

# Hyperglycemia Is a Major Determinant of Albumin Permeability in Diabetic Microcirculation

## The Role of $\mu$ -Calpain

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Increased permeability to albumin is a well-known feature of diabetic microvasculature and a negative prognostic factor of vascular complications. The mechanisms responsible for loss of the physiological albumin barrier in diabetic organs remain only partially understood. We have recently demonstrated that the protease  $\mu$ -calpain is activated in hyperglycemia, which causes endothelial dysfunction and vascular inflammation. In the present study, we investigated whether  $\mu$ -calpain is involved in the hyperpermeability of the diabetic vasculature. We also investigated the mechanistic roles of hyperglycemia and leukocyte adhesion in this process. Albumin permeability in the intact microcirculation of the Zucker diabetic fatty (ZDF) rat was quantified by intravital microscopy. Extravasation of albumin in the microcirculation of ZDF rats was significantly increased when compared with nondiabetic Zucker lean (ZL) rats. Microvascular albumin leakage was prevented by either antisense depletion of  $\mu$ -calpain or pharmacological inhibition of calpain *in vivo*. Calpain inhibition also attenuated urinary albumin excretion in ZDF rats. Glucose concentrations in the range of those found in the blood of ZDF rats increased albumin permeability in nondiabetic ZL rats. Thus, this demonstrates a mechanistic role for hyperglycemia in the hyperpermeability of diabetes. Depletion of polymorphonuclear leukocytes *in vivo* failed to prevent glucose-induced hyperpermeability, which suggests that hyperglycemia can disrupt the physiological endothelial cell barrier of the microcirculation, even in the absence of increased overt leukocyte-endothelium interactions. *Diabetes* 56:1842–1849, 2007

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IVM, intravital microscopy; ODN, oligodeoxynucleotide; PKC, protein kinase C; PMN, polymorphonuclear; Vrb, red blood cell velocity; ZLLal, benzoyloxycarbonyl-Leu-leucinal.

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**H**yperglycemia is associated with vascular complications. Because of its strategic position between blood and the vascular wall, the vascular endothelium is a primary target of the ravaging actions of hyperglycemia. Accordingly, there is consensus that hyperglycemia causes chronic endothelial dysfunction (1,2). Several studies have shown that increased endothelial permeability is an early manifestation of endothelial dysfunction in diabetes (3–5). Alterations in the physiological endothelial cell barrier are likely to impair organ function, due to the accumulation of plasma macromolecules in the interstitial compartment of body organs (6). Therefore, microalbuminuria is now viewed as an important clinical parameter and therapeutic target for the treatment of vascular complications in diabetic patients (7). However, the molecular mechanism(s) responsible for endothelial hyperpermeability in diabetes remains largely unknown, which limits effective therapeutic interventions.

Calpains are a family of calcium-dependent cysteine proteases found in mammals and many lower organisms (8). We have reported that acute experimental hyperglycemia, as well as diabetes, upregulate the endothelial-expressed  $\mu$ -calpain isoform in the microcirculation (a process that results in endothelial dysfunction and abnormal leukocyte trafficking) (9,10). Other studies have reported that inhibition of calpain activity improves organ function in diabetic animal models (11,12) and in acute cardiovascular events (13). *In vitro* studies have now implicated calpains in the regulation of endothelial cell barrier (14,15).

This study tests the hypothesis that calpain is involved in the abnormal permeability of the endothelial cell barrier of Zucker diabetic fatty (ZDF) rats, a relevant animal model of type 2 diabetes. We also evaluated the roles of calpain and leukocyte-endothelium interaction in the hyperpermeability of hyperglycemia, and we studied whether the inhibitory effect of calpain inhibition on albumin permeability is mediated through downregulation of leukocyte-endothelium interaction *in vivo*.

### RESEARCH DESIGN AND METHODS

**Experimental animal models of hyperglycemia.** All animal procedures were approved by the Thomas Jefferson University institutional animal care and use committee. We used 10- to 14-week-old male ZDF rats and age-matched nondiabetic Zucker lean (ZL) rats (Charles River Laboratories; Noblesville, IN). At this age, ZDF rats develop hyperglycemia with hypoinsulinemia (16), which permits study of the effect of chronic hyperglycemia alone

on microvascular function. Moreover, ZDF rats have increased vascular permeability (17), and increased calpain activity is in the microcirculation (9). Rats were randomly assigned to the one of the following experimental groups: 1) ZL rats injected with vehicle; 2) ZDF rats injected with vehicle; 3) ZDF rats treated with 1 mg/kg sense nucleotide to  $\mu$ -calpain (once a day for 4 consecutive days); 4) ZDF rats treated with 1 mg/kg antisense nucleotide to  $\mu$ -calpain (once a day for 4 consecutive days); or 5) ZDF rats injected intraperitoneally with 27  $\mu$ g/kg calpain inhibitor benzyloxycarbonyl-Leu-leucinal (ZLLal) (once a day for 4 consecutive days).

Acute experimental hyperglycemia of the mesenteric microvasculature in nondiabetic ZL rats was induced by a single intraperitoneal injection of 25 mmol/l D-glucose (Sigma, Saint Louis, MO), administered 12 h before study. We and others have demonstrated that this procedure triggers a sustained inflammatory response in mesenteric postcapillary venules, which lasts over a 24-h period (18,19). L-glucose (Sigma) was used at the concentration of 25 mmol/l as a control to exclude nonspecific osmolarity effects of D-glucose. In these experiments, upregulation of calpain activity was prevented by pretreatment of rats with a single intraperitoneal injection of 27  $\mu$ g/kg ZLLal, as previously described by our laboratory (10).

**In vivo microcirculatory parameters.** Quantitative intravital microscopy (IVM) of mesenteric microcirculation was used to measure vessel diameter, venular wall shear rates, leukocyte adhesion, vascular density, and albumin permeability in all experimental groups of rats.

**Preparation of animals for IVM.** Following anesthesia with 80 mg/kg i.p. pentobarbital, rats were prepared for IVM studies, as previously described (9). Briefly, four distal loops of ileal tissue, exteriorized through a midline incision laparotomy, were superfused with 37°C Krebs-Henseleit buffer in an IVM Plexiglas chamber attached on the stage of an Eclipse 80si microscope (Nikon). Three to four relatively straight unbranched segments of postcapillary venules with lengths of >100  $\mu$ m and diameters between 25 and 40  $\mu$ m were randomly studied in each rat. Observation of the mesenteric microcirculation was made with a 20 $\times$  salt water-immersion lens. Images were projected by high-resolution intensified video cameras (Evolution QEi or Photometric Cascade 512B) on to a high-resolution color video monitor (Multiscan 200-sf; Sony), and the image was recorded with a digital DVD recorder (Panasonic DMR-10). All data were analyzed using computerized imaging software (Image Pro Plus version 5.1; Media Cybernetics, Silver Spring, MD) on a Pentium-based International Business Machines (IBM) compatible computer.

**Venular diameter.** Venular diameter was measured online using a video caliper and Image Pro Plus version 5.1.

**Erythrocyte velocity and venular wall shear rates.** Red blood cell velocity (Vrbc) was measured online using an optical Doppler velocimeter (Microcirculation Research Institute, College Station, TX). Vrbc and venular diameter (D) were used to calculate the venular wall interfacial shear rate ( $\gamma$ ) by using the formula:  $\gamma = 4.9 \times 8 \times (\text{venular mean}/D)$ , where venular mean = Vrbc/1.6. (20).

**Leukocyte adhesion.** The number of adherent leukocytes was determined offline during video playback analysis, as previously described (9). Baseline leukocyte adherence was defined as the number of cells that remain stationary for >30 s in 100  $\mu$ m of vessel length.

**Vascular density.** Microvascular density was measured in random segments of the visceral peritoneum of three predefined ileal loops (i.e., the three most distal loops of ileum), according to a previously described method (21) (found in an online appendix "Supplemental Methods" [at <http://dx.doi.org/10.2337/db06-1198>]).

**Vascular permeability.** The permeability index of postcapillary venules was determined by intravital fluorescent microscopy using Texas Red-labeled albumin (15 mg/kg body wt) (TR-BSA; Molecular Probes), according to a previously described technique (Supplemental Methods) (22).

**Urinary albumin excretion.** ZL and ZDF rats and ZDF rats given 27  $\mu$ g  $\cdot$  kg<sup>-1</sup>  $\cdot$  day<sup>-1</sup> ZLLal for 4 consecutive days were placed in metabolic cages for a 24-h collection of urine. Urinary albumin excretion was analyzed with the Nephtraz enzyme-linked immunosorbent assay (Exocell, Philadelphia, PA). Briefly, 24-h urine samples stored at -20°C were thawed and centrifuged at 10,000 rpm for 15 min to remove debris, and samples were assayed according to the manufacturers' guide. Urinary creatinine levels were measured using the Creatinine Companion kit (Exocell). All albumin values were normalized to those of creatinine and expressed as milligrams albumin to milligrams creatinine.

**Quantification of calpain activity.** Calpain activity was measured using a standard fluorescent assay for calpain (23) that was recently adapted by our laboratory to measure calpain activity in vascular tissue (9). Briefly, highly vascularized mesenteric segments were isolated with the aid of a dissecting microscope. Tissue was snap frozen in liquid nitrogen and prepared for biochemical analyses of calpain activity, as previously described (24). For separation of soluble and total particulate mesentery fractions, the superna-

tant was subjected to 60-min centrifugation at 100,000g. Calpain activity in cytosolic tissue extracts was determined using the highly sensitive fluorescent calpain substrate Succ-LLVY-AMC (*N*-succinyl-L-leucyl-L-valyl-L-tyrosine-7-amino-4-methylcoumarin) (Molecular Probes, Eugene, OR), at the concentration of 30  $\mu$ mol/l. Mean fluorescence signals were measured using a Bio-Tek FLx 800 microplate fluorescence reader (Bio-Tek, Winooski, VT) (excitation  $\lambda$  360 nm and emission  $\lambda$  460 nm). Calpain activity levels in all experimental groups of rats were expressed as percentage change from control values.

#### **Inhibition of $\mu$ -calpain activity**

**Pharmacological inhibition.** The calpain inhibitor ZLLal (Biomol Research Laboratories, Plymouth Meeting, PA) was used because it is the highest selectivity for calpains over other proteasomal enzymes (25) and because of its efficacy in preventing activation of  $\mu$ -calpain in the microvasculature in response to acute hyperglycemia (10) and diabetes (9). ZLLal was dissolved in a final ethanol concentration of 0.01%. In control experiments, intraperitoneal injection of saline (0.9% NaCl) containing 0.01% ethanol to rats did not affect microvascular parameters (data not shown).

**Antisense depletion of  $\mu$ -calpain.** We used sense and antisense oligodeoxynucleotide (ODN) corresponding to exon 12 of  $\mu$ -calpain. Sense: 5' ATGCTTCTCGGGCACAAT 3'; antisense: 5' ATTTGCCCCGAGAAGCAT 3' (MWG Biotech, High Point, NC). The sense ODN was used as an inactive control to rule out nonspecific effects of ODN treatment. Three to five bases at both the 5' and 3' ends were modified with phosphorothioate to increase ODN stability in vivo; thus, pharmacokinetics and tissue distribution improved. ODNs were dissolved in sterile saline (0.9%) and administered to rats via intraperitoneal injection. We have previously demonstrated the efficacy of the oligonucleotides used in this study in suppressing  $\mu$ -calpain expression levels in rat mesenteric microvascular endothelial cells (9).

**Analysis of  $\mu$ -calpain expression level.** The extent and localization of  $\mu$ -calpain expression after antisense therapy were confirmed by Western blot analysis and by immunohistochemistry, respectively. For immunoblot studies, highly vascularized mesenteric segments were isolated with the aid of a dissecting microscope. Tissue was snap-frozen in liquid nitrogen and homogenized as previously described (10). Briefly, calpain expression levels were studied using an antibody against the stable Domain IV (RP3 calpain-I; Triple Point Biologics, Portland, OR) of the large subunit of  $\mu$ -calpain. Proteins were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL) and quantified by laser densitometry (Molecular Dynamics Personal Densitometer; Molecular Dynamics, Piscataway, NJ).

In additional studies, at the completion of IVM experiments, sections of mesentery and ileum were fixed in vivo, dehydrated in graded acetone washes, and embedded in plastic, as previously described (9). Briefly, immunohistochemical localization of  $\mu$ -calpain was accomplished using an antibody against Domain IV of  $\mu$ -calpain and the avidin/biotin immunoperoxidase technique.

**Depletion of polymorphonuclear leukocytes in vivo.** In some animals, the circulating pool of polymorphonuclear (PMN) leukocytes was depleted by intraperitoneal administration of 1 mg/kg antineutrophil serum (Accurate Chemical and Scientific Corporation, Wesbury, NY), according to a previously described method (26).

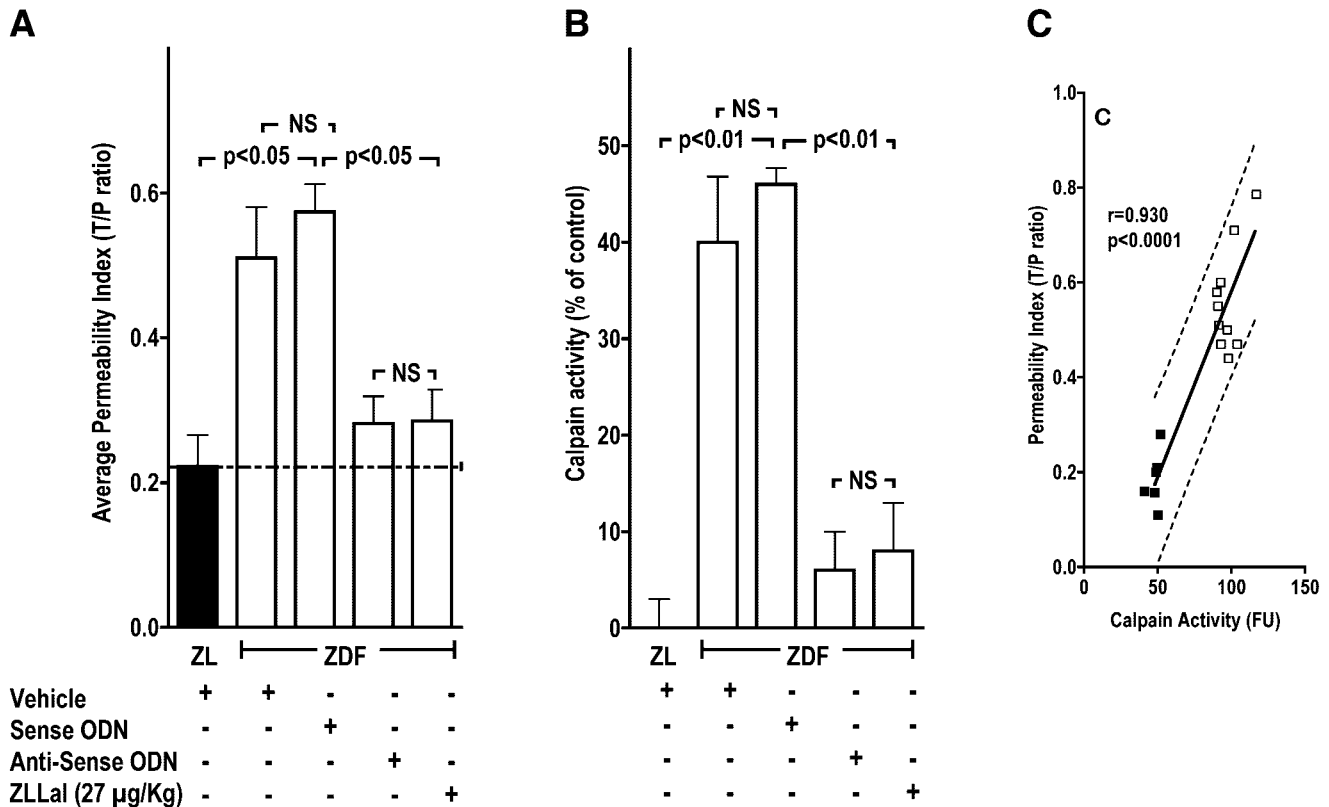
**Hematologic parameters.** Tail vein blood was used to obtain blood glucose levels and peripheral neutrophils counts. Blood glucose levels were measured using an Accu-Chek glucose meter (Roche Diagnostic, Indianapolis, IN). The peripheral neutrophil counts were evaluated manually using a Neubauer chamber, according to standard hematology procedures.

**Data analysis.** Data are presented as means  $\pm$  SEM and compared by ANOVA with post hoc analysis by Fisher's corrected *t* test. *P* values  $\leq$  0.05 were considered statistically significant.

## **RESULTS**

**Inhibition of calpain activity does not affect hyperglycemia in ZDF rats.** Compared with ZL rats, ZDF rats showed fasting hyperglycemia in the range of  $23 \pm 4.75$  mmol/l (*P* < 0.01 vs. ZL rats; blood glucose levels  $5.4 \pm 0.7$  mmol/l). Blood glucose levels were not changed by pharmacological inhibition of calpain activity with ZLLal or by  $\mu$ -calpain ODN therapy (blood glucose levels  $24 \pm 3.75$  mmol/l and  $22 \pm 6.7$  mmol/l, respectively; *P* > 0.05 vs. untreated ZDF rats).

**Downregulation of  $\mu$ -calpain reduces the albumin hypermeability of the diabetic microvasculature.** We measured albumin permeability in intact postcapillary venules of ZDF rats by IVM because postcapillary venules exhibit dramatic changes in permeability in response to



**FIG. 1.**  $\mu$ -Calpain is responsible for increased albumin permeability in the diabetic vasculature. **A:** The average permeability index to Texas Red-labeled albumin that was calculated during IVM studies in mesenteric postcapillary venules of nondiabetic ZL rats and diabetic ZDF rats.  $\mu$ -Calpain activity was blocked by either pharmacological inhibition with ZLLal or antisense ODN depletion. Control sense nucleotides were also given to the ZDF rats. **B:** Calpain activity levels in tissue extracts of vascular segments of mesenteries obtained from all groups of rats. Calpain activity in cytosolic tissue extracts was determined using the highly sensitive fluorescent calpain substrate Succ-LLVY-AMC using a microplate fluorescence reader. Calpain activity levels in all experimental groups of rats were expressed as percentage change from control values. **C:** Correlation analyses between calpain activity and the permeability index in nondiabetic ZL (■) and diabetic ZDF (□) rats. Data are expressed as means  $\pm$  SEM.  $n = 6$ –8 rats (average) in each group.

hyperglycemia and diabetes (5). Figure 1A (left panel) demonstrates that the microcirculation of ZDF rats is significantly more permeable to albumin than that of ZL rats. A 4-day treatment of ZDF rats with the calpain inhibitor ZLLal significantly attenuated albumin permeability in the diabetes microcirculation (Fig. 1A).

Both the m-calpain and  $\mu$ -calpain isoforms are constitutively expressed in endothelial cells (27), but the  $\mu$ -calpain isoform has been recently linked to microvascular alterations of hyperglycemia (9,10). Accordingly, we used antisense depletion of  $\mu$ -calpain to confirm whether  $\mu$ -calpain is the calpain isoform largely responsible for increased albumin permeability in diabetes. Intraperitoneal delivery of  $\mu$ -calpain antisense ODN for 4 consecutive days attenuated albumin permeability in the mesenteric microcirculation of ZDF rats. In contrast, administration of sense ODN sequences failed to prevent albumin leakage (Fig. 1A).

These data demonstrate a role for the  $\mu$ -calpain isoform in the hypermeability of the diabetic endothelium and indicate  $\mu$ -calpain as the molecular target of the endothelial protective action of pharmacological calpain inhibition in vivo.

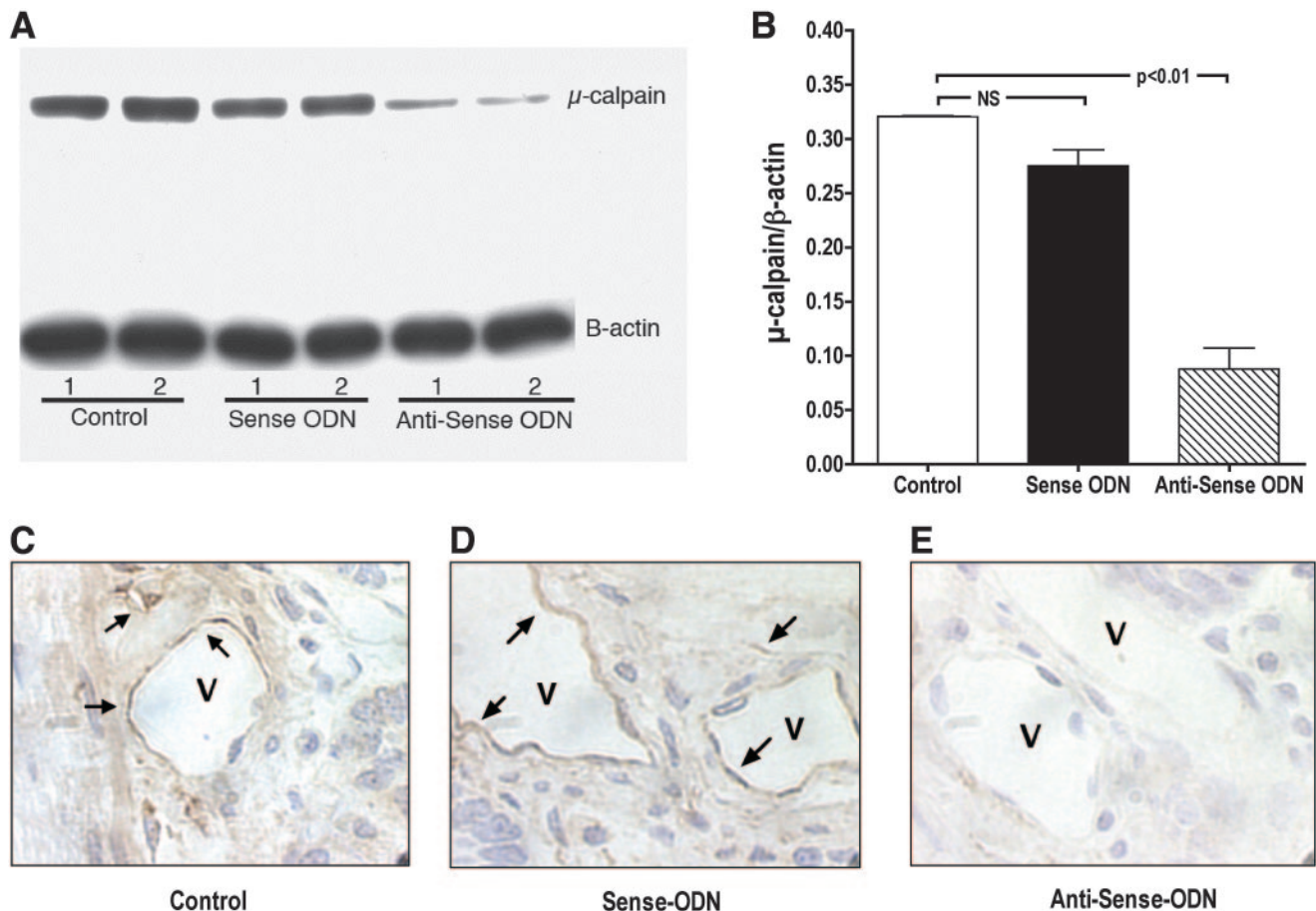
Biochemical analyses of calpain activity positively correlated with vascular permeability data (Fig. 1B). Compared with ZL rats, ZDF rats showed a 40% increase in calpain activity in highly vascularized regions of the mesentery that were also abnormally leaky to albumin (Fig. 1B). A comparable degree of calpain activity inhibition

was observed following intraperitoneal delivery of either  $\mu$ -calpain antisense ODN or the calpain inhibitor ZLLal. This indicates  $\mu$ -calpain as the calpain isoform responsible for increased endothelial calpain activity under our experimental conditions. Moreover, linear regression modeling confirmed that increased calpain activity positively correlates with increased vascular permeability in the diabetic microcirculation of the ZDF rat (Fig. 1C).

Immunoblot analyses of  $\mu$ -calpain demonstrated a threefold reduction in  $\mu$ -calpain expression levels in ZDF rats given antisense ODN (Fig. 2A and B). Immunohistochemical staining confirmed that  $\mu$ -calpain expression in the intestinal microvessels of ZDF rats was almost completely suppressed by antisense ODN therapy (Fig. 2C and E). In contrast, baseline staining for  $\mu$ -calpain was detected in the endothelial lining of blood vessels and in the extravascular space of intestinal tissue obtained from control ZDF rats and ZDF rats given sense ODN (Fig. 2C and D).

These data demonstrate the efficacy and specificity of  $\mu$ -calpain antisense ODN therapy for suppressing  $\mu$ -calpain expression in the rat mesenteric microcirculation.

We also studied the effect of systemic calpain inhibition on urinary albumin excretion. Data reported in Table 1 demonstrate that ZDF rats have elevated albuminuria when compared with nondiabetic ZL rats. A 4-day treatment with the calpain inhibitor ZLLal significantly attenuated albuminuria in ZDF rats, albeit urinary albumin levels



**FIG. 2.** Intrapерitoneal injection of antisense oligonucleotides for 4 consecutive days effectively suppresses  $\mu$ -calpain expression in the rat intestinal microvasculature. A primary antibody against  $\mu$ -calpain Domain IV was used to quantify total  $\mu$ -calpain content in vascularized tissue extracts of the rat mesentery (Immunoblot). *A* and *B*: Graphs demonstrate densitometric analysis of  $\mu$ -calpain Domain IV, after normalization to the expression levels of  $\beta$ -actin. Immunohistochemistry confirmed reduced expression level of  $\mu$ -calpain in the vascular wall of venules, in addition to the perivascular areas (arrows) of rats treated with antisense oligonucleotide (*E*), compared with control rats injected with either saline (*C*) or sense oligonucleotide to  $\mu$ -calpain (*D*). Original magnification 450 $\times$ . Three rats were studied in each group.

remained significantly elevated when compared with control ZL rats.

These data are consistent with those obtained in the mesenteric microcirculation and they further demonstrate the mechanistic contribution of calpain to the dysfunction of organs that witness important diabetes complications.

We also measured mean arterial blood pressure, blood flow, vessel diameter, vascular density, and leukocyte-endothelium interactions since they are important determinants of vascular permeability in vivo. No significant differences were observed in mean arterial blood pressure, vessel diameter, and vascular density under our experimental conditions (data not shown).

However, we found that calculated shear rate values in postcapillary venules of ZDF rat mesenteries were signif-

icantly decreased, despite remaining within physiologic range (Fig. 3A). This finding is in agreement with previous studies, demonstrating decreased shear rate values in the microcirculation of diabetic rats (28). These data indicate that the changes in albumin permeability seen in the microvasculature of ZDF rats are unlikely driven by abrupt alterations in hydrostatic pressures or vascular density.

In contrast, we found evidence of increased leukocyte adhesion in the microcirculation of ZDF rats. In particular, leukocyte adhesion was increased sixfold in postcapillary venules of ZDF rats ( $P < 0.01$  vs. ZL), a value which was equally attenuated by antisense depletion of  $\mu$ -calpain and pharmacological inhibition of calpain activity (Fig. 3B).

**Hyperglycemia and albumin permeability.** Albeit hyperglycemia is the most distinguished feature of ZDF rats, one must consider the possibility that complex metabolic alterations, and not hyperglycemia alone, can, in theory, play a role in the observed albumin hyperpermeability of ZDF rats. To answer this important question, we exposed the mesenteric microcirculation of nondiabetic ZL rats to glucose levels comparable with those found in ZDF rats. Figure 4 demonstrates that acute experimental hyperglycemia increases albumin leakage in nondiabetic postcapillary venules. The effects of D-glucose could not be attributed to changes in ambient osmolarity, as demonstrated by the fact that intraperitoneal administration of 25

**TABLE 1**  
The effect of calpain inhibition on urinary albumin excretion

Experimental group	Albumin/creatinine (mg/mg)	n
ZL	25.5 $\pm$ 1.5	5
ZDF + vehicle	298.9 $\pm$ 22.6*	5
ZDF + 27 $\mu$ g/kg ZLLal	167.3 $\pm$ 23.5†	5

Data are means  $\pm$  SEM. \* $P < 0.001$  vs. ZL rats; † $P < 0.01$  vs. untreated ZDF rats.

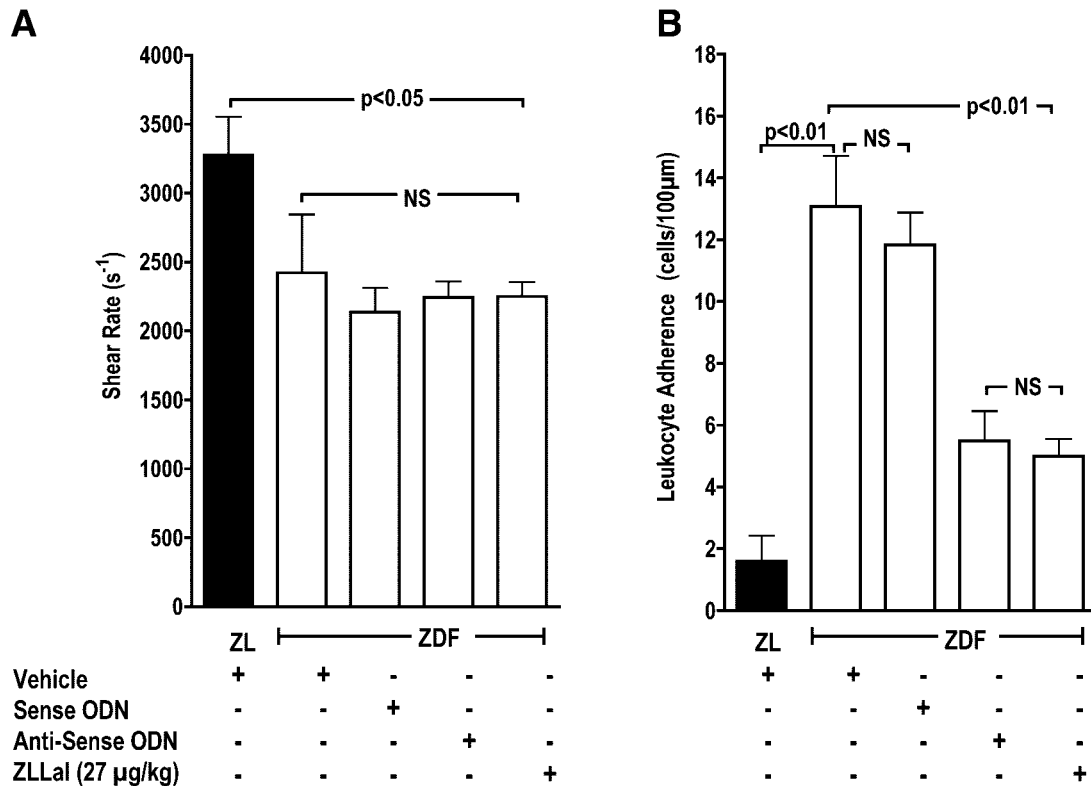


FIG. 3. Venular shear rate values are significantly reduced by hyperglycemia. **A:** Wall shear rate values were measured during IVM studies in mesenteric postcapillary venules of nondiabetic ZL (■) and diabetic ZDF (□) rats. Venular shear rates were significantly reduced in diabetic ZDF rats, albeit still found in the physiological range. No changes in venular shear rates were seen after the inhibition of calpain activity or its antisense depletion, indicating that inhibition of calpain does not affect blood flow velocity or vessel diameter in postcapillary venules. **B:** Baseline numbers of adherent leukocytes in all experimental groups of rats were quantified during IVM. Inhibition of calpain activity significantly attenuates leukocyte adhesion and extravasation in the diabetic microcirculation of ZDF rats. Data are expressed as means  $\pm$  SEM.  $n = 6-8$  rats (average) in each group.

mmol/l L-glucose did not increase albumin permeability (Fig. 4).

Thereafter, we studied whether the hypermeability response to acute experimental hyperglycemia was also calpain dependent. Figure 4 demonstrates that pharmacological inhibition of calpain activity completely prevented the increase in albumin permeability in response to D-glucose. Taken together, these data indicate that hyperglycemia alone is able to cause a calpain-dependent alteration of the endothelial cell barrier, which results in extravasation of circulating macromolecules into organ tissue.

**Role of leukocytes in the vascular permeability of hyperglycemia.** Leukocyte adhesion is an important determinant of vascular permeability in inflamed vessels. It is also widely accepted that PMN leukocytes are the leukocyte population largely responsible for increased albumin leakage in inflamed microvascular networks (29). We have demonstrated that increased leukocyte adhesion occurs in response to acute experimental hyperglycemia and diabetes and that this phenomenon is prevented by calpain inhibition (9,10). Accordingly, we embarked in further studies to understand whether the beneficial effect of calpain inhibition on microvascular permeability is secondary to its inhibitory action on PMN's adhesion.

The circulating pool of PMNs was depleted in control rats and rats injected with 25 mmol/l D-glucose by intraperitoneal injection of antineutrophil serum. Following antineutrophil serum treatment, the number of circulating

leukocytes was consistently reduced from the average value of  $1,900 \pm 70$  PMNs/ $\mu$ l to  $197 \pm 41$  PMNs/ $\mu$ l ( $P < 0.01$ ).

The effects of acute PMN depletion on leukocyte adhesion and albumin permeability in the hyperglycemic microcirculation were then studied by IVM. There was a marginal, but significant, attenuation of the basal vascular permeability following PMN depletion in control rats given antineutrophil serum alone (Fig. 4). This protective effect of antineutrophil serum on baseline vascular permeability is likely due to the prevention of nonspecific changes in the endothelial cell barrier caused by minimal activation of PMNs during the surgical procedures required for IVM. In contrast, acute elevation in ambient glucose dramatically increased vascular permeability (Fig. 4) and leukocyte adhesion (Fig. 5) in the microvasculature to levels comparable with those seen in chronically hyperglycemic ZDF rats (Figs. 1 and 3).

Leukocyte adhesion induced by D-glucose was drastically reduced by depletion of circulating PMNs, confirming previous observations that PMNs are the predominant leukocyte population that adheres in hyperglycemic microvascular networks, at least as seen by IVM (30) (Fig. 5). Of interest, the effect of high glucose on albumin permeability largely persisted even in the absence of adhered PMN (Fig. 4), which suggests that the vascular endothelium is a primary direct target of hyperglycemia. Similar results were observed following depletion of circulating leukocytes in ZDF rats (data not shown).

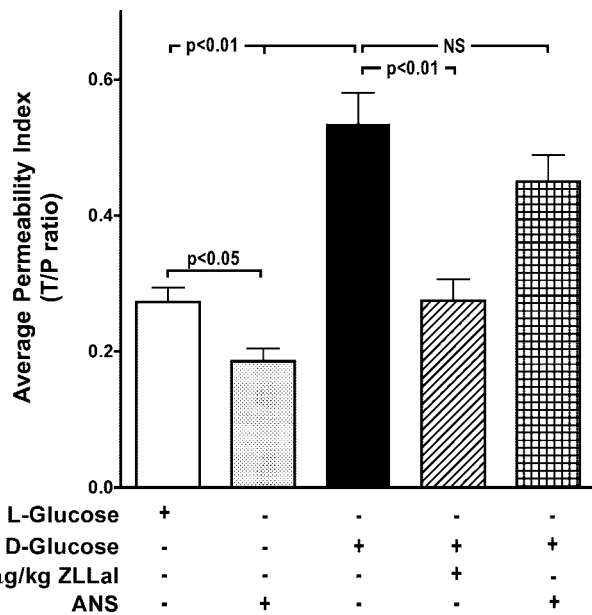


FIG. 4. Elevated ambient glucose increases albumin permeability in the nondiabetic microvasculature of ZL rats. The average permeability index to Texas Red-labeled albumin was calculated during IVM studies in mesenteric postcapillary venules of all experimental groups of ZL rats. A 12-h exposure of the mesenteric microcirculation to D-glucose, but not L-glucose, increases albumin permeability in a calpain-dependent manner. Depletion of circulating PMN leukocytes with antineutrophil serum (ANS) only marginally attenuated the increase in vascular permeability induced by D-glucose ( $P > 0.05$ ). In contrast, albumin permeability values were nearly normalized following inhibition of calpain activity. Data are expressed as means  $\pm$  SEM.  $n = 6-8$  rats (average) in each group.

These data further support the hypothesis that activation of calpain activity in hyperglycemia is responsible for primary alteration in the endothelial cell barrier, independent of overt neutrophil adhesion.

## DISCUSSION

Increased permeability to macromolecules is a well-described alteration of the diabetic microvasculature, which, along with inflammation, is causative of diabetes complications. Our data demonstrate that hyperglycemia increases albumin permeability via a  $\mu$ -calpain-dependent mechanism, independent of overt leukocyte adhesion.

The calpain system is a newly emerging signaling pathway in diabetic vascular disease. The gene encoding calpain-10 has now been associated with subclinical atherosclerosis in insulin-resistant humans (31). Studies have also demonstrated a role for calpains in key processes of inflammatory vascular remodeling, such as vascular smooth cell proliferation and migration (32) and platelet activation (33). We have recently reported that acute and chronic hyperglycemia increases leukocyte adhesion to the vascular endothelium of postcapillary venules and decreases nitric oxide production via a  $\mu$ -calpain-dependent mechanism (9,10); calpain inhibition prevents these actions and stabilizes basal levels of nitric oxide in the face of hyperglycemia and diabetes. Others have demonstrated that inhibition of calpain activity corrects endothelial dysfunction and penile nitric dysfunction in diabetic mice (11). Relevant to organ injury, overexpression of the endogenous calpain inhibitor calpastatin ameliorates renal injury and failure in diabetes (34). Finally, calpain has been implicated in the impaired nerve regenerations of hyperglycemic rats (12).

This study was undertaken to test whether calpain is implicated in the hypermeability of the hyperglycemic vasculature. Increased vascular permeability is commonly found in hyperglycemic vascular beds, and transcapillary escape of albumin is related to blood glucose control (35). Increased albumin permeability is a very early consequence of endothelial dysfunction in the diabetic retina (36), kidney (37), and rat mesentery (38). Overall, enhanced endothelial permeability leads to extravasation of plasma and macromolecules into the adjacent interstitial compartment. Chronic extravasation of albumin modifies the size and composition of the interstitium, which causes disturbances in the traffic of fluid and vital substrates to the cellular mass and in the removal of waste products in the opposite direction (6). Accordingly, capillary leakage contributes to the increased susceptibility to local and systemic injury of diabetic organs (39).

In the present study, we found evidence of a mechanistic link between  $\mu$ -calpain activity and albumin extravasation in the hyperglycemic microcirculation. One particularly intriguing aspect of our results is the fact that the effect of hyperglycemia on albumin permeability was found to be independent of leukocyte adhesion. It is widely accepted that the adhesion of leukocytes to the vascular endothelium is almost invariably associated with increased vascular permeability (40). Leukocyte adhesion is increased by hyperglycemia and diabetes (9,41). Inhibition of calpain activity prevents adhesion of leukocytes in the hyperglycemic microcirculation (10). Thus, it was reasonable to expect that the effect of calpain inhibition on vascular permeability would be secondary to reduced leukocyte-endothelium interactions. To the contrary, we found that leukocyte adhesion did not play an obligatory role in the albumin hyperpermeability of the hyperglycemic vasculature. Nonetheless, it should be noted that our study tested only the impact of acute neutrophil depletion on the

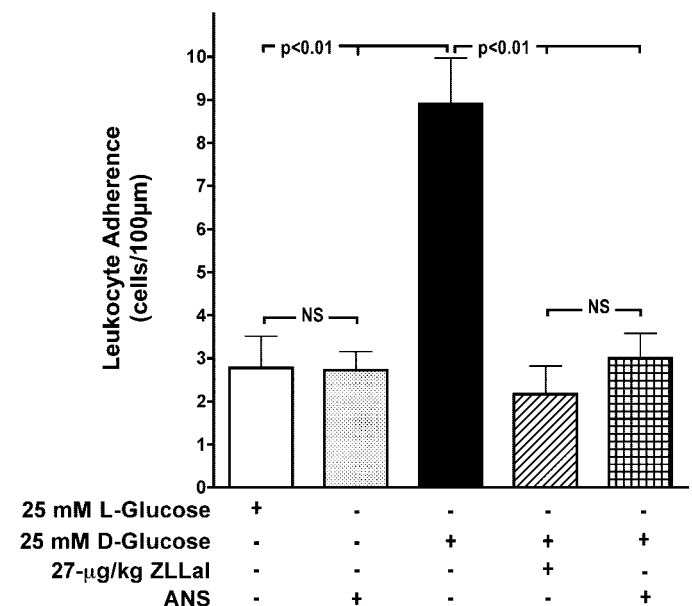


FIG. 5. Elevated ambient glucose increases the adhesion of circulating PMN leukocytes in the microcirculation of ZL rats. D-glucose levels in the range of those found in spontaneously hyperglycemic ZDF rats significantly increase the number of PMN leukocytes firmly adhering to the vascular endothelium of the microvasculature. Glucose-induced leukocyte adhesion was inhibited by calpain inhibition and PMN leukocyte depletion with antineutrophil serum (ANS). Data are expressed as means  $\pm$  SEM.  $n = 6-8$  rats (average) in each group.

hyperpermeability of the hyperglycemic vasculature. Thus, our results cannot entirely rule out the possibility that neutrophil-derived mediators that could have become bound to the vascular endothelium were, in part, still responsible for sustained experimental activation and vascular hyperpermeability under our experimental conditions. In support of this view, recent work has emphasized a role for endothelial-bound neutrophil-derived myeloperoxidase in the mechanism of endothelial dysfunction in humans with cardiovascular disease (42).

Under normal and pathologic conditions, several signaling pathways regulate the permeability of the vascular endothelium, and they have been recently reviewed by Mehta et al. (43). Relevant to diabetes, future research should focus on the roles of protein kinase C (PKC) and endothelial nitric oxide because of their relevance to vascular complications. In fact, several studies have implicated a role for PKC in the hyperpermeability of diabetes (44–46), and others have demonstrated the existence of cross-talk regulation between calpain and PKC (47).

The role of endothelial nitric oxide in the modulation of vascular permeability still remains controversial (43). However, recent work has suggested that basal endothelial nitric oxide is necessary to prevent albumin leakage through the vascular endothelium (48). Interestingly, we have reported that calpain reduces basal availability of endothelial nitric oxide in experimental hyperglycemia and diabetes (9,10). Overall, very little is available in scientific literature on the role of calpains in the regulation of endothelial cell barrier. Two recent studies have reported that under physiologic conditions, baseline  $\mu$ -calpain activity is important in maintaining the integrity of the endothelial cell barrier (15), whereas in vascular inflammation, increased calpain activity increases endothelial permeability (14). Obviously, further studies are needed to fully understand the molecular mechanism by which calpain increases vascular permeability during hyperglycemia and diabetes.

In terms of clinical application, albuminuria is now considered an independent risk marker for macro- and microvascular complications in diabetes (7). It has been hypothesized that loss of albumin through the kidneys reflects a generalized status of endothelial cell dysfunction in the hyperglycemic patient (49), acting through increased levels of biomarkers of inflammation (50). Conversely, decreasing albumin leakage is associated with improving cardiovascular and renal outcomes in diabetic patients (7). Our results support this view by demonstrating that a widespread calpain-dependent increase in albumin leakage occurs both in splanchnic circulation and in the kidney. Accordingly, calpains could represent an important molecular marker and pharmacological target for the prevention and treatment of diabetic vascular complications.

We have provided evidence that the inhibition of calpain activity attenuates the vascular hyperpermeability associated with acute and chronic hyperglycemia. These findings uncover a role for the calcium-dependent protease calpain in the pathophysiology of diabetic vascular disease.

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