Diabetes Induces an Impairment in the Proteolytic Activity Against Oxidized Proteins and a Heterogeneous Effect in Nonenzymatic Protein Modifications in the Cytosol of Rat Liver and Kidney

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It is assumed that increased oxidative stress contributes to the development of complications in diabetes. In this study, several markers of protein structural modifications directly induced by free radicals were investigated in the liver and kidney cytosolic fractions of rats with streptozotocin-induced diabetes. Sulfydryl residue and side-chain amino group analyses, as well as immunoblotting and chromatographic measurements of protein-bound carbonyl, suggest that protein oxidative modification is not increased by diabetes, with the exception of sulfydryl groups in renal cytosol. The levels of the glycation-derived carbonyl Nε-fructosyl-lysine are significantly increased by diabetes. Furthermore, unchanged proteolytic activity against in vivo–oxidized proteins, significant decreases both in activity against H_2O_2-modified proteins and in proteasome activity, measured by the degradation of a specific fluorogenic substrate, suggest that the unchanged oxidative protein modification in the diabetic state cannot be attributed to an increased cytosolic proteolytic activity in these tissues. These results provide evidence against a generalized increase in protein oxidative damage and demonstrate a diabetes-induced alteration in cytosolic proteolytic pathways, suggesting that proteasome activity may be impaired in these organs. *Diabetes* 48:2215–2220, 1999

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BSA, bovine serum albumin; DNP, dinitrophenylhydrazone; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FITC, fluorescein isothiocyanate; FL, Nε-fructosyl-lysine; FPLC, fast protein liquid chromatography; GC/MS, gas chromatography/mass spectrometry; PB, phosphate buffer; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene fluoride; s-LLVY-MCA, succinyl-leucine-leucine-valine-tyrosine-4-methylcoumarin-7-amide; SOD, superoxide dismutase; TFA, trifluoroacetic acid; 2,4-DNPH, 2,4-dinitrophenylhydrazine.

Present data suggest that oxidative stress increases in diabetes. This may be due to a decreased antioxidant status, increased free-radical production, alterations in enzymatic pathways related to the redox state, such as the polyol and glyoxalase pathways, and changes in the compartmentalization of metal ions, the overall effect of which is to promote increased oxidative stress in diabetes (1,2). However, the role of oxidative stress in the metabolic abnormalities of diabetes and in the development of diabetic complications is unclear, because the scant information available on the levels of oxidative stress markers is basically focused in lipid (1) and DNA (3,4) damage.

Although the protein modifications derived from hyperglycemia are clearly increased in diabetes (5–7), measurements of amino acid oxidation products such as methionine sulfoxide and ortho-tyrosine (8) argue against increased protein oxidative damage in diabetes. Since most information has been obtained using extracellular-matrix proteins, it remains unclear how these results may also apply to tissue protein oxidative damage.

To evaluate if diabetes induces changes in protein oxidative damage, we have measured the carbonyl content, side chain amino and sulfydryl groups, as well as Nε-fructosyl-lysine (FL) concentrations in the liver and kidney cytosol of streptozotocin-induced diabetic rats. It is known that protein turnover may modulate the steady-state levels of oxidative markers. For this reason, we have studied the cytosol proteolytic activity on H_2O_2-modified foreign proteins and on plasma proteins, as a model of in vivo oxidation and proteasome chymotrypsin-like activity, because it has been recently proven that mild oxidation of proteins increases their susceptibility to proteolysis by an ATP-independent proteasome pathway (9,10).

RESEARCH DESIGN AND METHODS

Animals. Experiments were carried out on 12-week-old Sprague-Dawley rats (300–350 g). Diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg/kg body weight in 20 mmol/l sodium citrate buffer, pH 4.5), and control rats were injected with sodium citrate buffer alone. One week after injection, all streptozotocin-injected rats showed glycemia >400 mg/dl. Throughout the experiment, they were maintained at 23 ± 2°C, 12:12 h (light:dark) cycle, 50 ± 10% relative humidity, and were fed ad libitum with a standard diet. After 3 months,

rats were fasted overnight and killed by decapitation. Blood was obtained by cardiac puncture, heparin was used as an anticoagulant, and plasma was separated by centrifugation (1,000*g*, 15 min). The abdomen was opened, the liver and kidneys excised, rinsed with phosphate-buffered saline (PBS) and, like the plasma samples, stored at –80°C until analysis.

Homogenization and subsequent procedures were performed at 4°C, unless stated otherwise. Samples (300 mg) were homogenized in 5 ml of 50 mmol/l Tris-HCl buffer, pH 7.5, containing 0.25 mol/l sucrose, 1 mmol/l EDTA, and 0.25 mmol/l phenylmethanesulfonyl fluoride (PMSF) using a Potter-Elvehjem device. The homogenate was centrifuged at 100,000*g* for 40 min, and the supernatant fraction, considered to be cytosol, was retained for study. For proteolysis measurements, cytosol was obtained as above but without the use of EDTA and PMSF. For FL measurements, samples were immediately dialyzed overnight, and proteins were precipitated with 10% trichloroacetic acid.

SDS-PAGE and immunodetection of protein-bound 2,4-dinitrophenylhydrazones. SDS-PAGE was performed according to Laemmli (11) with a 9% acrylamide as resolving gel, followed by staining with Coomassie Blue (12). Before electrophoresis, samples were derivatized with 2,4-dinitrophenylhydrazine (2,4-DNPH) as previously described by Shacter et al. (13). Briefly, to 15-µl cytosol samples adjusted to 4 mg/ml protein, SDS was added to a final concentration of 6%, and, after boiling for 3 min, 20 µl 10 mmol/l 2,4-DNPH in 10% trifluoroacetic acid (TFA) was added. After 15 min at room temperature, 20 µl of a solution containing 2 mol/l Tris base, 30% glycerol, and 15% β-mercaptoethanol was added for neutralization and sample preparation for loading onto SDS gels. As previously stated (13), times of derivatization longer than 45 min did lead to increases in nonspecific reactions. When appropriate, samples were reduced with sodium borohydride before derivatization, following previously described procedures (14). Briefly, cytosol samples were fully reduced by reacting a 4 mg/ml solution in Tris-HCl 50 mmol/l with 20 mg/ml sodium borohydride for 30 min, followed by neutralizing with HCl. After overnight dialysis against PBS, protein was ultrafiltrated and submitted to derivatization. Adequate controls without sodium borohydride were also processed.

For immunodetection, after SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA) with a semidry system (Semi-Phor; Hoefer Scientific Instruments, San Francisco, CA). Immunodetection with a rabbit anti-dinitrophenylhydrazone (DNP) antiserum as a primary antibody (V401; Dako, Carpenteria, CA) was performed using the Tropix chemiluminiscence kit (Bedford, MA). Luminiscence was recorded with Lumi-Imager equipment (Boehringer Mannheim, Mannheim, Germany).

Protein carbonyl quantification. Protein carbonyl was quantified by reaction with 2,4-DNPH (13). Nucleic acids were removed from homogenates by adding 10 µl 10% streptomycin sulfate to 90 µl of cytosol fractions, which were allowed to stand for 15 min, and centrifuged at 11,000*g* for 10 min. To 20 µl of the resulting supernatant, 20 µl 12% SDS was added during vortexing. Then, 40 µl of 20 mmol/l 2,4-DNPH in 10% TFA (without 2,4-DNPH for blanks) was added and mixed. The resulting mixtures were allowed to stand 30 min at room temperature. The samples were neutralized by adding 29 µl of 2 mol/l Tris/30% glycerol. Samples were filtered (0.4-µm PVDF filters; Whatman, Clifton, NJ) and 90 µl was injected onto the fast protein liquid chromatography (FPLC) system, consisting of a Waters 650 Protein Purification System (Waters Chromatography, Milford, MA) equipped with a Shodex protein KW-803 gel filtration column (Showa Denko K.K., Tokyo). The samples were analyzed using an isocratic elution in 200 mmol/l NaH_2PO_4, 1% SDS, pH 6.5, at 1 ml/min for 1 h. Ultraviolet absorbance spectra of eluate were monitored with a Waters W996 Diode Array Detector, scanning from 200 to 400 nm every 0.1 s. Sodium borohydride–reduced samples were processed in parallel, and the protein carbonyl content, expressed as nanomoles carbonyl per milligram protein, was obtained by subtracting the values of reduced samples.

Measurement of FL in cytosolic proteins. FL was measured in nonreduced protein samples (1–2 mg). Isotopically labeled internal standards ([$^2H_8$]lysine, purchased from MSD Isotopes [Rahway, NJ], and [$^{13}C_6$]FL, prepared as previously described [15]) were added, and the samples were hydrolyzed at 110°C for 24 h in 6 N HCl, then dried in vacuo. Residues were rehydrated in 1 ml 1% TFA and applied to a 1-ml C-18 solid extraction column (Supelco, Bellefonte, PA) equilibrated with the same solvent. The first milliliter of the flow-through and an additional 2 ml 1% TFA were all pooled and dried in vacuo. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described (16). Gas chromatography/mass spectrometry (GC/MS) analyses were carried out on a Hewlett-Packard model 5890 Series II gas chromatograph (Palo Alto, CA) equipped with a 30-m HP-5MS capillary column coupled to a Hewlett-Packard 5989A mass spectrometer as detector in the electron-impact mode. The injection port was maintained at 275°C; the temperature program was 2 min at 150°C, then 5°C/min to 225°C, then 25°C/min to 300°C, and finally held at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analyses were carried out by selected ion-monitoring GC/MS. The ions used were the following: lysine and [$^2H_8$]lysine, *m/z* 180 and 187, respectively; FL and [$^{13}C_6$]FL, *m/z* 110 and 116, respectively. The amount of products is expressed as the ratio nanomole FL/ per mole lysine.

Fluorescamine assay. Availability of side chain amino groups was measured as previously described (17). To 2.5-ml samples containing 6 µg of cytosolic protein in phosphate buffer (PB) at pH 9.5, 830 µl of 0.03% fluorescamine in dioxane was added while vortexing. After 10 min, fluorescence intensity at an emission wavelength of 470 nm and an excitation wavelength of 390 nm was recorded in a Shimadzu RF-500 Spectrofluorimeter (Shimadzu Europe, Duisburg, Germany). Quantitation was performed by external standardization using standard curve constructed from Nα-acetyl-lysine ranging from 1 to 20 nmol. The amount of side chain amino groups is expressed as micromole NH_2 per milligram protein.

Thiol assays. Protein thiol groups were quantified following a previously described procedure (17). To 100-µl samples containing 3 mg/ml of protein, 600 µl 1% SDS in 5 mmol/l PB pH 8.0 was added and mixed with 35 µl of 3 mmol/l dithiopyridine solution for assay samples or 35 µl distilled H_2O for blanks. The resulting mixture was incubated at 37°C for 15 min and at room temperature for 15 min. The absorbance of the samples was measured at 324 nm against its own blank in a Shimadzu UV-160A Spectrophotometer. Free thiol groups were quantified after protein precipitation. Briefly, supernatants obtained after 5-sulfosalicylic acid addition (1% v/v) were neutralized with 0.2 mol/l PB, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added to a final concentration of 0.1 mmol/l. After 1 h at 37°C, absorbance at 412 nm was measured against samples treated as above but without the use of DTNB. Quantification was performed by external standardization using a standard curve constructed from reduced glutathione ranging from 9.37 to 37 nmol. The amounts of thiol side chains are expressed as nmol sulfydryl/mg protein.

Oxidative modification of proteins. Bovine serum albumin (BSA) and superoxide dismutase (SOD) were used as model proteins for in vitro oxidation. Proteins were oxidized as described previously (10). Briefly, 600 µl of a 94-mmol/l H_2O_2 solution in PB was added to 3 ml 0.2 mol/l PB, pH 7.8, containing 2 mg BSA or SOD. After 1 h at room temperature, samples were concentrated by use of a Microsep Microconcentrator (Filtron Technology, Northborough, MA) equipped with a 10-kDa filter and immediately labeled with fluorescein isothiocyanate (FITC). Since plasma carbonyl content has been reported to be increased in diabetes (18), plasma proteins from diabetic and control animals were used as in vivo "oxidized" and "native" substrates, respectively.

Labeling of proteins and proteolytic activity measurement. The degradation of labeled foreign proteins by co-incubation with obtained cytosolic fractions was evaluated. To label proteins, 35 µl FITC (10 mg/ml) in DMSO was added to 0.6 ml 1 mol/l $NaHCO_3$ pH 9 containing 2 mg of native or oxidized proteins. After 1 h at room temperature, free FITC was quenched by adding 60 µl of a hydroxylamine solution (100 mg/ml, pH 9.8). FITC-labeled proteins were separated from low molecular contaminants using PD-10 columns (Pharmacia, Uppsala, Sweden) previously equilibrated in PBS. After protein quantification by the Bradford method (12), labeled proteins were stored frozen at –80°C until further analysis.

To quantify proteolytic activity, 100 µg of cytosol proteins and 1.87 µg of FITC-labeled native or oxidized proteins were added to 125 µl of proteolysis buffer, consisting of 50 mmol/l Tris-HCl (pH 7.8), 20 mmol/l KCl, 5 mmol/l magnesium acetate, and 0.5 mmol/l dithiothreitol (total volume 200 µl), and the resulting mixture was incubated at 37°C for 6 h, as previously described (9,10). An aliquot (100 µl) was then separated, and proteins in the remaining sample were precipitated using the alkaline zinc sulfate method (19). Fluorescence from both aliquots (495 nm excitation/515 nm emission) was measured in a Shimadzu RF-5000 spectrofluorimeter. Protein degradation was estimated as the ratio of fluorescence in the supernatant of the zinc-sulfate precipitated fraction to fluorescence of the sample without protein precipitation. Adequate controls in which the cytosolic fraction was boiled before addition of the labeled substrate were performed, and the results were corrected accounting for nonspecifically released fluorescence.

To test differences in proteolytic susceptibility of plasma proteins, 30 µg of protease type XIV from *Streptomyces griseus* and 15 µg of FITC-labeled plasma proteins from diabetic or control animals were added to 250 µl of proteolysis buffer (total volume 400 µl), and the resulting mixture was incubated for 1 h. Samples were withdrawn at several intervals and protein degradation was measured as described above.

Proteasome activity. Degradation of the fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-4-methylcoumarin-7-amide (s-LLVY-MCA) was measured as previously described (9). sLLVY-MCA was dissolved in 10% DMSO and used at a final concentration of 50 µmol/l in a buffer consisting of 50 mmol/l Tris-HCl pH 7.8, 20 mmol/l KCl, and 0.5 mmol/l magnesium acetate. Degradation was started by the addition of 100 µg of cytosolic proteins and incubation at 37°C. One hour later, the reaction was stopped by the addition of 2.8 ml 0.1 mol/l sodium borate pH 9.5 and 800 µl ethanol. Fluorescence intensity was recorded at an excitation wavelength of 360 nm and an emission wavelength of 460 nm in a Shimadzu RF-5000 spectrofluorometer.

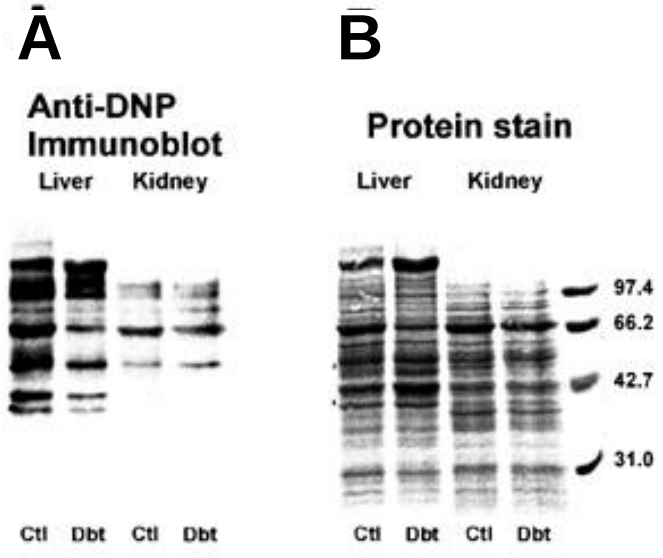

FIG. 1. Detection of oxidatively modified proteins in liver and kidney cytosol fractions. Liver and kidney cytosol (20 µg protein) from control (Ctl) and diabetic (Dbt) rats were analyzed by SDS-PAGE. *A*: Anti-dinitrophenyl immunoblotting; *B*: Coomassie Blue protein staining. Molecular weight markers are shown at the right side of the protein stain panel.

Statistical analysis. For comparisons, Student's *t* test was used when normality of variables was confirmed with the one-sample Kolmogorov-Smirnov Test, using the SPSS-PC program (SPSS, Chicago, IL). Otherwise, the Mann-Whitney *U* test was used. In all analyses, $P < 0.05$ was considered significant.

RESULTS

Proteins in the cytosolic fraction of liver and kidney homogenates from control rats and streptozotocin-induced diabetes rats were separated by SDS-PAGE, after 2,4-DNPH derivatization. Oxidized proteins were visualized by immunoblotting (Fig. 1). No difference was evident in the diabetic group, in either liver or kidney, and, in any case, several bands seemed to be less immunostained in samples from diabetic specimens. This finding was confirmed upon measuring several oxidative modification markers.

Table 1 illustrates the results obtained for the carbonyl content quantified as the 2,4-DNPH derivatives by FPLC. The content of amino and sulfydryl groups is also shown, as markers of protein structural modifications produced by free radicals. The results indicate that the carbonyl content in cytosolic proteins from liver of diabetic rats was significantly lower than that obtained from control rats ($P < 0.01$), whereas contents of amino and sulfydryl groups remained unchanged. For kidney, only the protein sulfydryl content was significantly lower compared with the control group ($P < 0.01$), whereas carbonyl and amino group content did not show significant differences. To further explore the effect of diabetes on sulfydryl status, this was also measured in protein-free cytosolic fractions. The results demonstrate that diabetes does not induce significant differences in nonprotein sulfydryl content, in the liver (3.07 ± 0.17 vs. 3.77 ± 0.41 nmol sulfydryl/mg protein, control versus diabetic, mean ± SE) or in the kidney (7.86 ± 0.26 vs. 8.18 ± 0.41 nmol sulfydryl/mg protein, control versus diabetic, mean ± SE). Finally, since Amadori products, arising from nonenzymatic glycosylation, could contribute to protein 2,4-DNPH reactive carbonyls, the concentrations of FL, an indicator of this reaction, were measured. The results demonstrated that FL levels are significantly increased in the diabetic state, both in liver and kidney ($P < 0.001$, in both cases).

The decreased or unchanged protein oxidative carbonyl modification in the diabetic group may reflect an increase in the proteolytic activity on oxidatively modified proteins. To test this possibility, the degradation of native or H_2O_2-modified foreign proteins, such as BSA and SOD, was evaluated. The results (Fig. 2) indicate that the degradation of H_2O_2-modified proteins is significantly increased in both the control and diabetic groups and in liver and kidney ($P < 0.001$). When comparing the control and diabetic groups, the results indicate small but significant changes induced by diabetes, impairing the degradation of H_2O_2-modified foreign proteins (Fig. 2). When these analyses were performed to less oxidatively damaged proteins, such as plasma proteins from diabetic specimens, the results further reinforce the fact that cytosol proteolytic activity is not increased by diabetes (Fig. 3*A*), even considering that diabetes induces changes in the anti-DNP profile of plasma, with an increase in oxidation in some bands and a decrease in others (Fig. 3*B*). Plasma proteins from diabetic specimens exhibit, despite their oxidative damage, resistance to in vitro proteolysis (Fig. 3*C*).

To test if the decreased proteolytic activity on oxidatively modified proteins is linked to the proteasome system activity, the rate of degradation of the specific substrate sLLVY-MCA was measured. The results, shown in Fig. 4, indicate that proteasome activity in both liver and kidney of diabetic rats is significantly decreased compared with the control group ($P < 0.001$).

DISCUSSION

Oxidative attack on proteins can covalently modify amino acid residues, leading to a variety of products (20). Introduction of carbonyl groups (aldehydes or ketones) into amino

TABLE 1
Steady-state levels of protein carbonyl content, side chain amino groups, sulfydryl groups, and N^{ε}-fructosyl-lysine content in cytosolic protein extracts from liver and kidney of control and diabetic rats

	Liver		Kidney	
	Control	Diabetic	Control	Diabetic
Carbonyl content (nmol/mg protein)	1.028 ± 0.135	0.87 ± 0.07*	3.06 ± 0.27	3.44 ± 0.28
Amino groups (µmol NH_2/mg protein)	1.08 ± 0.13	0.88 ± 0.16	1.41 ± 0.12	1.38 ± 0.07
Sulfydryl groups (nmol sulfydryl/mg protein)	93.38 ± 13.24	100.2 ± 15.57	132.46 ± 6.00	107.66 ± 5.24*
N^{ε}-fructosyl-lysine content (mmol/mol lysine)	0.52 ± 0.07	2.59 ± 0.09†	0.25 ± 0.08	2.90 ± 0.18†

Data are means ± SE from seven animals per group. *$P < 0.05$; †$P < 0.001$.

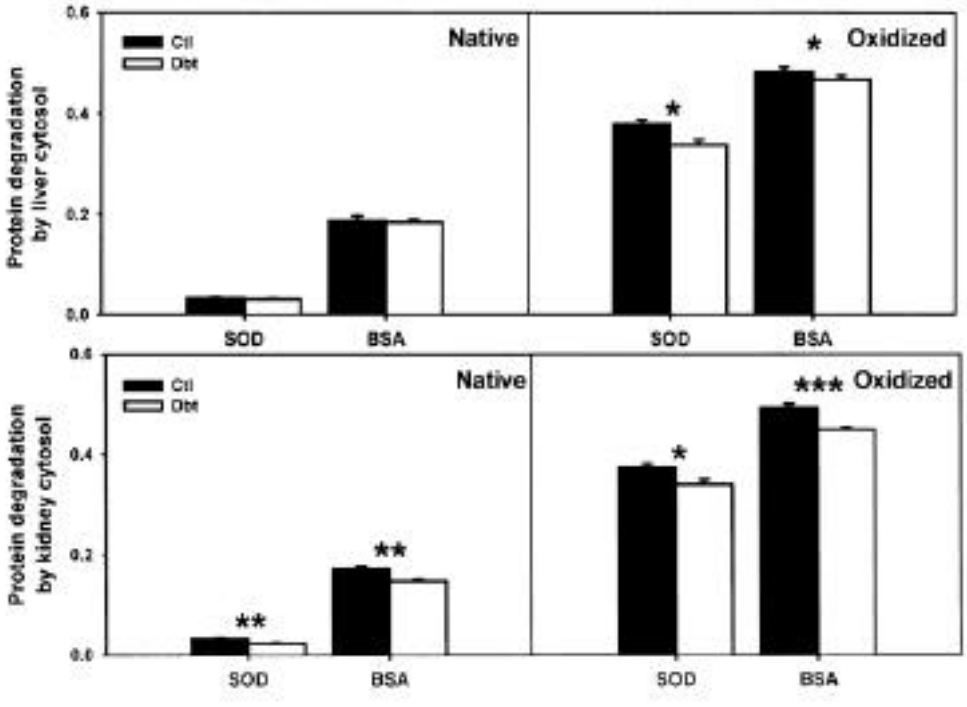

FIG. 2. Effect of diabetes on cytosolic proteolytic activity on H_2O_2-modified proteins. FITC-labeled BSA or SOD (native or previously oxidized with H_2O_2) was added to cytosol of liver and kidney from control and diabetic rats. Proteolysis was measured as described in RESEARCH DESIGN AND METHODS. Bars represent means ± SE from seven animals per group. Differences between control and diabetic specimens: *P < 0.05; **P < 0.01; ***P < 0.001.

acid structure is a frequent consequence of protein oxidation. Thus, these carbonyl groups are commonly accepted as an oxidative modification marker (20–22). Accordingly, increases in the protein carbonyl content have been demonstrated in tissues after a variety of pro-oxidant stresses (20). However, despite the fact that an increase in oxidative stress has been invoked in the development of diabetic complications (1,2,23), the information available on tissue protein carbonyl-oxidative damage is limited to ocular tissues (24,25). Using an immunoblotting assay with anti-dinitrophenyl antibodies to detect protein-bound carbonyl groups, the results presented here do not support an increase in protein oxidative damage of liver and kidney cytosolic fractions from diabetic rats. Reaction of protein carbonyls with 2,4-DNPH under the conditions described here is rapid and reaches half-completion after 2 min. As previously described (13), longer times for derivatization resulted in an increased nonspecific immunostaining. Sodium borohydride reduction was performed to obtain a "zero carbonyl response," and this experiment further demonstrated the lack of differences in cytosol immunostaining for DNP-proteins (data not shown). The lack of differences between control and diabetic specimens has been also confirmed by quantitating the protein carbonyl content by means of gel filtration FPLC, demonstrating unchanged steady-state levels in kidney proteins and even a significant decrease in diabetic liver. The interorgan differences in the absolute amount of carbonyls contrasted with that present in the immunoblot analyses. This fact could be derived from the use of "zero-carbonyl" controls in the FPLC analyses, and the molecular weight selectivity derived from the SDS-PAGE technique used here. The use of 2,4-DNPH–reactive protein carbonyl as a marker for oxidative damage has been recently revisited (26,27) and criticized, especially when absolute values are examined, because these are subject to some uncertainty. However, the use of carbonyl assay has been applied to experimental studies and clinical samples (28,29), and the putative lack of specificity arising from the colorimetric method may be overcome with the Western blot or high-performance liquid chromatography techniques (30–32), especially when comparing samples analyzed with a standard system (14).

Furthermore, both the side chain amino and sulfydryl groups, as biomarkers for the detection of protein structural modifications induced by free radicals (17), also indicate, in liver, unchanged or decreased steady-state protein oxidative damage in the diabetic state. In contrast, protein sulfydryl content in kidney cytosol is diminished, suggesting increased protein oxidative damage. This latter finding may be relevant in the development of diabetic nephropathy, due to the critical importance of sulfydryl group oxidation in cellular signaling (33). Thus, from a pathophysiological viewpoint, the decreased sulfydryl groups might be more deleterious than the higher states of oxidation represented by DNP reactivity. These apparent contradictions in the data may arise from the use of different sources of the markers measured here, stressing the concept that protein oxidative modification assessment could not be restricted to a single marker. Nevertheless, these data do not support a generalized increase of protein oxidative damage. Accordingly, in the extracellular compartment from diabetic compared with nondiabetic subjects, unchanged levels of several biomarkers of amino acid oxidation have been reported recently by independent groups (8,34). In contrast with these reports, recent studies demonstrate increased protein carbonyl concentration in diabetic retinopathy (24,25). These differences may arise from tissue-

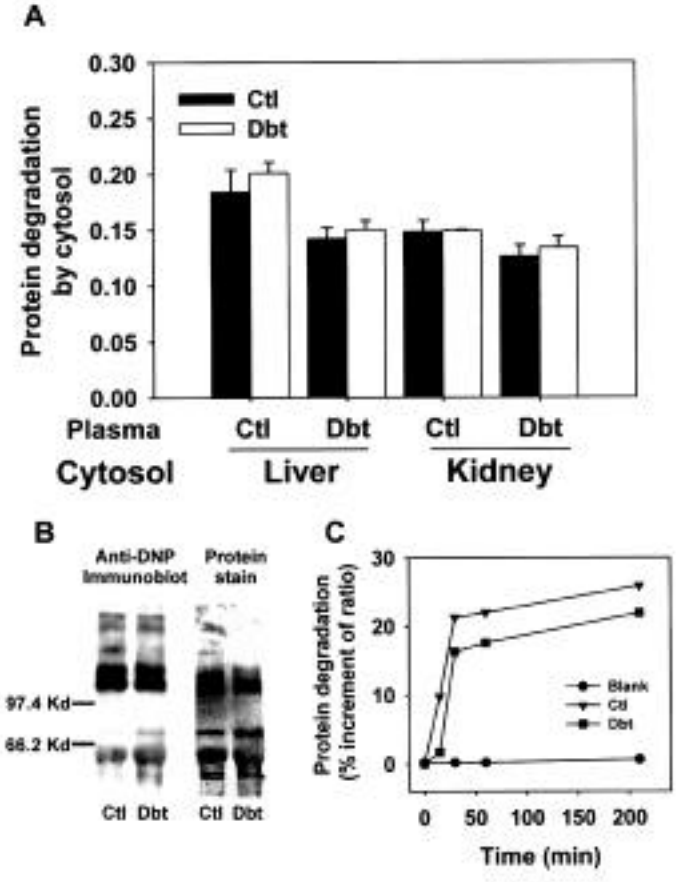

FIG. 3. Effect of diabetes on cytosolic proteolytic activity on plasma proteins. *A*: Plasma was pooled from control (Ctl) and diabetic (Dbt) specimens (n = 7 for each group), FITC labeled, and added to cytosol of liver or kidney from control or diabetic rats. Proteolysis was then measured as described in RESEARCHDESIGNANDMETHODS. Bars represent means ± SE from seven animals per group. *B*: Detection of oxidatively modified proteins in plasma from control (Ctl) and diabetic (Dbt) rats, analyzed by SDS-PAGE and anti-DNP immunoblotting. *C*: Time course proteolysis of FITC-labeled plasma protein from diabetic (Dbt) or control (Ctl) specimens by type XIV protease from *Streptomyces griseus*. (Blank, FITC-labeled plasma proteins treated like the other samples but without enzyme.) Values represent the means of three independent experiments for which standard errors were always <10%.

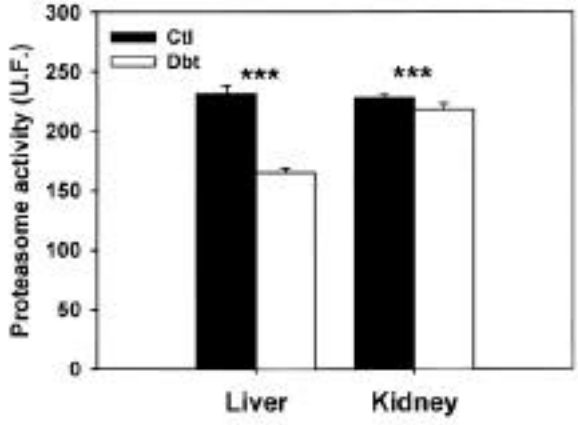

FIG. 4. Effects of diabetes on liver and kidney proteasome activity. Rates of s-LLVY-MCA degradation by cytosol of liver and kidney from control (Ctl) and diabetic (Dbt) rats. Values, expressed as units of fluorescence (U.F.), are means ± SE from seven animals per group. ***P < 0.001.

specific antioxidant mechanisms and from differences in the experimental model.

The present results can be explained assuming that insulin is one of the main factors that regulate overall protein catabolism in mammals (35). Accordingly, average rates of protein catabolism are accelerated 30–150% in liver cells, fibroblasts, adipocytes, skeletal muscle, and heart muscle in insulin-deficient states (35). Thus, it is expected that an increased rate of protein catabolism may give rise to proteins with decreased extent of oxidative modification. In fact, it has been demonstrated that oxidative stress enhances intracellular proteolysis by increasing the susceptibility of cellular proteins to proteolysis (9,10). To test whether increased proteolysis may explain the unchanged levels of oxidative stress markers in cytosolic proteins, we have measured the proteolytic activity of the cytosolic protein extracts from liver and kidney of diabetic rats on the H_2O_2-modified foreign proteins BSA and SOD. The results obtained indicate that oxidation of foreign proteins significantly increases their proteolytic susceptibility, both in liver and kidney, independently of the presence of diabetes. Nevertheless, when comparing control with the diabetic group, unchanged or even decreased proteolytic activity has been observed. Because protein oxidation with H_2O_2 under the conditions exposed here may be more severe than that present in physiological oxidative stress conditions, plasma proteins from diabetic and control specimens were derivatized and used as substrates of cytosolic proteolytic activity. The results of this analysis confirm the lack of increased proteolytic activity in liver or kidney cytosol from diabetic specimens and demonstrate a striking resistance to in vitro proteolysis of plasma proteins, which might be related to the resistance of nonenzymatically glycated proteins to proteolytic degradation (36).

Furthermore, the analysis of the proteasome substrate sLLVY-MCA degradation (10) indicates a significantly decreased proteolytic activity in both the liver and kidney of the diabetic group. These findings suggest that the unchanged oxidatively modified protein in the diabetic state is not the result of an increased cytosolic proteolytic activity. It remains to be elucidated whether the activity of other proteolytic systems (such as lysosomal or ATP-dependent proteolytic pathways), increased during diabetes (37), may explain the low steady-state levels of protein oxidation. It should be remembered, however, that the major pathway for clearing oxidized proteins is an ATP-independent proteasome system (9,10), located in cytosol. Also, the possibility cannot be excluded that in vivo, oxidatively modified cytosolic proteins may be preferentially degraded. If such is the case, the present methodology would not detect increased proteolysis. However, the decreased proteasome activities suggest that this system for removal of oxidatively damaged proteins is impaired.

All of these findings argue against a diabetes-induced generalized increase in the oxidative modification of proteins, in accordance with the hypothesis that extensive free-radical–derived damage may be a late event in the pathogenesis of diabetes complications (38). Nevertheless, it could be also considered that a high intracellular glucose concentration may drive protein damage to other kinds of modifications such as Maillard reaction, as demonstrated by the FL measurements, which indicate a clear increase in this type of modification. These data are in accordance with reports of advanced gly-

cation in endothelial cell cultures in hyperglycemic conditions (39,40). Furthermore, the marked rise in FL levels argues against increased proteolysis, since it may be viewed as a surrogate for protein half-life (41). Thus, decreased cytosolic proteolysis might contribute to the hyperglycemia-induced increased levels of FL, although the precise pathways of intracellular disposal of glycated proteins, if different from that for their unmodified counterparts, remain unknown. Recent data demonstrate that lipid peroxidation, but not glycation, leads to carbonyl modification in proteins (42). Since lipid peroxidation is increased in diabetes, this should result in an increase in protein carbonyl. The reasons for this apparent discrepancy are the focus of current investigation, but they may be related to secondary antioxidant defenses, such as the presence of low molecular weight amines or other substances able to cope with increased aldehyde production.

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