Kidney International, Vol. 60 (2001), pp. 358–363

Albumin is the major plasma protein target of oxidant stress in uremia

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Albumin is the major plasma protein target of oxidant stress in uremia.

Background. Patients with uremia are exposed to increased oxidative stress. Examination of the oxidation of individual plasma proteins may be useful in establishing specific pathways of oxidative stress in vivo and in determining functional consequences of oxidant stress exposure. We therefore examined oxidative modification of plasma proteins by carbonyl formation using Western blot immunoassay and enzyme-linked immunosorbent assay (ELISA) techniques in patients with chronic renal failure (CRF) and on chronic hemodialysis therapy (HD).

Methods. Plasma was obtained from 25 HD, 20 CRF, and 20 healthy volunteers, derivatized with 2,4 dinitrophenylhydrazine (DNP) and electrophoresed on duplicate 4 to 12% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, transferred to nitrocellulose, and stained for DNP for carbonyls and amido black for protein content. Data are recorded as DNP area/protein area and are reported in densitometry units. Total plasma carbonyls were determined by ELISA.

Results. Plasma albumin is substantially more oxidized in HD than in healthy volunteers (1.22 ± 0.14 densitometry units vs. 0.60 ± 0.08, $P = 0.002$). There were no significant differences in oxidation of plasma transferrin, immunoglobulin, and fibrinogen in HD versus healthy volunteers. In CRF patients, plasma albumin is more oxidized compared with normal volunteers (1.36 ± 0.20 densitometry units vs. $0.94 + 0.08$, $P = 0.09$). There were no differences in oxidation of plasma transferrin, fibrinogen, and immunoglobulin in CRF patients versus healthy volunteers. An increased plasma protein carbonyl concentration in CRF patients compared with healthy volunteers was confirmed by ELISA (0.31 ± 0.07 vs. 0.04 ± 0.01 nmol/mg protein ($P = 0.001$).

Conclusion. Albumin is the major plasma protein target of oxidant stress in CRF and HD patients.

Proteins are among the main targets of oxidation in the plasma [1, 2]. We have previously demonstrated that oxidative stress in patients on chronic hemodialysis (HD) is manifested by an increase in plasma protein oxidation, including thiol group oxidation, and protein carbonyl formation [3]. We have also demonstrated that plasma proteins from patients on chronic dialysis contain 3-chlorotyrosine, a biomarker for myeloperoxidase catalyzed oxidative reactions [4]. Miyata et al have used the term "carbonyl stress" to describe the excess oxidative formation of carbonyl groups (aldehydes and ketones) detectable in proteins, lipids, and carbohydrates in patients with chronic renal failure (CRF) [5]. Carbonyl formation may be important in the development of β_2-microglobulin amyloidosis and accelerated atherosclerosis in patients with renal failure [5–7].

To date, investigations of plasma protein oxidation in patients with CRF have utilized methods that measure cumulative oxidation and do not assess oxidation of individual plasma proteins. Examination of individual plasma proteins may be useful in establishing specific pathways of oxidative stress in vivo and in determining the potential functional consequences of oxidant stress exposure [1]. Recently, Shacter et al developed a Western blot immunoassay that takes advantage of the ability to derivatize carbonyl groups with 2,4 dinitrophenylhydrazine (DNP) [8, 9]. Derivatized proteins can then be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoassay with anti-DNP antibodies, thus providing a highly sensitive technique for detecting oxidation of individual proteins. Using this immunoassay technique, we have demonstrated that oxidation of albumin accounts for almost all of the excess plasma protein oxidation in patients with uremia.

METHODS

Patient characteristics

Twenty-five patients were on chronic maintenance HD. Twenty patients with CRF did not receive renal replacement therapy, and 20 healthy volunteers were studied. Blood was drawn into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) supplemented with 1000 U/mL catalase and centrifuged at $1700 \times g$ for

Key words: carbonyl stress, plasma protein oxidation, chronic renal failure, hemodialysis, dialysis, Western blot, ELISA.

Received for publication July 31, 2000
and in revised form February 7, 2001
Accepted for publication February 19, 2001

Table 1. Characteristics of the patient groups

	Normal	CRF	HD
Age	62 ± 4	62 ± 4	72.6 ± 2.0
Sex *M/F*	17/3	17/3	13/12
Diabetes	8/20	8/20	10/25
Albumin *g/dL*		3.8 ± 0.1	3.4 ± 0.1
Total protein *g/dL*		6.7 ± 0.2	6.0 ± 0.1
Cholesterol *mg/dL*		191 ± 11	151 ± 5.5
Creatinine *mg/dL*		4.3 ± 0.3	7.8 ± 0.5
BUN *mg/dL*		63 ± 4	59 ± 3.8
URR			72 ± 3

Abbreviations are: BUN, blood urea nitrogen; CRF, chronic renal failure; HD, hemodialysis; URR, urea reduction ratio.

15 minutes. For CRF patients, the mean serum creatinine was 4.3 ± 0.3 mg/dL, and creatinine clearance was 23.5 ± 2.1 mL/min. All HD patients were routinely receiving treatment with high-flux polysulfone membranes and a bicarbonate-based dialysate with heparinization. For HD patients, blood was drawn predialysis. Informed consent was obtained from all subjects, and the protocol was approved by the Institutional Review Board. Table 1 displays the characteristics of the patient groups.

Western blotting technique

Oxidation of individual plasma proteins was measured by analysis of Western blots according to the method of Shacter et al [8]. Total protein was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Plasma was diluted to 2 mg/mL with phosphate-buffered saline (PBS) and derivatized with DNP using the OxyBlot Kit (Oncor, Gaithersburg, MD, USA). Samples were diluted to 1 mg/mL by the addition of an equal volume of 2 × nonreducing sample buffer, and 15 μL samples were electrophoresed according to the method of Laemmli on duplicate 4 to 12% gradient SDS-PAGE gels (Novex, San Diego, CA, USA) for 90 minutes at 125 V. Following overnight electroblotting to nitrocellulose at 30 V, one blot was stained for DNP using OxyBlot Kit reagents. Bands were visualized with chemiluminescent chemicals and captured on film at four exposure times (15 sec, 1 min, 5 min, and 10 min). The second blot was stained with amido black for protein. Blots were scanned on an StudioStar scanner (Agfa Corporation, Ridgefield Park, NJ, USA) and analyzed for band area using NIH Image software.

Analysis of blots

Each Western blot included both patient and healthy volunteer samples so that in all experiments, patient samples were compared with healthy volunteers developed under the same conditions. For each experiment, carbonyl density was determined from the blot with the shortest possible exposure time that produced clearly visible bands. DNP and protein blots were scanned using the same size section of the blot in each scan. The analysis box included 14 lanes for each analysis. The uniform window size and analysis box ensured that data were being analyzed consistently from band to band and from blot to blot. Because the concentration of various plasma proteins differs between patient groups and healthy volunteers, densitometry data for the area of the DNP blot band were divided by the densitometry data for the area of the protein blot band obtained under identical gel loading and electrotransfer conditions. These data are recorded as DNP area/protein area and are reported in densitometry units. The mean was calculated for each subject group on each blot.

Total plasma protein carbonyl measurement

Plasma protein carbonyl concentration was determined by enzyme-linked immunosorbent assay (ELISA) using the Zentech PC Test Kit (Zenith Technology, Dunedin, New Zealand).

Statistics

Data were analyzed using analysis of variance (ANOVA) and the Student *t* test comparing the means of patients versus controls on each blot. Results are reported as mean ± SEM.

RESULTS

Figure 1 shows representative Western blots using anti-DNP antibodies to measure carbonyl formation and amido black for analysis of protein concentration, respectively. It can readily be appreciated that there is considerably more carbonyl detected in chronic hemodialysis patients (lanes 1 through 4, 6, and 7) as compared with healthy volunteers (lanes 5 and 8) in Figure 1A.

Figure 2 summarizes the extent of plasma protein carbonyl formation in chronic hemodialysis patients compared with healthy volunteers for the major plasma proteins. In chronic hemodialysis patients, plasma albumin is substantially more oxidized than albumin in healthy volunteers (1.22 ± 0.14 densitometry units vs. 0.60 ± 0.08, $P = 0.002$). In contrast, there are no significant differences in the extent of oxidation of plasma transferrin (1.43 ± 0.34 vs. 1.21 ± 0.18 densitometry units, $P = 0.59$), immunoglobulin (1.48 ± 0.08 vs. 1.29 ± 0.11 densitometry units, $P = 0.19$), and fibrinogen (1.46 ± 0.34 vs. 1.6 ± 0.59 densitometry units, $P = 0.84$) in chronic hemodialysis patients versus healthy volunteers, although there was a trend toward increased immunoglobulin oxidation versus healthy volunteers. These results demonstrate that oxidation of albumin accounts for virtually all of the increased plasma protein carbonyl formation found in chronic hemodialysis patients.

Figure 3 analyzes the extent of plasma protein carbonyl formation in patients with CRF compared with

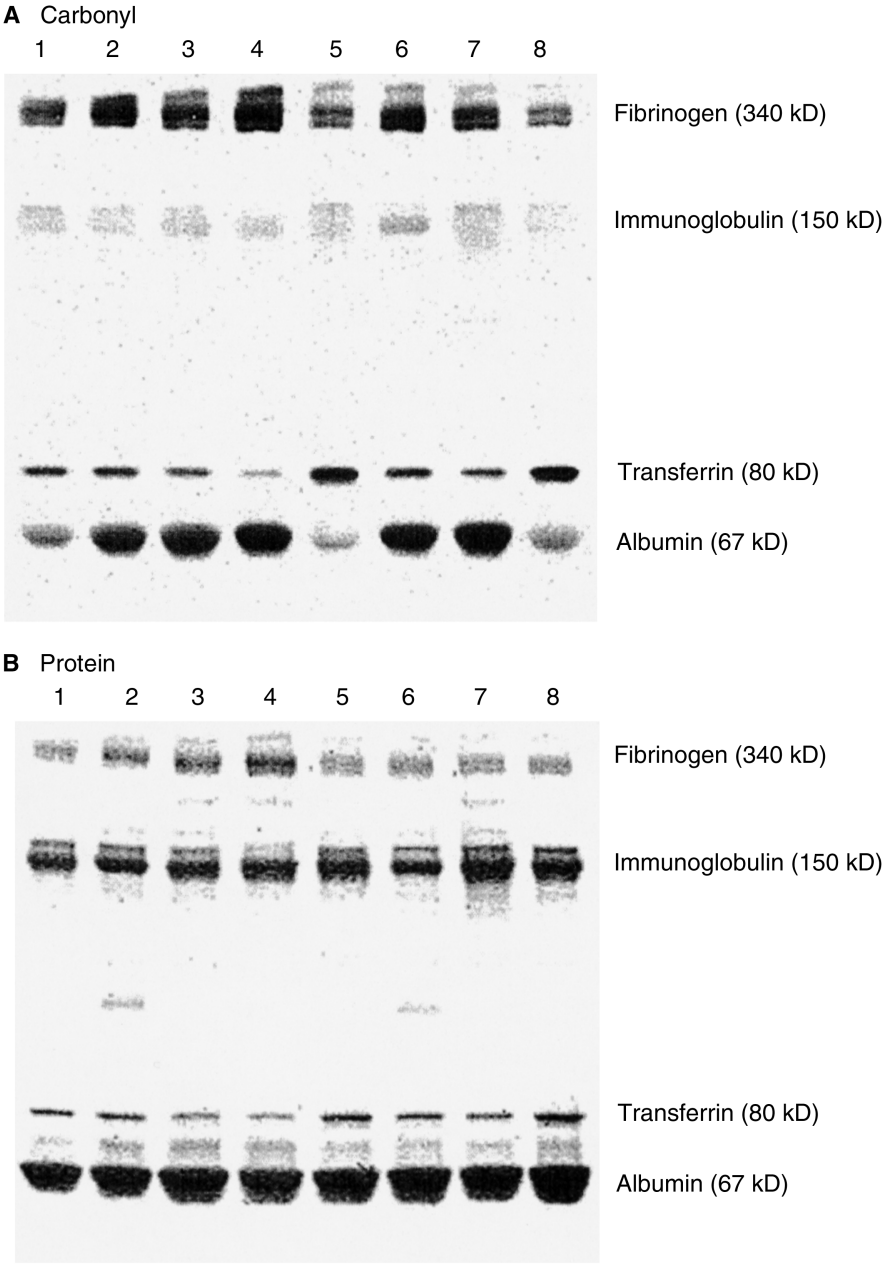

Fig. 1. Western blots of plasma from healthy volunteers (lanes 5 and 8) and chronic hemodialysis patients (lanes 1 through 4, 6, 7) after derivatization and staining with Oxyblot Kit reagents using a 15-second film exposure (*A*) or with amido black (*B*).

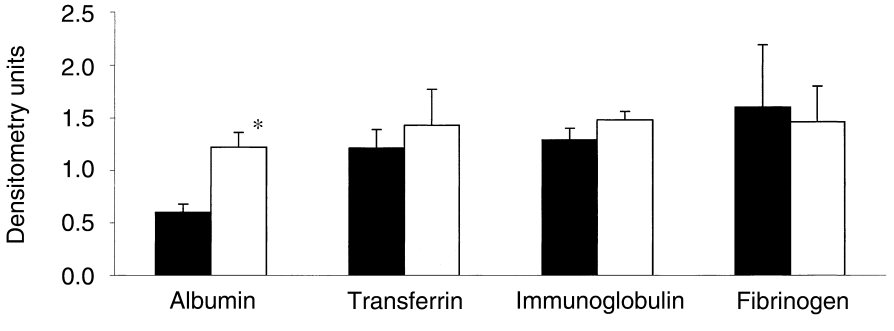

Fig. 2. Plasma protein carbonyl formation measured by Western blot chronic hemodialysis patients (□; $N = 25$) versus healthy volunteers (■; $N = 6$). $^*P < 0.05$ vs. healthy volunteers.

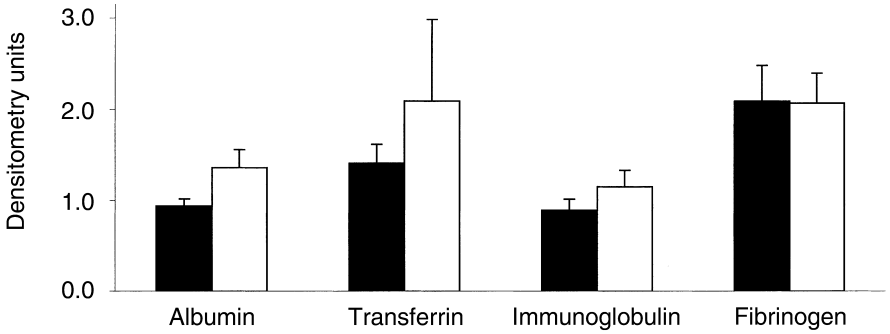

Fig. 3. Plasma protein carbonyl formation measured by Western blot in CRF patients (□; N = 20) versus healthy volunteers (■; N = 20). *P = 0.09 vs. healthy volunteers.

Table 2. Comparison of two subject groups with versus without diabetes

	Healthy		CRF	
	No diabetes	Diabetes	No diabetes	Diabetes
Albumin	1.04 ± 0.22	1.20 ± 0.15	1.66 ± 0.22	1.75 ± 0.33
Transferrin	1.89 ± 0.48	1.50 ± 0.22	3.44 ± 1.11	4.04 ± 1.45
Immunoglobulin	0.86 ± 0.26	0.85 ± 0.12	1.29 ± 0.18	1.29 ± 0.29
Fibrinogen	2.11 ± 0.59	1.18 ± 0.10	2.23 ± 0.30	2.82 ± 0.85

Values are from 2 blots with matched sets of subjects, N = 6 in each group. Values are in densitometry units and expressed as mean ± SEM.

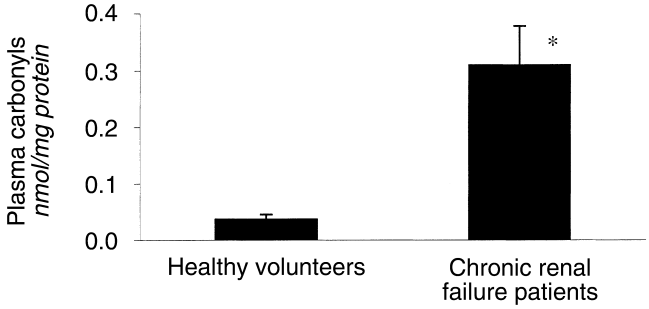

Fig. 4. Plasma protein carbonyl formation measured by ELISA techniques in age-, sex-, and diabetes-matched healthy volunteers (N = 20 for healthy volunteers and N = 20 for CRF patients, *P < 0.001 vs. healthy volunteers).

healthy volunteers. Similar to chronic hemodialysis patients, in patients with CRF, plasma albumin is more oxidized compared with healthy volunteers (1.36 ± 0.20 densitometry units vs. 0.94 ± 0.08), although of borderline significance (P = 0.09). In patients with CRF, as in patients on hemodialysis, the extent of plasma transferrin (2.09 ± 0.90 vs. 1.41 ± 0.21 densitometry units, P = 0.48), immunoglobulin oxidation (1.15 ± 0.18 vs. 0.89 ± 0.12 densitometry units, P = 0.26), and fibrinogen (2.07 ± 0.33 vs. 2.09 ± 0.39 densitometry units, P = 0.97) was not significantly different from healthy volunteers.

As many patients with CRF have diabetes mellitus and because diabetes mellitus has been associated with oxidative stress independent of the development of renal failure, we compared plasma protein carbonyl formation in our study group patients with CRF and healthy volunteers with and without diabetes mellitus (Table 2). For healthy volunteers with diabetes mellitus (N = 6), there was no significant difference in oxidation of albumin (1.20 ± 0.15 densitometry units vs. 1.04 ± 0.22, P = 0.55), transferrin (1.49 ± 0.22 densitometry units vs. 1.89 ± 0.48, P = 0.48), immunoglobulin (0.85 ± 0.12 densitometry units vs. 0.86 ± 0.26, P = 0.98), or fibrinogen (1.18 ± 0.10 densitometry units vs. 2.11 ± 0.59, P = 0.18) compared with healthy volunteers without diabetes mellitus (N = 6). In patients with CRF, there were also no significant differences in oxidation of albumin, transferrin, immunoglobulin, or fibrinogen between patients with (N = 6) and without diabetes mellitus (N = 6; Table 2).

Since oxidative stress increases in patients with CRF prior to the initiation of renal replacement therapy, we wished to examine whether there was a correlation between the level of creatinine clearance and plasma protein carbonyl formation in this patient population. Western blotting analysis allows only an accurate comparison of plasma protein carbonyl formation of plasma samples run on the same gel, and thus is less suitable for overall group correlations. Therefore, the total plasma protein carbonyl formation in patients with CRF were compared with healthy volunteers using an ELISA technique. Patients with CRF (range of creatinine clearance 16 to 46 mL/min) had a significantly elevated total plasma protein carbonyl concentration compared with healthy volunteers (0.31 ± 0.07 nmol per mg protein in CRF patients vs. 0.04 ± 0.01 nmol per mg protein healthy volunteers, P < 0.001; Fig. 4). Figure 5 illustrates that for the 20 patients with CRF, there was no significant relationship between creatinine clearance and levels of plasma protein carbonyl concentration (R^2 = 0.01).

DISCUSSION

This study used Western blotting to demonstrate that plasma albumin is the major plasma protein target of excess oxidant stress in CRF and chronic hemodialysis patients. To our knowledge, the present study is the first

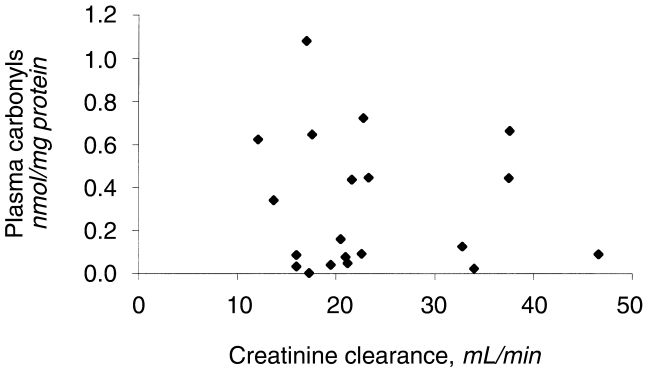

Fig. 5. Relationship between creatinine clearance and total plasma protein carbonyls measured by ELISA in 20 CRF patients ($R^2 = 0.01$).

to use Western blotting techniques to detect differential susceptibility of plasma proteins to carbonyl formation in vivo in any pathophysiologic disease state.

Cardiovascular disease continues to be the major cause of both morbidity and mortality for patients on chronic dialysis therapy. For chronic hemodialysis patients, the annual mortality caused by cardiovascular causes is approximately 9%, which is 10- to 20-fold higher than the general population, even when adjusted for age, sex, race, and the presence or absence of diabetes [10]. Additional risk factors for cardiovascular disease in the general population have been shown to have only modest predictive power in dialysis patients, suggesting the need for "nontraditional" markers of cardiovascular risk in this patient population [11]. A recent study has suggested that antioxidant therapy may lessen cardiovascular complications in end-stage renal disease (ESRD) patients, suggesting that oxidant stress may be an important "nontraditional" factor [12].

A potential link between inflammation, hypoalbuminemia, and subsequent cardiovascular risk may be through the process of oxidative stress. Chronic inflammatory stimuli result in phagocytic cell activation with subsequent increased production of reactive oxygen species. Phagocyte-derived oxidants are now believed to be central to the mechanisms of atherosclerosis formation [13, 14]. Since albumin is known to be an important plasma antioxidant [15–18], the finding of a predisposition of albumin to oxidation in renal failure also suggests a mechanism by which hypoalbuminemia contributes to the development of cardiovascular risk.

Plasma is known to contain a wide range of important antioxidants, including albumin, ascorbate, and urate. In contrast, concentrations of superoxide dismutase, reduced glutathione, and catalase, all of which are known to be important intracellular antioxidants, are low in the plasma and are not likely to be important plasma antioxidants [16, 19]. In plasma, free thiol groups are quantitatively the most important scavengers of hypochlorous acid and other oxidants and are known to be largely located on albumin [20, 21]. While ascorbate is an important extracellular antioxidant, albumin, via its thiol groups, provides quantitatively almost tenfold greater antioxidant protection against hypochlorous acid in human plasma [18]. The demonstration of albumin carbonyl formation in this study, in conjunction with our previous work demonstrating plasma protein thiol group oxidation in patients with uremia [3], indicates that this patient population is at considerable risk of tissue oxidant injury because of decreased plasma antioxidant defense.

Another important finding in this study is that patients with advanced CRF not receiving renal replacement therapy already have evidence of exposure to increased oxidative stress manifested by plasma protein carbonyl formation. The predominance of albumin as the target of protein oxidation in patients with CRF is similar to patients on chronic HD therapy, although of borderline statistical significance. These data are in accordance with our study demonstrating that plasma protein thiol group oxidation is also present in patients with CRF prior to the introduction of renal replacement therapy [3]. Recent investigators have emphasized that both markers of inflammation and hypoalbuminemia frequently begin in the pre-ESRD period [22]. Other investigators have pointed out the importance of the pre-ESRD period in the generation of cardiovascular risk factors that contribute to the high cardiovascular mortality of patients on renal replacement therapy [23]. To the extent that oxidative stress is involved in the genesis of atherosclerotic complications in uremia, the data in this study would suggest that the process begins substantially prior to the initiation of renal replacement therapy.

Shacter et al have used an in vitro iron/ascorbate hydroxyl radical generating system to study plasma protein oxidation using the same Western blotting assay for carbonyl groups. Of interest, they were able to demonstrate an increased susceptibility of fibrinogen to oxidative modification compared with other proteins [8, 9]. Fibrinogen contains a number of calcium-binding sites that may also bind to iron; this may be the explanation for increased fibrinogen susceptibility to oxidation in a metal-dependent oxidizing system. The lack of excess fibrinogen carbonyl formation in chronic hemodialysis patients or CRF patients compared with healthy volunteers in the present study suggests that metal-catalyzed oxidative chemistry may not be as important in vivo as myelperoxidase-catalyzed reactions and other pathways in these patients.

In summary, we have used Western blotting techniques measuring carbonyl formation to demonstrate that albumin is the predominant oxidatively modified plasma protein in patients with CRF and patients on HD. Oxidation of albumin will decrease plasma antioxidant defenses and increase the likelihood of oxidant stress-induced tis-

sue injury and cardiovascular disease in this patient population.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grant DK45610. We gratefully acknowledge the assistance of Joel Botler, M.D., Mr. Gary Banker, and Ms. Pam Hicks in providing blood samples from healthy volunteers with and without diabetes mellitus. We also acknowledge the secretarial assistance of Ms. Karen A. Kinne in the preparation of this manuscript.

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