The Calcium-Dependent Protease Calpain Causes Endothelial Dysfunction in Type 2 Diabetes

Timothy J. Stalker, Yulan Gong, and Rosario Scalia

Cardiovascular complications are the leading cause of morbidity and mortality in diabetic patients. Endothelial dysfunction with impaired endothelial nitric oxide (NO) synthase (eNOS) activity is a widely accepted cause of diabetic vasculopathy. The mechanisms of endothelial dysfunction in diabetes remain elusive, thus limiting effective therapeutic interventions. We report novel evidence demonstrating that the calcium-dependent protease calpain causes endothelial dysfunction and vascular inflammation in the microcirculation of the ZDF (Zucker diabetic fatty) rat, a genetic rat model of type 2 diabetes. We found evidence of increased calpain activity and leukocyte trafficking in the microcirculation of ZDF rats. Inhibition of calpain activity significantly attenuated leukocyte-endothelium interactions in the vasculature of ZDF rats. Expression of cell adhesion molecules in the vascular endothelium of ZDF rats was consistently increased, and it was suppressed by calpain inhibition. In vivo measurement of endothelial NO availability demonstrated a 60% decrease in NO levels in the microcirculation of diabetic rats, which was also prevented by calpain inhibition. Immunoprecipitation studies revealed calpain-dependent loss of association between eNOS and the regulatory protein heat shock protein 90. Collectively, these data provide evidence for a novel mechanism of endothelial dysfunction and vascular inflammation in diabetes. Calpains may represent a new molecular target for the prevention and treatment of diabetic vascular complications. *Diabetes* **54:**1132–1140, 2005

The prevalence of type 2 diabetes is increasing dramatically in the U.S., with the number of patients diagnosed with type 2 diabetes escalating across all age-groups (1). Although vascular complications are the leading cause of morbidity and mortality in diabetic patients (1), the mechanisms responsible for diabetic vascular dysfunction remain poorly understood. Metabolic hallmarks of type 2 diabetes include hyperglycemia, obesity, and dyslipidemia, all of which are known risk factors for the development of vascular disease. All of these factors, especially hyperglycemia, have been associated with endothelial dysfunction (2). Overall, it is widely accepted that the endothelial dysfunction present in diabetic patients is a primary contributing factor to vascular complications.

Several studies in both animals (3,4) and humans (5,6) have demonstrated that hyperglycemia/diabetes causes endothelial dysfunction characterized by a loss of endothelium-derived nitric oxide (NO) and increased oxidative stress. Endothelial NO is an important regulator of vascular homeostasis. Loss of NO results in endothelial dysfunction, leading to increased vascular tone and abnormal endothelial adhesiveness, which increases platelet aggregation and leukocyte trafficking at the vessel wall (7). Thus, the diabetic vasculature experiences increased oxidative stress and abnormal inflammatory signals (8). Among other approaches, the mechanisms responsible for the loss of NO in the hyperglycemic vasculature are being intensely investigated with the aim to uncover novel therapeutic strategies for the treatment of diabetic vasculopathies.

The calpains are a family of calcium-dependent proteases that have previously been implicated in the pathophysiology of several inflammatory disorders of the cardiovascular system, including myocardial reperfusion injury (9), cerebral ischemia/reperfusion (10), and circulatory shock (11). In the current study, we have used the ZDF (Zucker diabetic fatty) rat, a genetic rat model of type 2 diabetes (12), to test the hypothesis of a role for calpains in the endothelial dysfunction of diabetes.

RESEARCH DESIGN AND METHODS

This study was performed in accordance with the National Institutes of Health and Thomas Jefferson University institutional animal care and use committee guidelines for the use of experimental animals. We used ZDF rats (Charles River Laboratories, Noblesville, IN) of 10–14 weeks of age, the age range at which they develop overt diabetes with hypoinsulinemia and permanent hyperglycemia (12). ZDF rats have a homozygous mutation in their leptin receptor gene (*fa/fa*) that causes them to develop a metabolic disorder similar to type 2 diabetes in humans (12). Zucker lean (ZL) rats are either homozygous wild-type or heterozygous at the leptin receptor gene (+/?), do not develop diabetes, and are used as control animals because of the common genetic background. Rats were divided into one of three groups: *1*) control ZL rats injected with vehicle, *2*) ZDF rats injected with vehicle, and *3*) ZDF rats injected with 27 μg/kg i.p. calpain inhibitor benzyloxycarbonyl-leucyl-leucinal (ZLLal) once a day for 5 consecutive days. The microcirculation of the mesentery was used for these studies because it is highly amenable to intravital microscopy and because it expresses a typical inflammatory phenotype in response to both hyperglycemia (13) and diabetes (14). In a preliminary study, administration of ZLLal 60 min before intravital microscopy, as well as 120 min local superfusion of ZLLal during intravital microscopy

experiments, failed to inhibit leukocyte-endothelium interaction in the diabetic microcirculation (data not shown).

Western blot analysis of calpain activity in the rat mesentery in vivo. Mesenteric segments with dense microvascular networks were dissected under microscopy. Sections were snap-frozen in liquid nitrogen and homogenized as previously described (15). Calpain activity was studied by Western blot analysis using polyclonal antibodies against either the NH_2 terminus domain (RP1 calpain-1 or RP2 calpain-2) or domain IV (RP3 calpain-1 and RP3 calpain-2; Triple Point Biologics, Portland, OR) of the large subunit of m- and μ-calpain, respectively. Once activated, m- and μ-calpains autolytically cleave their NH_2-terminal ends, respectively, resulting in the loss of NH_2 terminus antibody recognition, which can be used as a measure of calpain activation. Quantification of the stable domain IV was used to measure total m- and μ-calpain content in mesenteric extracts. Proteins were detected by chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL) and quantified by laser densitometry (personal densitometer; Molecular Dynamics, Piscataway, NJ).

Intravital microscopy. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and prepared for intravital microscopy as previously described (13). Leukocyte rolling, adhesion, and transmigration were studied in mesenteric postcapillary venules. Leukocyte rolling is defined as the number of leukocytes rolling past a fixed point per minute; leukocyte adherence is defined as the number of leukocytes firmly adhered to a 100-μm length of endothelium for at least 30 s. The number of leukocytes extravasated within a 5-μm perivascular area were counted and normalized with respect to the area. Erythrocyte velocity was determined online using an optical Doppler velocimeter obtained from the Microcirculation Research Institute (College Station, TX). Erythrocyte velocity (V_{rbc}) and venular diameter (D) were used to calculate the venular wall shear rate (g) using the formula: $g = 8(V_{mean}/D)$, where $V_{mean} = V_{rbc}/1.6$.

Immunohistochemistry. At the completion of intravital microscopy experiments, sections of mesentery and ileum were fixed in vivo, dehydrated using graded acetone washes, and embedded in plastic as previously described (13). Immunohistochemical localization of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) was accomplished using monoclonal antibodies (BD Transduction Laboratories, San Diego, CA) and the avidin/biotin immunoperoxidase technique. The percentage of positively staining venules was determined by examining 5–10 tissue sections per rat, with 50 venules analyzed per tissue section.

NO release from rat mesenteric venules. NO levels in mesenteric postcapillary venules were measured during intravital microscopy experiments, using an ISO-NO microelectrode (100-nm diameter at the tip) connected to an ISO-NO Mark-II NO meter (World Precision Instruments, Sarasota, FL), according to a previously described technique (13,16). Briefly, the microelectrode was positioned directly over a mesenteric postcapillary venule at a distance of <5 μm from the venular wall. To calculate the amount of NO released by the vascular endothelium, background currents obtained at a distance of 0.5–1 cm from the venular wall were subtracted from currents measured at the venular wall. Endothelial NO values were normalized to the size of the blood vessel studied and reported as nanomolar NO/1,000 μm^2 vessel area.

Association of endothelial NO synthase and heat shock protein 90. The association of endothelial NO synthase (eNOS) and heat shock protein 90 (hsp90) in the mesentery of all experimental groups of rats was studied by immunoprecipitation and Western blotting according to a technique described previously (15). Briefly, mesentery extracts containing ~750 μg protein were incubated with anti-eNOS monoclonal antibody in immunoprecipitation buffer for 1 h followed by incubation with prewashed protein G-agarose (Sigma) for 2 h. The resulting pellet was washed three times in PBS, boiled in SDS sample buffer, and resolved by SDS-PAGE. Immunoblot analysis was performed using primary antibodies against eNOS (BD Transduction) and hsp90 (Santa Cruz Biotechnology, Santa Cruz, Ca).

Isolation of mesenteric microvascular endothelial cells and quantification of calpain in vitro. Densely vascularized segments of mesentery were isolated. Mesenteric microvascular endothelial cells (MMECs) were then isolated using magnetic microbeads (Miltenyi Biotech, Auburn, CA) ligated with anti-rat platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody (17). MMECs were phenotyped by fluorescence-activated cell sorting analysis of PECAM-1 and by functional uptake of acetylated LDL. For fluorescence-activated cell sorting studies, MMECs were incubated with monoclonal CD31 antibody conjugated with phycoerythrin (BD Pharmingen) for 30 min at 4°C and analyzed using a Becton Dickinson flow cytometer (17). Uptake of labeled acetylated LDL (10 μg/ml; Biomedical Technologies, Stoughton, MA) by MMECs was visualized by fluorescent microscopy.

Rat MMECs were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 150 μg/ml endothelial cell growth supple-

TABLE 1
Blood glucose levels and main hemodynamic values

	n	Blood glucose (mmol/l)	Mean arterial blood pressure (mmHg)	Venular shear rate (s^{-1})
ZL rat	5	5.5 ± 0.2	126.6 ± 4.4	581 ± 60.1
ZDF rat	5	17.2 ± 1.4*	125.8 ± 3.0	335 ± 26.7†
ZDF rat + 27 μg/kg ZLLal	5	22.0 ± 3.7*	128.5 ± 2.1	461 ± 88.2

*$P < 0.01$, †$P < 0.05$ vs. ZL rat.

ment (Sigma, St. Louis, MO) on gelatin-coated tissue culture dishes. A twofold approach was used to minimize changes in the diabetic phenotype of MMECs. First, we only used primary or second-passage MMEC cultures. Second, glucose concentrations in the culture medium were adjusted to match the average glucose levels detected in the plasma of donor ZDF rats (Table 1). Thus, MMECs from ZL rats were cultured in 5.5 mmol/l D-glucose (normal glucose) medium, and MMECs from ZDF rats were cultured in 20 mmol/l D-glucose. In additional control experiments, MMECs from nondiabetic ZL rats were incubated with 20 mmol/l D-glucose.

Calpain activity in intact MMECs was determined with the highly sensitive fluorescent calpain substrate t-BOC-Leu-Met-CMAC (Molecular Probes, Eugene, OR), as described previously (18). Mean fluorescence signals from MMECs grown to confluence in a 24-well plate were measured using a Cyto-Fluor plate reader (excitation $\lambda = 360$ nm, emission $\lambda = 460$ nm). Specificity of the t-BOC for calpain was confirmed by both pharmacological inhibition and antisense depletion of calpain activity. Sense (ATGCTTCTCGGGCACAAT) and antisense (ATTGTGCCCGAGAAGCAT) thioated μ-calpain oligonucleotides were obtained from MWG (High Point, NC).

Pharmacological inhibition of calpain activity. The calpain inhibitor ZLLal (Biomol Research Laboratories, Plymouth Meeting, PA) was used because, in the concentration range used in this study, it has highest selectivity for calpains than for other proteosomal enzymes (19). 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 in rats did not affect baseline leukocyte rolling, adherence, or transmigration (data not shown).

Data analysis. Data are presented as the means ± SE and compared by ANOVA with post hoc analysis by Fisher's corrected t test. Probabilities of ≤0.05 were considered statistically significant.

RESULTS

Blood glucose levels and hemodynamics in ZDF rats. Compared with nondiabetic ZL rats, diabetic ZDF rats displayed fasting hyperglycemia that was not lowered by calpain treatment (Table 1). Mean arterial blood pressures were not significantly different among the three groups of rats studied (Table 1). However, venular shear rate values in postcapillary venules of ZDF rat mesenteries were significantly decreased, despite remaining within physiological range (Table 1). This finding is in agreement with previous studies demonstrating decreased shear rates (14) and increased vascular permeability (20) in the microcirculation of diabetic rats. Thus, 10- to 14-week-old ZDF rats develop overt hyperglycemia and hemodynamic perturbations in the microcirculation that are highly relevant to diabetic vascular disease.

Assessment of calpain activity in ZDF rats. To investigate the effect of diabetes on calpain activity in vivo, we measured autoproteolysis of m- and μ-calpain, a well-established indicator of calpain proteolytic activity in biological systems (21). On activation, calpains undergo autoproteolysis with removal of the NH_2 9–14 amino acids from the large 80-kDa subunit (22,23). Accordingly, evaluation of m- and μ-calpain autoproteolysis in freshly isolated vascular segments of mesenteric tissue from ZL and ZDF rats was assessed by immunoblot analysis, using primary antibodies to the NH_2 terminus domains of the m-
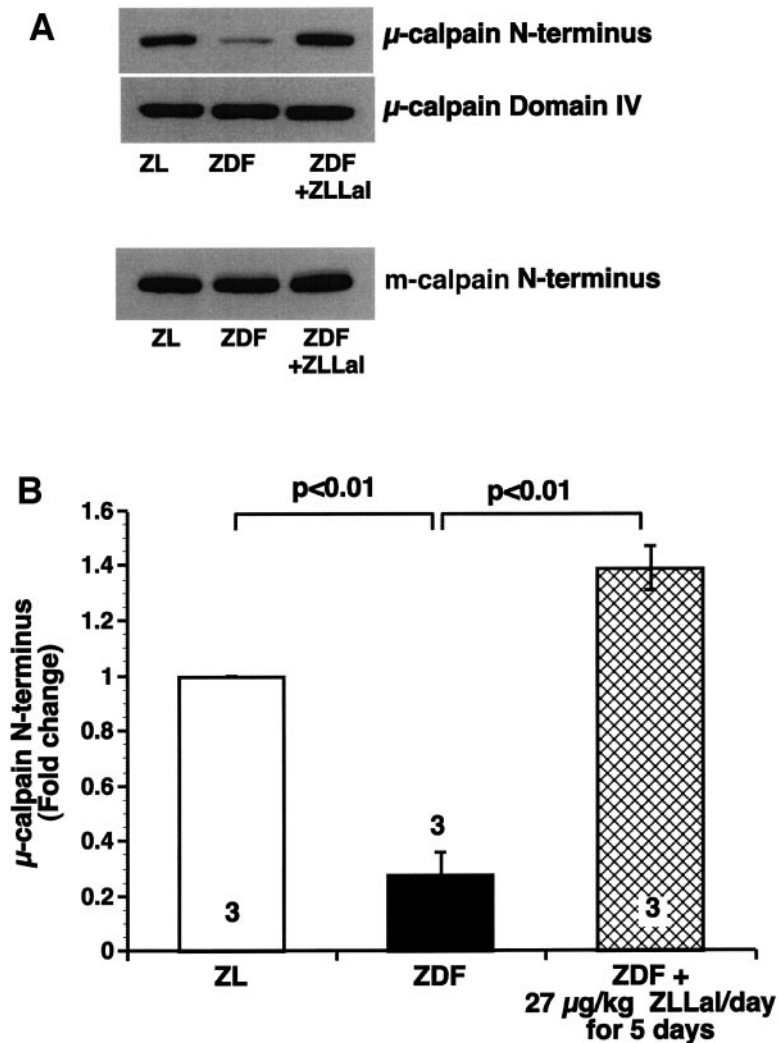
FIG. 1. *A*: μ-Calpain activity was assessed by immunoblot analysis using a primary antibody that recognizes the μ-calpain NH_2 terminus domain. Increased calpain activation is demonstrated by loss of antibody recognition. A primary antibody against μ-calpain domain IV, which recognizes both inactive and active μ-calpain, was used to quantify total μ-calpain content. A primary antibody against the *m*-calpain NH_2 terminus domain was used to assess *m*-calpain activity. *B*: Densitometric analysis of μ-calpain NH_2 terminus domain autolysis. Numbers at the base of the columns indicate the number of rats studied in each group.

and μ-calpain large subunits (Fig. 1*A*). We found increased proteolytic activity of μ-calpain in ZDF rats, as demonstrated by 75% loss in NH_2 terminus antibody binding (Fig. 1). Administration of 27 μg/kg i.p. of the calpain inhibitor ZLLal to ZDF rats for 5 consecutive days completely blocked μ-calpain autoproteolysis in the rat mesentery (Fig. 1). Total μ-calpain content was quantified using a primary antibody against domain IV of the large subunit, which recognizes both unautolyzed and autolyzed μ-calpain. No significant differences in total μ-calpain protein expression were found in ZDF rats (Fig. 1*A*).

In contrast, the proteolytic activity of *m*-calpain was not increased in ZDF rat mesenteries, as demonstrated by a lack of changes in the *m*-calpain NH_2 binding domain expression level (Fig. 1). No significant changes were observed in total *m*-calpain expression, as detected using a primary antibody against *m*-calpain domain IV (data not shown). These findings demonstrate that type 2 diabetes selectively increases the proteolytic activity of μ-calpain in densely vascularized tissues.

Intravital microscopy. Nondiabetic ZL rats exhibited a low baseline leukocyte rolling value of 24.1 ± 2.4 cells/min in mesenteric postcapillary venules (Fig. 2*A*). In contrast, ZDF rats had a threefold increase in leukocyte rolling ($P < 0.05$ vs. ZL). Injection of 27 μg/kg i.p. ZLLal once a day for 5 consecutive days to ZDF rats attenuated leukocyte

rolling to 47.8 ± 10.0 cells/min, a value that did not reach statistical significance.

There were a low number of leukocytes firmly adhering to the vascular endothelium of mesenteric postcapillary venules in ZL rats (Fig. 2*B*). Leukocyte adherence was increased fivefold in ZDF rats ($P < 0.01$ vs. ZL rats). Administration of 27 μg/kg i.p. ZLLal once a day for 5 days significantly attenuated the number of adherent leukocytes in mesenteric postcapillary venules of ZDF rats (Fig. 2*B*).

The number of leukocytes that had transmigrated into the perivascular area was also studied. Few leukocytes were found in the perivascular area of postcapillary venules in nondiabetic ZL rats (Fig. 2*C*). In contrast, increased numbers of transmigrated leukocytes were observed within 5 μm of the postcapillary venular wall in ZDF rats ($P < 0.01$ vs. ZL rats). Inhibition of calpain activity with ZLLal greatly reduced the number of leukocytes extravasated in the mesentery of ZDF rats (Fig. 2*C*).

These data demonstrate that the diabetic microcirculation of ZDF rats experiences chronic inflammatory signals with increased leukocyte trafficking and that inhibition of calpain activity acutely attenuates vascular inflammation in the face of chronic hyperglycemia.

Immunohistochemistry. To investigate the molecular mechanisms of increased leukocyte trafficking interactions in the diabetic microcirculation, we studied endothe-
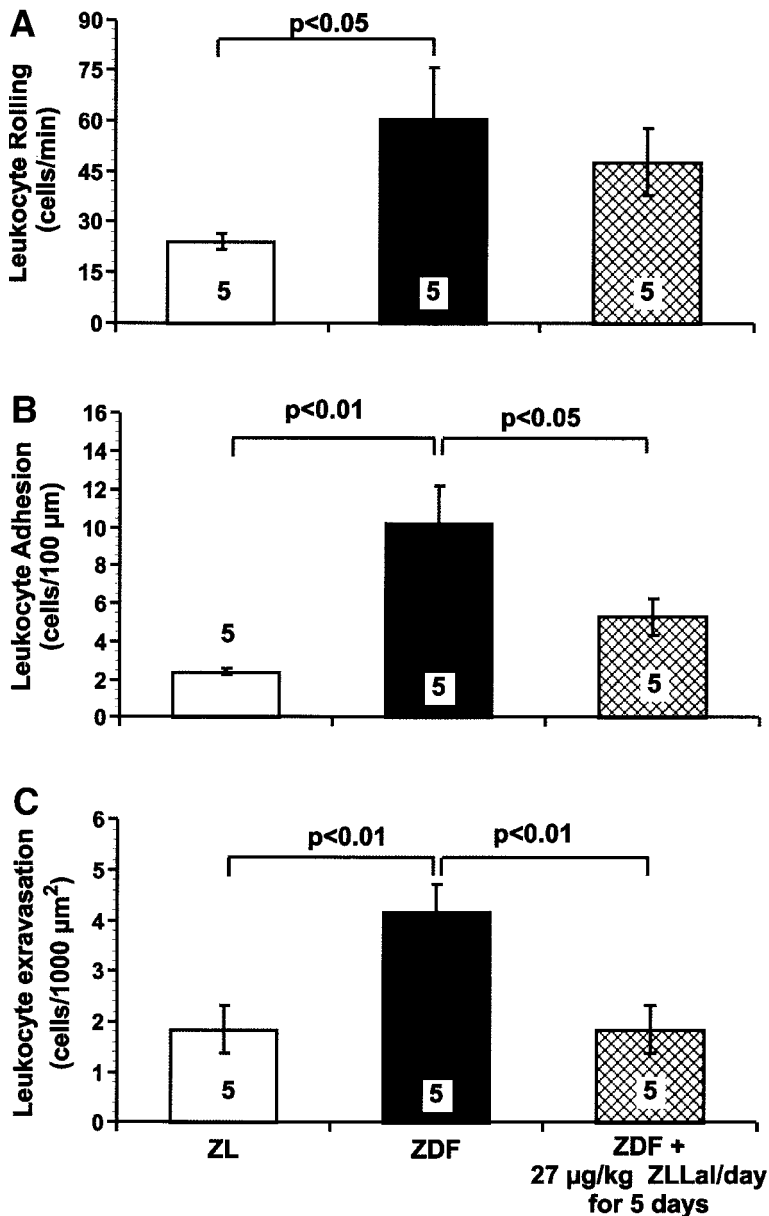
FIG. 2. Baseline number of rolling (*A*), adherent (*B*), and extravasated leukocytes (*C*) in all experimental groups of rats was quantified after a 60-min stabilization period. Inhibition of calpain activity significantly attenuates leukocyte adhesion and extravasation in the diabetic microcirculation of ZDF rats. Data are expressed as the means ± SE. Numbers at the base of the columns indicate the number of rats studied in each group.

lial cell surface expression of ICAM-1 and VCAM-1 (Fig. 3), two cell adhesion molecules relevant to vascular inflammation and atherogenesis. Compared with nondiabetic ZL rats, expression of ICAM-1 increased 2.5-fold in the vascular endothelium of ZDF rats ($P < 0.01$), and it was attenuated to control values by ZLLaL treatment ($P < 0.01$ vs. untreated ZDF rats). Similarly, we found evidence of a 2.8-fold increase in VCAM-1 expression in the vascular endothelium of ZDF rats. Treatment of ZDF rats with the calpain inhibitor ZLLal attenuated VCAM-1 expression levels to control values ($P < 0.01$ vs. untreated ZDF rats). These data demonstrate that increased calpain activity plays a role in the upregulation of endothelial cell adhesion molecules in diabetes.

Basal release of endothelial NO in postcapillary venules of ZDF rats. Endothelial NO suppresses VCAM-1 expression at the molecular level (24). Accordingly, we measured levels of endothelial NO in mesenteric postcapillary venules to further study the molecular mechanisms of vascular dysfunction in diabetes. We found that levels of NO decreased by 2.4-fold in the inflamed postcapillary venules of ZDF rats (Fig. 6). Inhibition of calpain activity with ZLLal significantly restored endothelial NO levels in mesenteric venules of ZDF rats ($P < 0.05$ vs. untreated ZDF rats) (Fig. 4). Thus, loss of endothelial NO in the diabetic vasculature is at least in part calpain dependent.

Association of eNOS and hsp90. Previous studies have indicated that increased calpain activity impairs NO production by decreasing the association of the regulatory protein hsp90 with the eNOS complex (25). Accordingly, we measured total hsp90 content in the mesentery of ZL and ZDF rats using Western blot analyses. We also performed immunoprecipitation studies to determine the extent of eNOS/hsp90 association in vascular segments of the ZDF rat mesentery. Densitometric analysis revealed a $32 \pm 1.7\%$ reduction in hsp90 expression levels in ZDF rat mesenteries, compared with nondiabetic ZL rats ($P < 0.05$). Furthermore, less hsp90 was associated with eNOS in the vasculature of ZDF rats as compared with ZL rats (Fig. 5). Inhibition of calpain activity signifi-
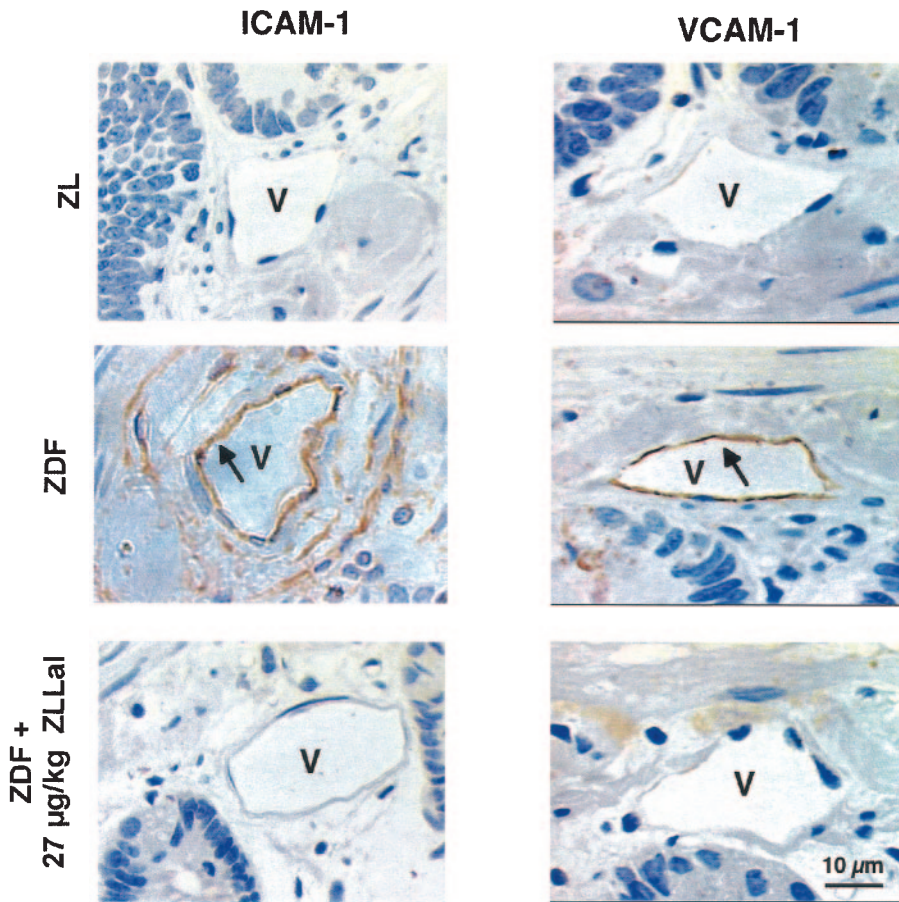
FIG. 3. Representative photomicrographs illustrating the effect of calpain inhibition on ICAM-1 (*left panels*) and VCAM-1 (*right panels*) expression in rat ileal venules. Brown immunoperoxidase reaction product (arrows) indicates positive staining. ICAM-1 and VCAM-1 were significantly upregulated in the vascular endothelium of ZDF rats (*middle panels*). Calpain inhibition attenuates endothelial cell surface expression of ICAM-1 and VCAM-1 in diabetic rats (*lower panels*). V, venule.

cantly attenuated the loss of hsp90 from the eNOS complex (Fig. 5). These data demonstrate that increased calpain activity impairs the posttranslational assembly of eNOS in the diabetic vasculature, and they also correlate with the evidence of calpain-dependent loss of NO illustrated in Fig. 4.

Increased calpain activity in MMECs isolated from diabetic ZDF rats. Measuring of calpain activity in mesenteric tissue homogenates does not provide absolute evidence of increased calpain activity in the vascular endothelium. We isolated MMECs from ZL and ZDF rats to further confirm that diabetes does increase calpain activity
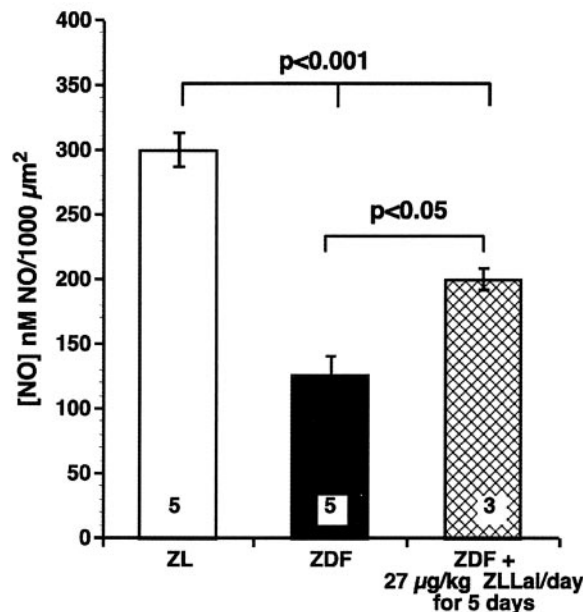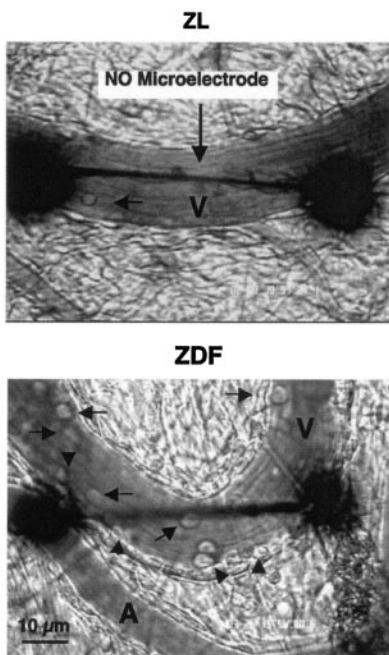

FIG. 4. Measurement of NO in mesenteric venules from ZL (*upper left panel*) and ZDF (*lower left panel*) rats by an NO polarographic microelectrode. The NO electrode was placed parallel to the long axes of the venule and advanced to a 5-μm distance from the vessel wall under direct microscopy observation. Large black dots are the optical projection of the optical Doppler velocimeter used to measure blood flow velocity and venular shear rates during intravital microscopy. Arrows indicate rolling and adhering leukocytes. All values are means ± SE. Basal NO release is expressed as nanomolar NO/1,000 μm². Numbers at the base of the columns indicate the number of rats studied in each group.
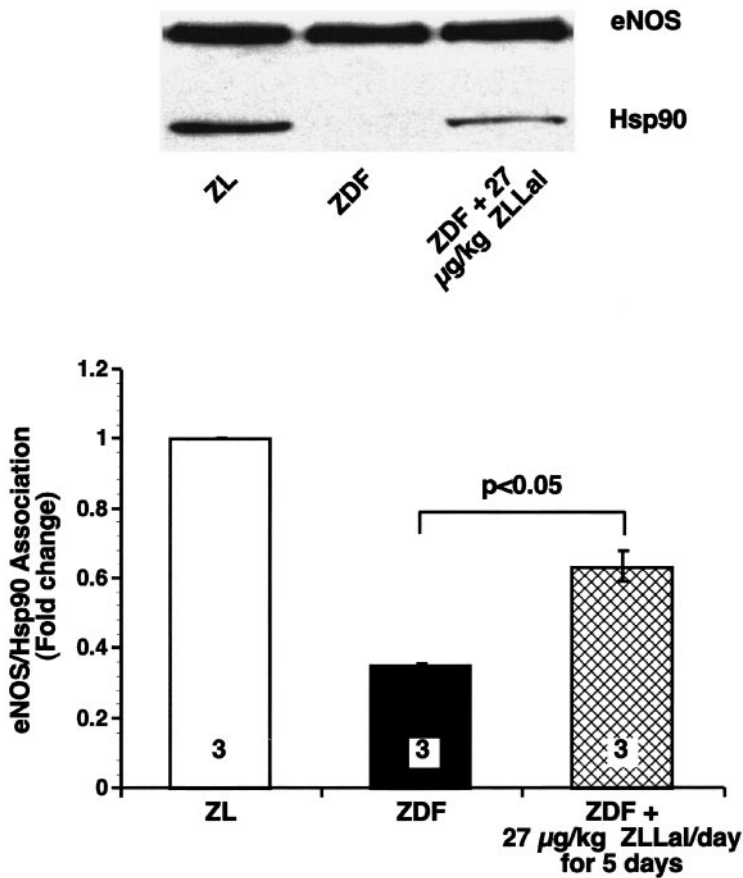
FIG. 5. Representative immunoblot from immunoprecipitation experiments examining the association of eNOS and hsp90. *C*: Densitometric analysis of the association of hsp90 with eNOS. All values are means ± SE. Numbers at the base of the columns indicate the number of rats studied in each group.

in the endothelium of the microcirculation. Microvascular endothelial cells were phenotyped by uptake of acetylated LDL and flow cytometry analyses of PECAM-1 (Fig. 6). Calpain activity in normal and diabetic MMECs was measured with the use of the fluorescent probe *t*-BOC (Fig. 7 *upper panel*). We detected a 70% increase in basal calpain activity in MMECs isolated from ZDF rats, compared with MMECs from nondiabetic ZL rats (Fig. 7, *lower panel*). In additional control experiments, a comparable degree of calpain activation in the range of 1.6 ± 0.19-fold change was detected in nondiabetic MMECs incubated with 20 mmol/l D-glucose for 48 h ($P < 0.05$ vs. nondiabetic MMECs cultured under normal glucose conditions). This result points to hyperglycemia as the leading cause of increased calpain activity in the diabetic vasculature.

Treatment of diabetic MMECs with 10 µmol/l antisense oligonucleotides to µ-calpain for 48 h attenuated calpain activity to values found in nondiabetic control MMECs (Fig. 7). A comparable degree of calpain activity attenuation was also obtained after treatment of diabetic MMECs with ZLLal for 24 h (Fig. 7), a result that strengthens the significance of our in vivo data. Taken together with the biochemical results reported in Fig. 1, these data provide strong evidence that hyperglycemia and diabetes increase calpain activity in the vascular endothelium.

DISCUSSION

The present study demonstrates a novel role for the calcium-dependent protease calpain in the pathophysiology of diabetic vascular disease. We provide novel evidence implicating µ-calpain in the endothelial dysfunction and vascular inflammation of the ZDF rat, a genetically occurring animal model of type 2 diabetes.

Oxidative stress, upregulation of endothelial cell adhesion molecules, and leukocyte trafficking has been linked with damage to blood vessels and organ tissue in diabetes (8,26,27). In vivo studies have demonstrated that accumulation of adherent leukocytes is increased in the aortic endothelium of alloxan-induced diabetic rabbits (28) and in the retinal microcirculation of diabetic rats (27), where capillary occlusions by leukocytes appears to precede destruction of the retinal capillary bed (29). The relationship between diabetes and cell surface expression of adhesion molecules has been and continues to be the subject of several in vivo and in vitro studies. Upregulation of cell adhesion molecules occurs in spontaneously hyperglycemic mice (30). Circulating levels of soluble ICAM-1 and VCAM-1 are increased in diabetic patients (31) and in response to transient hyperglycemia (6). Monocytes isolated from diabetic patients are more adhesive to cultured human endothelium (32), and they are in a proinflammatory state (33). Overall, the proinflammatory effects of diabetes on endothelial cell adhesion molecules in humans is associated with glucose-driven oxidative stress (34) and reduced release of NO (2,8).

It is now well appreciated that inflammation of the microcirculation can increase the risk of developing cardiovascular disease and atherosclerosis (35). Thus, inflammatory microangiopathy is considered an additional contributing factor to accelerated atherogenesis in diabetes. In fact, infiltration of leukocytes through the micro-
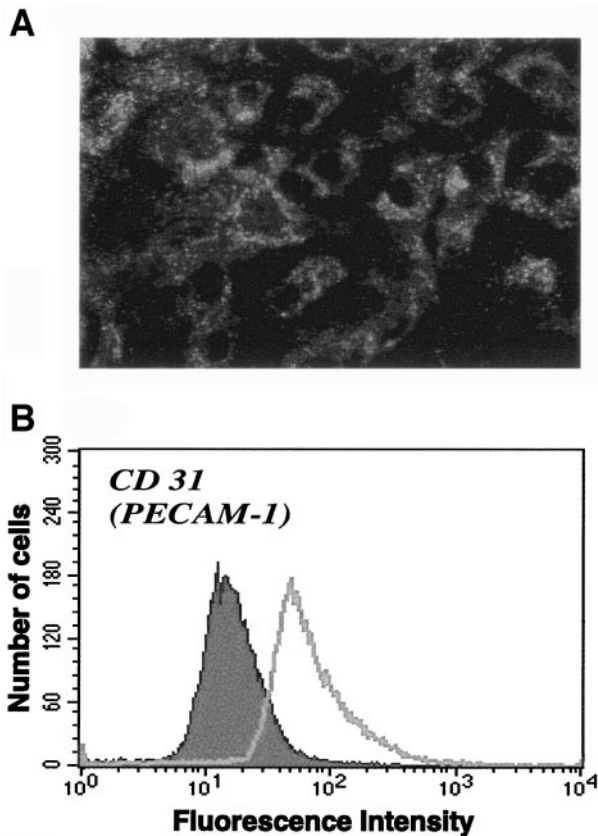
FIG. 6. The phenotype of isolated MMECs was verified by uptake of acetylated LDL (*A*) and PECAM-1 staining (*B*). First-passage MMECs were 98 and 99% positive to acetylated LDL and PECAM-1 staining, respectively.

vascular network of vasa vasorum may actually favor development of atherosclerosis (36).

Indeed, the cellular mechanisms responsible for the upregulation of leukocyte-endothelium interactions in the hyperglycemic vasculature remain elusive, which limits therapeutic interventions in diabetic patients. In the present study we provide novel evidence linking the calcium-dependent cysteine protease calpain with increased leukocyte trafficking in a relevant animal model of type 2 diabetes.

The calpains are a family of calcium-dependent proteases that act independently of the proteosome pathway and cleave a number of cellular substrates, including kinases, phosphatases, transcription factors, and cytoskeletal proteins (37). We focused our research on the well-characterized, ubiquitously expressed μ- and m-calpain isoforms, which have been isolated in a protein form. We found that diabetes selectively increases μ-calpain activity in vascular sections of the rat mesentery as well as in microvascular endothelial cells. The difference in isoform activation observed in the current study may be related to selective regulation of calpains under different pathological conditions. In support of this view, it has been suggested that whereas m-calpain is activated in response to large calcium fluxes, such as during apoptosis, μ-calpain may be more important in the setting of cell signaling under physiological and pathological conditions (21). Future studies addressing the signaling cascade(s) responsible for calpain activation in states of insulin resistance and

diabetes may offer additional insight on the contribution of calpains to endothelial dysfunction and vascular disease.

Our study demonstrates that increased levels of endothelial NO and attenuated expression of ICAM-1 and VCAM-1 are key mechanisms of the anti-inflammatory action of calpain inhibition in the diabetic vasculature in vivo. Of note, it has been established in the literature that obesity, insulin resistance, and type 2 diabetes cause vascular inflammation with endothelial dysfunction (8), loss of endothelial NO (2), and activation of circulating leukocytes (27,33). Activated leukocytes adhere to the vascular endothelium, where they quench NO, increase oxidative stress, and release proinflammatory cytokines. Given the important role played by oxidative stress in the cardiovascular complications of obesity and diabetes (33,34), further studies are needed to better understand the impact of calpain inhibition on the production of oxidant species and inflammatory cytokines in the vascular wall during states of hyperglycemia and insulin resistance.

There is a general consensus in the literature that endothelial NO exerts potent anti-inflammatory effects in the cardiovascular system. Accordingly, endothelial dysfunction with impaired release of NO has been demonstrated in experimental animal models of hyperglycemia (4,13) and diabetes (14,30), as well as in diabetic humans (2,5). Notable to diabetes, insulin exerts important anti-inflammatory effects during acute experimental hyperglycemia of the microcirculation in the rat (13). In human endothelial cells, insulin increases release of NO (38), induces eNOS expression (39), and suppresses levels of ICAM-1 (40). Interestingly, the NO-potentiating effect of insulin is inhibited by tumor necrosis factor-α, a leukocyte-derived proinflammatory cytokine elevated in states of insulin resistance (41). Thus, it is likely that recurrent transient inflammatory events of the vascular wall initiated by primary loss of NO may exacerbate insulin resistance and cardiovascular disease. Interestingly, statins have been recently proven to exert cardiovascular protective actions, which are independent of their cholesterol-lowering activity because of direct anti-inflammatory and NO-potentiating effects (42). Recent studies have demonstrated reduced expression of eNOS in cultured endothelial cells exposed to elevated ambient glucose (43) or dysfunctional eNOS under hyperglycemic conditions (44). The anti-inflammatory action of NO is largely based on its inhibitory effect on leukocyte-endothelium interactions. At the molecular level, NO suppresses leukocyte-endothelium interactions by modulating the activity of nuclear factor-κB, a nuclear transcription factor that increases transcription of proinflammatory cell adhesion molecules such as VCAM-1 (24). Loss of basal release of NO in postcapillary venules induces inflammatory responses in the microcirculation characterized by increased leukocyte-endothelium interactions and upregulation of endothelial cell adhesion molecules (42). Thus, it is likely that after calpain inhibition, the attenuation in VCAM-1 expression levels observed in the present study were a consequence of increased availability of NO in the vascular endothelium.

The production of NO by eNOS is dependent, among other factors and regulatory proteins (45), on the molecular chaperone hsp90 (46). There is compelling evidence
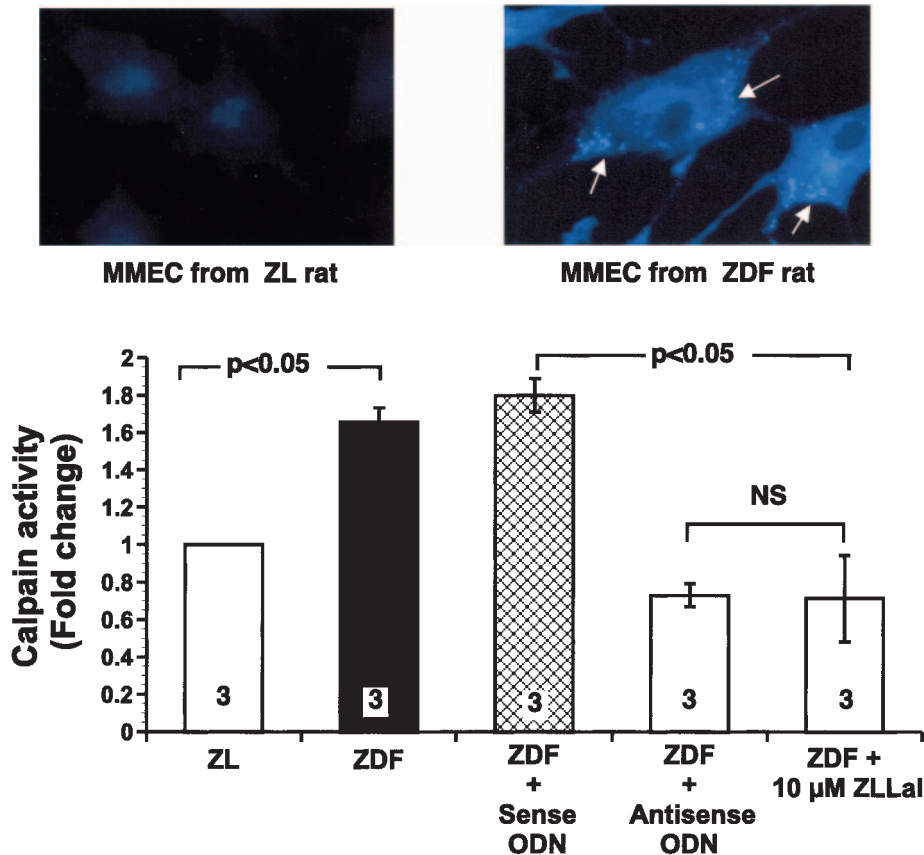
FIG. 7. Measurement of calpain activity with the fluorescent substrate t-BOC. White arrows indicate increased calpain activity in primary culture of MMECs isolated from ZDF rats. Treatment of diabetic MMECs with antisense oligonucleotides (ODNs) (10 μmol/l) to μ-calpain for 48 h or with 10 μmol/l calpain inhibitor ZLLal attenuates calpain activity to values found in MMECs from nondiabetic ZL rats (bar graph). All values are means ± SE of three independent experiments, and 18 replicates were studied in each experimental group.

that hsp90 plays a key role in activating eNOS, as suggested by a recent study showing that the interaction of hsp90 and eNOS permits hsp90 to serve as a docking site for Akt-dependent phosphorylation of eNOS (47). Thus, decreased association of hsp90 with the eNOS complex decreases eNOS activity and endothelial NO release (46). Interestingly, it has been shown that the neutral cysteine protease calpain degrades hsp90 in several cell systems (48,49). Recently, a study by Su and Block (25) demonstrated that calpain-mediated degradation of hsp90 decreases NO production in pulmonary artery endothelial cells. These observations agree with our finding of reduced availability of endothelial NO and decreased hsp90/eNOS association in the microcirculation of diabetic rats. Indeed, inhibition of calpain activity in ZDF rats restored the association of eNOS with hsp90 along with increased NO release and attenuation of leukocyte trafficking in the microcirculation.

In conclusion, we have demonstrated that the activity of the calcium-dependent protease μ-calpain is increased in the diabetic vasculature and that inhibition of calpain activity attenuates the vascular dysfunction associated with chronic diabetes. These findings uncover a role for the calcium-dependent protease calpain in the pathophysiology of diabetic vascular disease.

REFERENCES

1. US Department of Health and Human Services, Centers for Disease Control and Prevention: National Diabetes Fact Sheet: national estimates and general information on diabetes in the United States [article online], 2002. Available from http://www.cdc.gov/diabetes/pubs/factsheet.htm
2. De Vriese AS, Verbeuren TJ, Van De Voorde J, Lameire NH, Vanhoutte PM: Endothelial dysfunction in diabetes. *Br J Pharmacol* 130:963–974, 2000
3. Booth G, Stalker TJ, Lefer AM, Scalia R: Mechanisms of amelioration of glucose-induced endothelial dysfunction following inhibition of protein kinase C in vivo. *Diabetes* 51:1556–1564, 2002
4. Lash JM, Nase GP, Bohlen HG: Acute hyperglycemia depresses arteriolar NO formation in skeletal muscle. *Am J Physiol* 277:H1513–H1520, 1999
5. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, Creager MA: Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. *Circulation* 97:1695–1701, 1998
6. Marfella R, Esposito K, Giunta R, Coppola G, De Angelis L, Farzati B, Paolisso G, Giugliano D: Circulating adhesion molecules in humans: role of hyperglycemia and hyperinsulinemia. *Circulation* 101:2247–2251, 2000
7. Lefer AM, Scalia R: Nitric oxide in inflammation. In *Physiology of Inflammation*. Ley K, Ed. New York, Oxford University Press, 2001, p. 447–472
8. Dandona P, Aljada A, Chaudhuri A, Mohanty P: Endothelial dysfunction, inflammation and diabetes. *Rev Endocr Metab Disord* 5:189–197, 2004
9. Ikeda Y, Young LH, Lefer AM: Attenuation of neutrophil-mediated myocardial ischemia-reperfusion injury by a calpain inhibitor. *Am J Physiol Heart Circ Physiol* 282:H1421–H1426, 2002
10. Rami A, Krieglstein J: Protective effects of calpain inhibitors against neuronal damage caused by cytotoxic hypoxia in vitro and ischemia in vivo. *Brain Res* 609:67–70, 1993
11. McDonald MC, Mota-Filipe H, Paul A, Cuzzocrea S, Abdelrahman M, Harwood S, Plevin R, Chatterjee PK, Yaqoob MM, Thiemermann C: Calpain inhibitor I reduces the activation of nuclear factor-kappaB and organ injury/dysfunction in hemorrhagic shock. *FASEB J* 15:171–186, 2001
12. Peterson RG: The Zucker diabetic fatty (ZDF) rat. In *Animal models of Diabetes: A Primer*. Sharif E, Ed. Newark, NJ, Hardwood Academic Publishers, 2001, p. 109–128
13. Booth G, Stalker TJ, Lefer AM, Scalia R: Elevated ambient glucose induces

acute inflammatory events in the microvasculature: effects of insulin. *Am J Physiol Endocrinol Metab* 280:E848–E856, 2001

14. Panes J, Kurose I, Rodriguez-Vaca D, Anderson DC, Miyasaka M, Tso P, Granger DN: Diabetes exacerbates inflammatory responses to ischemia-reperfusion. *Circulation* 93:161–167, 1996

15. Shah V, Wiest R, Garcia-Cardena G, Cadelina G, Groszmann RJ, Sessa WC: Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. *Am J Physiol* 277:G463–G468, 1999

16. Stalker TJ, Skvarka CB, Scalia R: A novel role for calpains in the endothelial dysfunction of hyperglycemia. *FASEB J* 17:1511–1513, 2003

17. Marelli-Berg FM, Peek E, Lidington EA, Stauss HJ, Lechler RI: Isolation of endothelial cells from murine tissue. *J Immunol Methods* 244:205–215, 2000

18. Rosser BG, Powers SP, Gores GJ: Calpain activity increases in hepatocytes following addition of ATP: demonstration by a novel fluorescent approach. *J Biol Chem* 268:23593–23600, 1993

19. Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S: Differential inhibition of calpain and proteasome activities by peptidyl aldehydes of di-leucine and tri-leucine. *J Biochem* (*Tokyo*) 119:572–576, 1996

20. Bonnardel-Phu E, Wautier JL, Schmidt AM, Avila C, Vicaut E: Acute modulation of albumin microvascular leakage by advanced glycation end products in microcirculation of diabetic rats in vivo. *Diabetes* 48:2052–2058, 1999

21. Goll DE, Thompson VF, Li H, Wei W, Cong J: The calpain system. *Physiol Rev* 83:731–801, 2003

22. Zimmerman UJ, Schlaepfer WW: Two-stage autolysis of the catalytic subunit initiates activation of calpain I. *Biochim Biophys Acta* 1078:192–198, 1991

23. Brown N, Crawford C: Structural modifications associated with the change in Ca2+ sensitivity on activation of m-calpain. *FEBS Lett* 322:65–68, 1993

24. De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MAJ, Shin WS, Liao JK: Nitric oxide decreases cytokine-induced endothelial activation: nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 96:60–68, 1995

25. Su Y, Block ER: Role of calpain in hypoxic inhibition of nitric oxide synthase activity in pulmonary endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 278:L1204–L1212, 2000

26. Ribau JC, Hadcock SJ, Teoh K, DeReske M, Richardson M: Endothelial adhesion molecule expression is enhanced in the aorta and internal mammary artery of diabetic patients. *J Surg Res* 85:225–233, 1999

27. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP: Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. *Proc Natl Acad Sci U S A* 96:10836–10841, 1999

28. Hadcock S, Richardson M, Winocour PD, Hatton MW: Intimal alterations in rabbit aortas during the first 6 months of alloxan-induced diabetes. *Arterioscler Thromb* 11:517–529, 1991

29. Miyamoto K, Ogura Y: Pathogenetic potential of leukocytes in diabetic retinopathy. *Semin Ophthalmol* 14:233–239, 1999

30. Lefer DJ, Scalia R, Jones SP, Sharp BR, Hoffmeyer MR, Farvid AR, Gibson MF, Lefer AM: HMG-CoA reductase inhibition protects the diabetic myocardium from ischemia-reperfusion injury. *FASEB J* 15:1454–1456, 2001

31. Dandona P, Aljada A, Mohanty P, Ghanim H, Hamouda W, Assian E, Ahmad S: Insulin inhibits intranuclear nuclear factor kappaB and stimu-lates IkappaB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? *J Clin Endocrinol Metab* 86:3257–3265, 2001

32. Kim JA, Berliner JA, Natarajan RD, Nadler JL: Evidence that glucose increases monocyte binding to human aortic endothelial cells. *Diabetes* 43:1103–1107, 1994

33. Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P: Circulating mononuclear cells in the obese are in a proinflammatory state. *Circulation* 110:1564–1571, 2004

34. Mohanty P, Hamouda W, Garg R, Aljada A, Ghanim H, Dandona P: Glucose challenge stimulates reactive oxygen species (ROS) generation by leuco-cytes. *J Clin Endocrinol Metab* 85:2970–2973, 2000

35. Ridker PM: Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation* 107:363–369, 2003

36. Kwon HM, Sangiorgi G, Ritman EL, McKenna C, Holmes DRJ, Schwartz RS, Lerman A: Enhanced coronary vasa vasorum neovascularization in exper-imental hypercholesterolemia. *J Clin Invest* 101:1551–1556, 1998

37. Sorimachi H, Ishiura S, Suzuki K: Structure and physiological function of calpains. *Biochem J* 328 (Pt. 3):721–732, 1997

38. Zeng G, Quon MJ: Insulin-stimulated production of nitric oxide is inhibited by wortmannin: direct measurement in vascular endothelial cells. *J Clin Invest* 98:894–898, 1996

39. Aljada A, Dandona P: Effect of insulin on human aortic endothelial nitric oxide synthase. *Metabolism* 49:147–150, 2000

40. Aljada A, Saadeh R, Assian E, Ghanim H, Dandona P: Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothe-lial cells through stimulation of nitric oxide. *J Clin Endocrinol Metab* 85:2572–2575, 2000

41. Aljada A, Ghanim H, Assian E, Dandona P: Tumor necrosis factor-alpha inhibits insulin-induced increase in endothelial nitric oxide synthase and reduces insulin receptor content and phosphorylation in human aortic endothelial cells. *Metabolism* 51:487–491, 2002

42. Scalia R, Stalker TJ: Microcirculation as a target for the anti-inflammatory properties of statins. *Microcirculation* 9:431–442, 2002

43. Morigi M, Angioletti S, Imberti B, Donadelli R, Micheletti G, Figliuzzi M, Remuzzi A, Zoja C, Remuzzi G: Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-kB-dependent fashion. *J Clin Invest* 101:1905–1915, 1998

44. Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB: Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. *Diabe-tes* 47:945–952, 1998

45. Papapetropoulos A, Rudic RD, Sessa WC: Molecular control of nitric oxide synthases in the cardiovascular system. *Cardiovasc Res* 43:509–520, 1999

46. Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC: Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature* 392:821–824, 1998

47. Fontana J, Fulton D, Chen Y, Fairchild TA, McCabe TJ, Fujita N, Tsuruo T, Sessa WC: Domain mapping studies reveal that the M domain of Hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. *Circ Res* 90:866–873, 2002

48. Minami Y, Kimura Y, Kawasaki H, Suzuki K, Yahara I: The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function in vivo. *Mol Cell Biol* 14:1459–1464, 1994

49. Bellocq A, Doublier S, Suberville S, Perez J, Escoubet B, Fouqueray B, Puyol DR, Baud L: Somatostatin increases glucocorticoid binding and signaling in macrophages by blocking the calpain-specific cleavage of Hsp 90. *J Biol Chem* 274:36891–36896, 1999