodies were used in every experiment as internal reference, i.e., the expression of each enzyme on the protein level was normalized to β-actin protein expression of the same cells. After incubation with IRDye800CW-infrared fluorescent dye-conjugated goat anti-rabbit secondary antibodies, imaging was performed at 800-nm emission with an excitation wavelength of 780 nm.

The term protein amount used here throughout the article summarizes all protein species present in the cell at the time of measurement that were detected by SOD1 antibody in accordance with the protein species concept by Jungblut et al. (2008).

RNA isolation and quantitative real-time PCR

RNA isolation and quantitative real-time PCR were performed as recently described by our group (Thilo et al. 2008). Primer design was (order: enzyme/reference sequence database accession number: primer sequence $5' \rightarrow 3'$ F: forward, R: reverse primer; fragment length):

SOD1/NM_000454:

F: CAGTGCAGGTCCTCACTTTA; R: CCTGTCTTTG TACTTTCTTC; 240 bp;

β-actin/NM_001101:

F: GGACTTCGAGCAAGAGATGG; R: AGCACTGT GTTGGCGTACAG; 234 bp.

Quantitative real-time PCR was performed with a LightCycler 2.0 Instrument (Roche Diagnostics). Reaction conditions were the following: denaturation 95°C, primer annealing SOD1: 55°C, β-actin: 60°C, elongation 72°C, number of cycles 40. Normalized ratios of antioxidant enzyme mRNA expression were calculated relative to housekeeping gene β-actin mRNA expression including efficiency correction and calibrator normalization. PCR products were also size fractionated on 1.0% agarose gels, and cDNA was visualized by ethidium bromide staining.

Statistics

Data are reported as mean $\pm$ SEM. Data between groups were compared using one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison post hoc test. Analyses were performed with GraphPad prism software (version 5.0, GraphPad Software, San Diego, CA). All statistical tests were two-sided. A two-sided value of P less than 0.05 was considered statistically significant.

Results

We analyzed proteomic profiles in human monocytes from chronic HD patients, patients with CKD, and control subjects without kidney disease. As shown in Fig. 1a–c, total protein samples from human monocytes were separated on a 2-DE gel (pH range from 3 to 11 and molecular weight range from 10000 to 150000). A standardized rectangle was used to mark identical regions which were compared between a chronic HD patient, a patient with CKD, and a control subject. These regions are enlarged in Fig. 1a–c. We analyzed nine protein spots detected by silver staining.

In the CKD patient, the protein amount obtained for spot #1, 2, 3, 4, 5, 6, 7, 8, and 9 was −9%, +54%, +24%, +11%, +11%, +48%, +58%, +69%, and +54%, respectively.

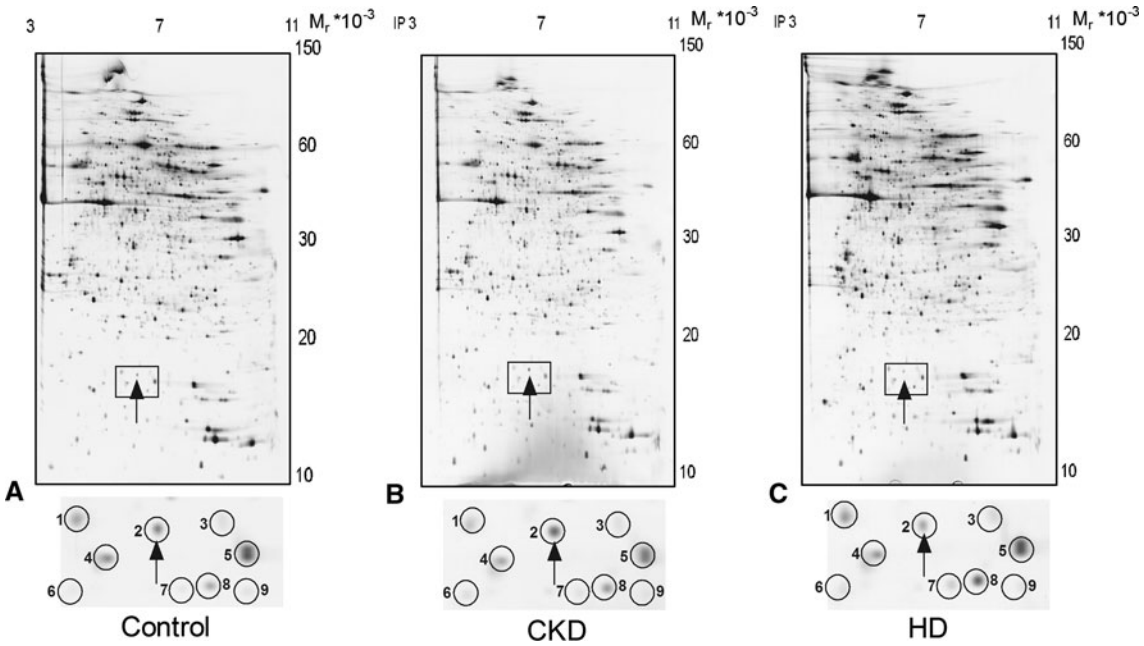

Fig. 1 Two-dimensional gel electrophoresis (2-DE) of proteins from human monocytes from a control subject without kidney disease (**a**, control), a patient with chronic kidney disease (**b**, CKD), and a chronic hemodialysis patient (**c**, HD). Proteins were separated by pH 3–11 in the first dimension and by 7.5–17.5% linear gradient SDS-PAGE in the second dimension. The investigated region and the nine analyzed proteins therein are shown on the original silver-stained 2-DE gels in an enlarged view. The *arrowed spot* depicts superoxide dismutase 1 (SOD1; calculated pI = 5.86; M_r 15868)

In the HD patient, the protein amount obtained for spot #2 was reduced by 27%. The changes obtained for spot #1, 2, 3, 4, 5, 6, 7, 8, and 9 were −7%, −27%, +5%, −10%, −7%, +11%; −4%, +26%, and +46%, respectively. Analysis of faint visible spot #3, 6, 7, and 9 may be limited due to limited resolution.

As shown in Fig. 2, protein spot #2 was identified as superoxide dismutase [Cu-Zn] (SOD1; EC 1.15.1.1; calculated pI value of 5.86; M_r 15868) by mass-spectrometry. The identified protein (Fig. 2a) matching the Mascot database entry accession number P00441 (Mowse score 132; $P < 0.05$; three peptides matched) had a sequence coverage of 23%. Tryptic fragments of SOD1 protein were identified by MS/MS mass-spectrometry. As indicated in Fig. 2b–e, MS/MS fragmentation of GDGPVQGIINFEQK, MS/MS fragmentation of HVGDLGNVTADK, and MS/MS fragmentation of LACGVIGIAQ could be found in the protein sequence of gi|1237406 according to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acids 11–24 (sequence, GDGPVQGIINFEQK) showed a M_r (experimental/calculated) of 1500.7/1500.7; amino acids 81–92 (sequence, HVGDLGNVTADK) showed a M_r (experimental/calculated) of 1224.6/1224.6, and amino acids 145–154 [sequence, LACGVIGIAQ-Propionamide (C)] showed a M_r (experimental/calculated) of 1014.6/1014.5.

The remaining spots in the investigated gel region were identified as follows: spot #1, transgelin 2 (gi|4507357, Mowse score 313; $P < 0.05$; four peptides matched); spot #3, adenylate cyclase-associated protein 1(gi|119627647, Mowse score 159; $P < 0.05$; three peptides matched); spot #4, actin-related protein 2/3 complex subunit 5 (gi|5031593, Mowse score 85, $P < 0.05$; two peptides matched); spot #5, cofilin 1 (gi|5031635, Mowse score 159; $P < 0.05$; four peptides matched) and spot #6, transgelin 2 (gi|4507357, Mowse score 187; $P < 0.05$; three peptides matched). Spots 7, 8, and 9 could not be identified.

As shown in Fig. 3a, we identified SOD1 protein from 2-DE gels by immunoblotting using specific primary antibodies against SOD1 and fluorescence-labeled secondary antibodies. To allow protein location, each gel was silver stained after blotting. Protein staining of the blot membranes was performed with SYPRO Ruby, Ponceau S, or colloidal gold (Fig. 3a). Protein staining of the blot membranes was performed with SYPRO Ruby, Ponceau S, or colloidal gold. Landmark protein spots were marked on the silver-stained gels and the corresponding blot membrane to enable comparative analysis and for determination of the investigated gel region on the blot membranes. Immunostaining using specific SOD1 antibodies and counterstaining for proteins were each performed on the same membrane to illustrate the position of SOD1 immunoreactive spots within the 2-DE pattern.

Immunoblottings of monocytes obtained from chronic HD patients, patients with CKD, and control subjects were

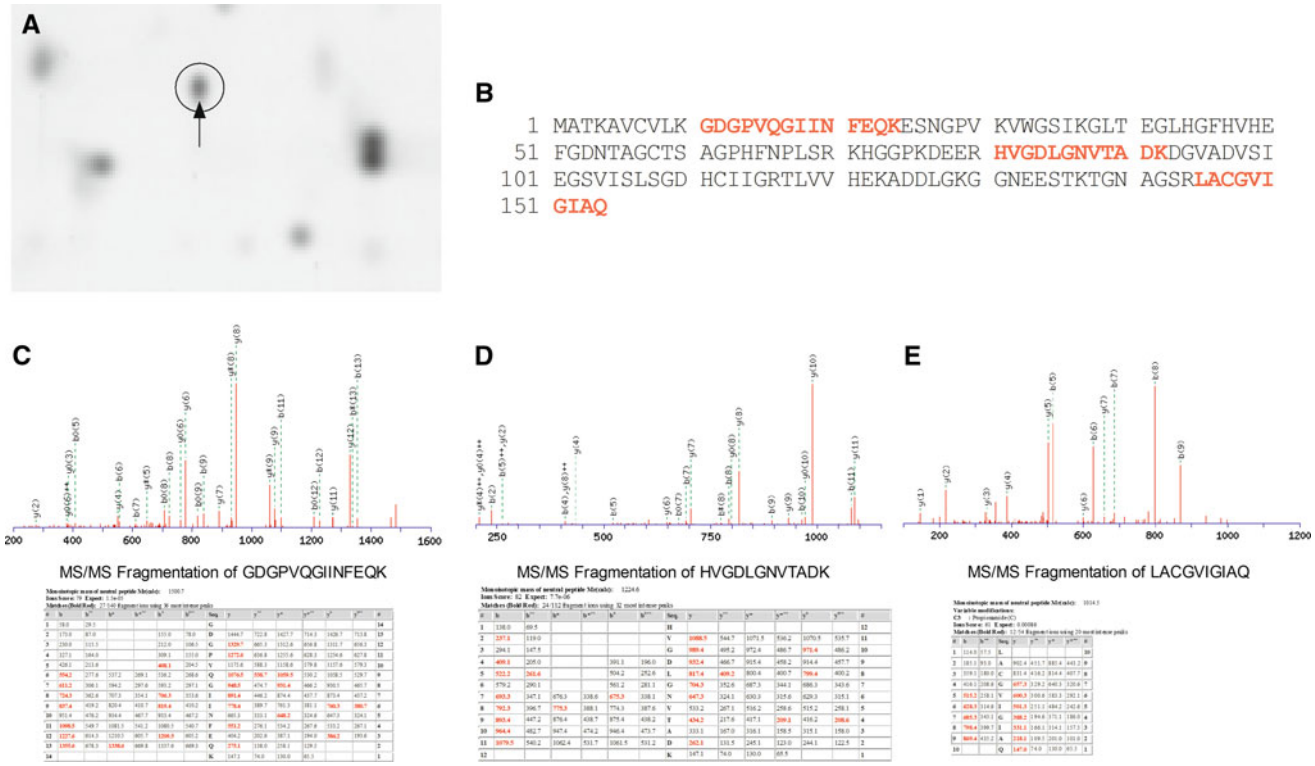

Fig. 2 **a** Enlarged view of the silver-stained two-dimensional electrophoresis gel of proteins from human monocytes showing a protein spot (*arrow*) that was identified as superoxide dismutase [Cu-Zn] (SOD1; EC 1.15.1) by MS/MS mass-spectrometry of tryptic fragments and Mascot search (Mascot accession no. P00441). **b** Protein sequences as observed by mass-spectrometry matching BLAST search of gi|1237406 are shown in *bold red*. **c** MS/MS fragmentation of GDGPVQGIINFEQK; table shows matches in *bold red*: 27 from 140 fragment ions using 36 most intense peaks were observed. **d** MS/MS fragmentation of HVGDLGNVTADK; table shows matches in *bold red*: 24/112 fragment ions using 32 most intense peaks were observed. **e** MS/MS fragmentation of LACGVIGIAQ; table shows matches in *bold red*: 12/54 fragment ions using 20 most intense peaks were observed (color figure online)

analyzed (Fig. 3b). The immunoreactive spot pattern appeared similar between the groups ($n = 16$ individual immunoblottings). Six spots could be distinguished, immunoreactive spot #2 showed the largest signal in all samples (mean, 163-fold over detection limit). There was no significant difference between the groups concerning immunoreactive spot intensities relative to main immunoreactive spot #2 for immunoreactive spot #1 ($P = 0.87$); immunoreactive spot #3 ($P = 0.67$); immunoreactive spot #5 ($P = 0.90$); and immunoreactive spot #6 ($P = 0.60$ by ANOVA). Immunoreactive spot #4, which was relatively faint, showed a significant lower relative immunofluorescence intensity in controls compared to CKD and HD patients ($P < 0.001$ by ANOVA).

Furthermore, we compared values of SOD1 immunofluorescence from immunoreactive spot #2 between the three groups. Control subjects (2373 ± 731 arbitrary units; $n = 3$) showed significantly more SOD1 protein in immunoreactive spot #2 compared to patients with CKD (1307 ± 177 arbitrary units; $n = 6$) or patients on chronic HD (645 ± 231 arbitrary units; $n = 3$; $P < 0.0001$ by ANOVA; Fig. 3c).

Changes of SOD1 protein amount in patients with CKD were quantified using in-cell Western assay and SOD1 gene expression by quantitative real-time PCR in 343 subjects, including 98 patients on chronic HD treatment, 211 patients with CKD, and 34 age-matched control subjects. The according clinical and biochemical characteristics are summarized in Table 1.

Figure 4a shows representative immunoblottings of SOD1 and housekeeping gene β-actin in monocytes with the expected molecular weight for the two proteins. Figure 4b shows a representative in-cell Western assay for quantification of SOD1 and housekeeping gene β-actin in monocytes from a HD patient, a patient with CKD, and a control subject. Summary data of quantitative in-cell Western assays for all patients are given in Fig. 4c.

We compared the SOD1 protein amount from a total of 214 subjects, including 56 HD patients, 148 patients with CKD, and 20 control subjects, using quantitative in-cell Western assay. The SOD1 protein amount was significantly lower in monocytes from HD patients (normalized SOD1 protein amount, 27.2 ± 2.8; $n = 56$) compared to patients with CKD (normalized SOD1 protein amount, 34.3 ± 2.8;

$n = 148$), or control subjects (normalized SOD1 protein amount, 48.0 ± 8.6; $n = 20$; $P < 0.05$ by ANOVA). These data indicated a significant reduction of SOD1 protein amount in chronic HD patients by 43% compared to control subjects. In patients with CKD, the SOD1 protein amount was reduced by 29%.

SOD1 gene expression was investigated by quantitative real-time PCR. Typical melting curves of PCR products of SOD1 or the housekeeping gene β-actin in monocytes from a HD patient, from a patient with CKD, and a control subject are shown in Fig. 5a. The melting curve analysis confirmed the presence of one single peak. Figure 5b shows representative amplification curves of quantitative real-time PCR for SOD1 and housekeeping gene β-actin in monocytes from a HD patient, from a patient with CKD, and a control subject. The shift of the fluorescence curve to lower crossing points indicated increased SOD1 gene expression in HD patients. Summary data of quantitative real-time PCR of SOD1 for all patients are given in Fig. 5c.

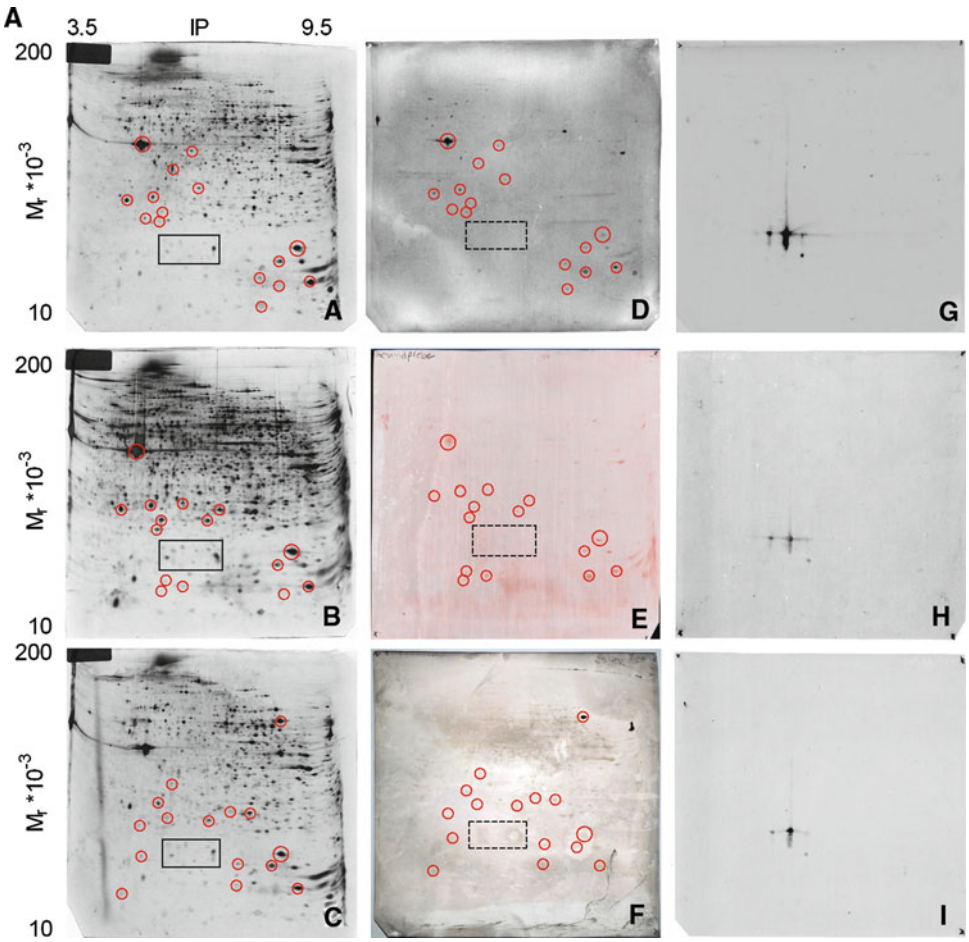

Fig. 3 **a** Two-dimensional electrophoresis gels (**a**–**c**) of monocyte proteins from control subjects. Proteins were separated by pH 3.5–9.5 in the first dimension and by 15% SDS-PAGE in the second dimension. The areas corresponding to the region shown on the gels in Fig. 1 are indicated by the *box*. To allow protein location, each gel was silver stained after blotting. Protein staining of the blot membranes was performed with SYPRO Ruby (**d** before immunostaining), Ponceau S (**e** before immunostaining), or colloidal gold (**f** after immunostaining). Landmark protein spots are marked by *circles* on the silver-stained gel and the corresponding blot membrane to allow comparison. These landmark spots were also used to determine the position of the investigated region on the blot membranes (*dotted box*). Immunostaining using specific SOD1 antibodies (**g**–**i**) and counterstaining for protein (**d**–**f**) were each performed on the same membrane to illustrate the position of immunoreactive spots within the 2-DE pattern. **b** Representative immunoblottings and summary data of the quantification of immunoblottings from monocytes obtained from control subjects (control, *open bars*), patients with chronic kidney disease (CKD, *hatched bars*), and chronic hemodialysis patients (HD, *filled bars*). The localizations of the analyzed immunoreactive spots (spots 1–6) are indicated. Spot intensities were calculated relative to immunoreactive spot #2. **c** Bar graph showing SOD1 immunofluorescence of spot #2 quantified from the spot intensity in arbitrary units from control subjects without kidney disease (control, *open bars*, $n = 3$) from patients with CKD (*hatched bars* $n = 6$) and chronic HD patients (*filled bars* $n = 3$). Total protein loading for each gel was 100 µg. *** $P < 0.0001$ by one-way analysis of variance and Bonferroni's multiple comparison post hoc test

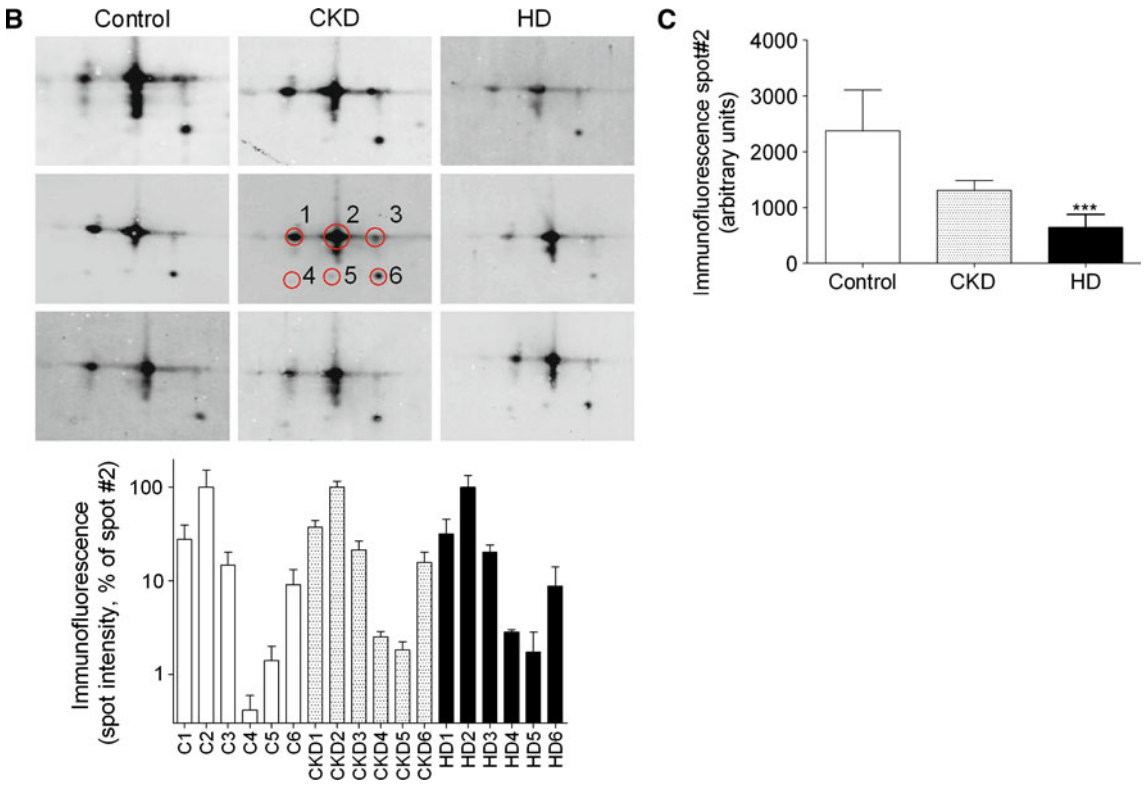

Fig. 3 continued

SOD1 gene expression was significantly higher in monocytes from chronic HD patients (normalized SOD1 gene expression, 17.8 ± 2.3; $n = 60$) compared to patients with CKD (normalized SOD1 gene expression, 9.0 ± 0.7; $n = 133$), or control subjects (normalized SOD1 gene expression, 5.5 ± 1.0; $n = 19$; $P < 0.0001$ by ANOVA).

These data indicated a significant increase of SOD1 gene expression in HD patients by 98% compared to patients with CKD and by 224% compared to control subjects without kidney disease, respectively.

Discussion

The major finding of the present study is that quantification of proteins using quantitative in-cell Western assay showed a reduction of SOD1 protein amount in CKD, most pronounced in HD patients, and the existence of several SOD1 protein species in human monocytes.

Protein species can be differentially regulated in disease. Analyses of changes of protein amount or changes of protein species may help to uncover the underlying pathophysiological changes. The present study indicates reduced SOD1 protein amount in patients with CKD, which can contribute to increased oxidative stress, which is commonly observed in these patients. Increased oxidative stress will cause increased oxidative modifications of proteins or lipids. Evaluation of the causes of the observed protein changes will help to establish novel therapeutic strategies. Our present study ruled out a decrease of gene transcription as reason for decreased SOD1 protein amount. Therefore, further investigations should aim at mechanisms of SOD1 protein degradation in these patients.

We used three different approaches to characterize SOD1 protein in CKD and HD patients and controls. First, spot analysis in silver-stained 2-DE gels, second, analysis of immunostaining intensities in 2-DE gel blots, and third, quantitative in-cell Western assays of SOD1 protein using specific antibodies. Different staining intensities of 2-DE gels and immunoblottings may complicate the task to observe differences between the groups. Therefore, statistical analyses were performed mainly on data from quantitative in-cell Western assay or quantitative real-time PCR.

The SOD1 immunostaining of 2-DE gel blot membranes revealed six distinguishable immunoreactive spots. Immunoreactive spots 1–3 and 6 are the main immunoreactive SOD1 spots with spot #2 being the most pronounced. A similar pattern of SOD1 immunofluorescence resembling immunoreactive spots 1–3 was described by Choi et al. (2005) in human brain. Our immunoreactive spot #6 showed a similar IP as spot #3 but had a lower

Table 1 Clinical and biochemical characteristics of 34 control subjects without kidney disease, 211 patients with chronic kidney disease (CKD), and from 98 patients on chronic hemodialysis treatment (HD)

Parameter	Control	CKD	HD
Age (years)	50 ± 2	65 ± 1	66 ± 1
Men/women	16/18	132/79	64/34
Body mass index (kg/m^2)	24.0 ± 0.4	27.2 ± 0.4	24.6 ± 0.4
Systolic blood pressure (mmHg)	130 ± 1	137 ± 2	131 ± 3
Diastolic blood pressure (mmHg)	73 ± 2	74 ± 1	68 ± 2
Pulse pressure (mmHg)	57 ± 2	63 ± 1	63 ± 2
Heart rate (min^{-1})	63 ± 3	74 ± 1	77 ± 1
Serum creatinine (mg/dl)	0.9 ± 0.0	1.8 ± 0.1	7.0 ± 0.3
Hemoglobin (g/dl)	14.6 ± 0.2	12.5 ± 0.1	10.9 ± 0.2
Total protein (g/dl)	7.4 ± 0.2	7.0 ± 0.1	6.2 ± 0.1

Continuous data are shown as mean ± SEM

molecular weight. The SOD1 species which was described by Choi et al. in brain tissue at IP5 was not detected in human monocytes used in our present study (Choi et al. 2005). It cannot be excluded from the present investigation that there are further protein species of SOD1 which were not detected by the used antibody.

The immunoreactive spot pattern was similar between the patients with CKD, HD patients and controls probably indicating rather an overall reduction of SOD1 protein amount than the reduction of a certain SOD1 protein species. Nevertheless, the faint immunoreactive spot #4 showed a significant lower relative immunofluorescence intensity in controls compared to CKD and HD patients pointing to a differential regulation of different SOD1 protein species. Since immunoreactive spot #4 was inconstantly present in our investigations, a reliable analysis was precluded. Also, the SOD1 pattern detected by immunostaining was not observed in the silver-stained 2-DE gel pattern so that a definite analysis of the SOD1 protein species by mass-spectrometry was impossible. To this purpose, additional approaches including protein fractionation and post-translational modification enrichment techniques will be necessary (Zhao and Jensen 2009).

Given the contribution of SOD1 to the cellular antioxidant defense system, the reduction of SOD1 protein amount is important (Himmelfarb et al. 2002; Vaziri 2004). Oxidative stress, which increases with weakening antioxidative defense, has been linked to accelerated arteriosclerosis and cardiovascular mortality in patients with CKD (Roberts et al. 2006; Locatelli et al. 2003). SOD1 catalyzes the reaction of superoxide radicals to hydrogen peroxide and oxygen. Yilmaz et al. (2005) and Zwolinska et al. (2006) reported reduced SOD1 activity in red blood cells from patients with CKD. Monocyte-derived oxidative

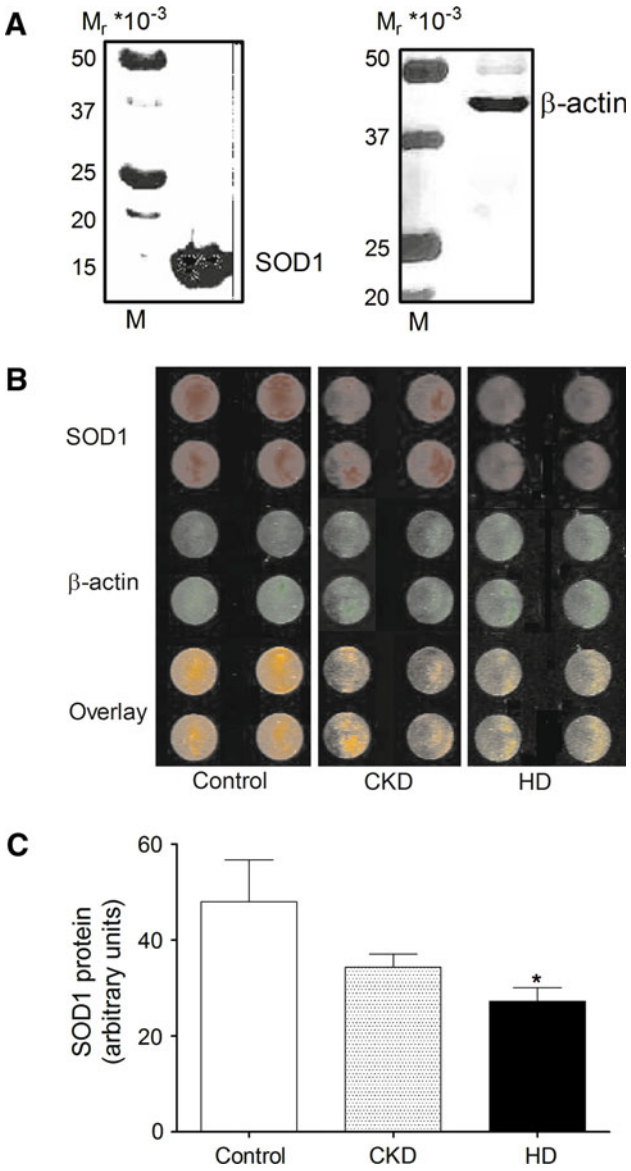

Fig. 4 **a** Representative immunoblottings of SOD1 and housekeeping gene *β*-actin in monocytes from a control subject. The predicted molecular weight of SOD1 is 15868 and molecular weight of *β*-actin is 41737; *M* marker. **b** Representative in-cell Western assay for quantification of SOD1 protein amount in monocytes from a control subject (control), a patient with chronic kidney disease (CKD), and from a hemodialysis patient (HD). *Upper panels* show expression of SOD1 (red fluorescence), *middle panels* of the housekeeping gene *β*-actin (green fluorescence), and *lower panels* show overlay. Fluorescence intensities were analyzed in quadruplicate for each sample. **c** Summary data showing SOD1 protein expression using quantitative in-cell Western assay normalized to *β*-actin expression in monocytes from control subjects ($n = 20$), patients with CKD ($n = 148$), and from HD patients ($n = 56$). *$P < 0.05$ by ANOVA

stress due to impaired antioxidant defense contributes to the progression of atherosclerotic lesions in the arterial vessel wall. According to recent literature, the enzymatic regulation of oxidative stress by SOD1 plays an important

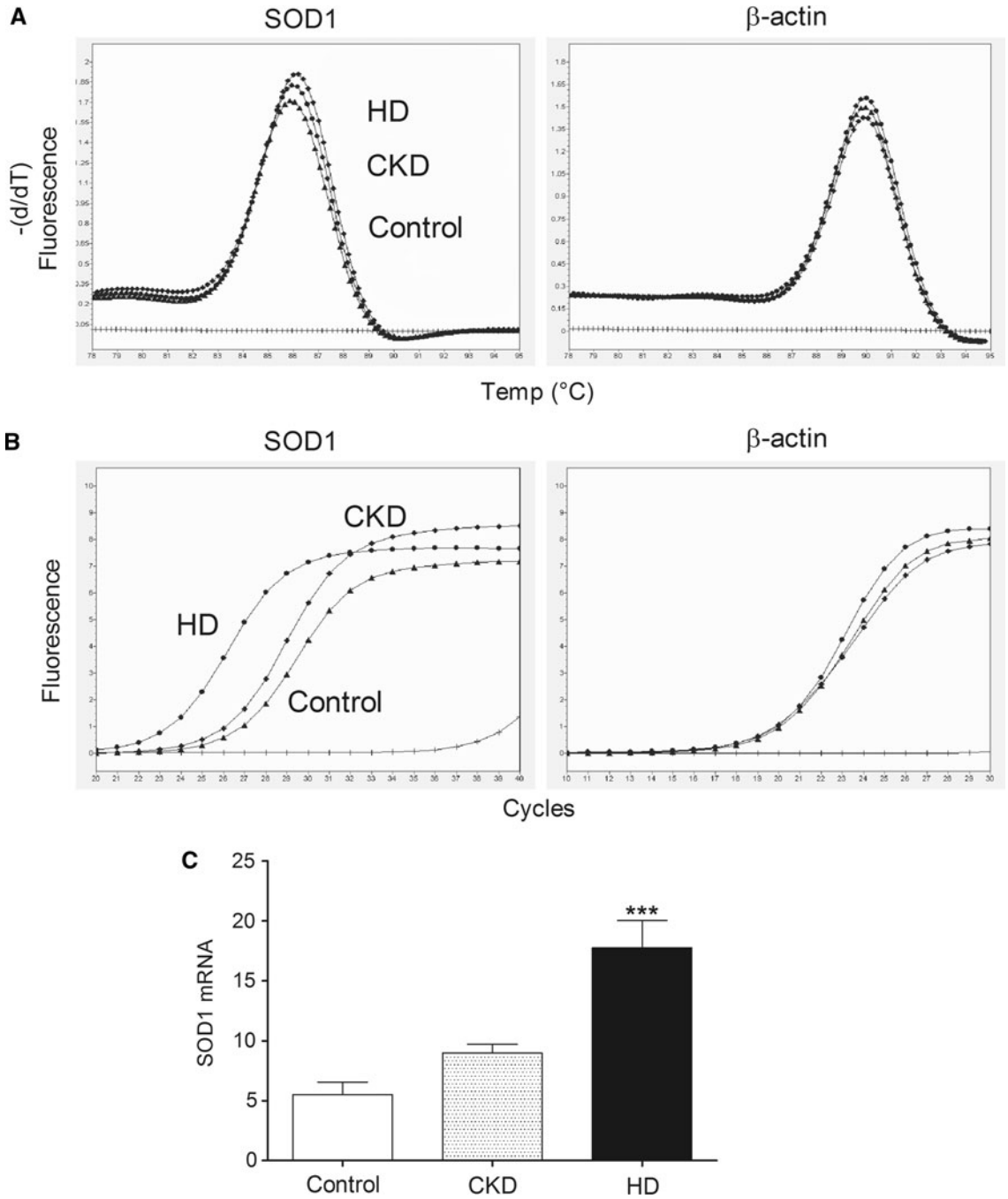

Fig. 5 **a** Melting analysis after amplification of SOD1 or the housekeeping gene β-actin. The melting curve analysis confirmed the presence of one single peak in control subjects (control; *triangles*), patients with chronic kidney disease (CKD, *squares*), and in hemodialysis patients (HD, *circles*). Curves with *x-symbols* indicate the absence of RNA (-RNA). **b** Representative amplification curves of the quantitative real-time PCR for SOD1 or for the housekeeping gene β-actin in control subjects (control; *triangles*), patients with CKD (*squares*), and in HD patients (*circles*). Curves with *x-symbols* indicate the absence of RNA (-RNA). The shift of the fluorescence curve to the left indicates increased SOD1 gene expression in HD. **c** Summary data showing SOD1 gene expression analyzed by quantitative real-time PCR normalized to β-actin in monocytes from control subjects ($n = 19$), patients with CKD ($n = 133$), and from HD patients ($n = 60$). ***$P < 0.0001$ by ANOVA

role for oxidative stress related vascular damage. Deficiency of SOD1 in heterozygous CuZnSOD(+/−) mice caused increased superoxide levels in aorta and marked impairment of endothelial function with aging (Didion et al. 2006). The lifespan of SOD1-deficient CuZnSOD(−/−) mice was shortened (Elchuri et al. 2005).

The mechanisms leading to a reduction of SOD1 protein amount and increased SOD1 gene expression in CKD

patients as shown in our study are not entirely clear. Protein species occurrence is regulated by a multiplicity of ways, including transcriptional regulation, regulation of mRNA stability, translational regulation, regulation of protein degradation, post-translational proteolytic processing, and finally regulated trafficking (Jungblut et al. 2008; Knepper 2002). Transcriptional activity of SOD1 gene is regulated by antioxidant response elements, i.e., an enhancer sequence that mediates transcriptional activation of genes in cells exposed to oxidative stress (Park and Rho 2002; Nguyen et al. 2003). Since the increased oxidative stress in HD and CKD patients is well appreciated, increased SOD1 gene expression in CKD patients as shown in the present study can be explained (Himmelfarb et al. 2002).

Increased SOD1 gene expression and reduced SOD1 protein amount in CKD suggest either diminished protein production or enhanced protein degradation. The latter is of interest since the combination of increased gene expression and reduced protein amount as shown in our present study has been shown for post-transcriptional regulation of different proteins by several authors (Monteleone et al. 2005; Tan et al. 2006; Santibanez et al. 2007). For example, that mechanism has already been observed for downregulation of SnoN expression in obstructive nephropathy (Tan et al. 2006). An increased SOD1 gene expression as observed in the present study may indicate increased protein degradation in patients with CKD and compensatory increase of SOD1 gene expression.

Two conditions in CKD can contribute to enhanced protein degradation: uremia and oxidative stress. Oxidative damage of SOD1 protein has been described in Alzheimer's and Parkinson's diseases (Choi et al. 2005). Concerning uremia, it was shown in several studies that glycation of SOD1 protein results in aggregation and decrease of protein amount (Jabeen et al. 2006; Jabeen et al. 2007). Increased protein glycation is a well-recognized condition in uremia. For example, glyoxal and methylglyoxal, which both can modify SOD1 protein, have been recognized as uremic toxins (Jabeen et al. 2007; Vanholder et al. 2008).

In summary, assessing SOD1 protein in human monocytes including 2-DE, mass-spectrometry, immunoblotting, and quantitative in-cell Western assay, we observed a reduction of SOD1 protein amount in CKD, especially in HD patients, although this patient group shows a significantly upregulated SOD1 gene expression.

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Conflict of interest All authors declare that there is no conflict of interest.

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