Calpains: a physiological regulator of the endothelial barrier?

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THE ASSIGNMENT OF CLEAR physiological functions and pathophysiological roles to the family of enzymes known as calpains has proven to be elusive (28). Calpains are neutral cysteine proteases needing Ca²⁺ for activation and proteolytic activity. Activated, the ubiquitous isoforms µ-calpain (calpain I) and m-calpain (calpain II) are capable of invoking limited proteolysis of numerous proteins, including cytoskeletal proteins (e.g., β-integrin cytoplasmic domains, talin, filamin, and fodrin) (5, 9, 32), protein phosphatases (e.g., PTP-1B), and protein kinases (e.g., PKC, FAK, Src, and Syk) (2, 24). Because of the modulated cleavage of their substrates, calpains are presumed to be indirectly involved in many cellular processes associated with cytoskeletal remodeling, such as cell spreading and migration, or integrin signaling (3).

Endothelial cells regulate vascular permeability to fluid, solutes, macromolecules, and transmigrating cells by forming contacts to neighboring cells and to extracellular matrix constituents. Because inhibition of calpains in various cell types is associated with an inability of the cells to form focal complexes, focal adhesions, or actin filament networks (3, 18), any loss of calpain activity should impair flexibility and function of the vascular endothelium. In the coronary system, the physiological integrity of the endothelium can be disturbed during posts ischemic reperfusion (13, 21), leading to fluid extravasation and edema formation. Ischemia-reperfusion is also accompanied by a dramatic increase of intracellular Ca²⁺ (4, 9), suggesting that an activation of coronary endothelial calpains may participate in the change of vascular integrity. On the basis of the literature concerning beneficial effects of calpain inhibitors during reperfusion, inhibition of these proteases should result in a reduction of edema and coronary leak (15, 32).

In view of these contradictory expectations (leak arising both from activation and inhibition of calpain), the present study was designed to elucidate the role of endothelial calpains in the development of vascular leak and to identify a mechanism/candidate substrate employed by calpains in this process. Experiments were conducted on human umbilical vein endothelial cells (HUVEC) and isolated guinea pig hearts. HUVEC contain µ-calpain, m-calpain, and calpastatin (the chief endogenous inhibitor of both enzymes) (9, 29). To gain precise information about endothelial cell morphology subsequent to calpain inhibitor treatment, we performed phase-contrast microscopy and electrical capacitance measurements on HUVEC. Furthermore, the putative signaling kinase Syk and the small GTPase RhoA were quantified in the absence and presence of calpain inhibitors, as was the membrane translocation of Rac. In addition to calpain inhibitor I (CI), a newly constructed, specific, and cell-permeable inhibitor was employed: a combination of the calpain inhibitory peptide calpastatin with penetratin (CPP). The latter is the membrane-permeable fragment of the homeodomain of the Drosophila transcription factor Antennapedia (22).

MATERIALS AND METHODS

Materials

Calbiochem (Schwalbach, Germany) supplied the peroxidase-conjugated secondary antibody for Western blotting, µ-calpain from human erythrocytes, calpain inhibitor I (ALLN/CI), and calpastatin peptide [specific calpain inhibitor, 27-amino acid (aa) peptide]. The monoclonal antibody against Syk (4D10) and the polyclonal antibodies against Rac1 (C-11) and RhoA (Sc-179-G) were bought from Santa Cruz Biotechnology (Heidelberg, Germany). The antibody against µ-calpain (9A4/H8D3) was obtained from Alexis Biochemicals (Grünberg, Germany). The pull-down assay for determining active RhoA was obtained from Upstate-Biomanl (Hamburg, Germany). For immunofluorescence experiments, phalloidin (labeled with AlexaFluor 633) and a secondary antibody (anti-mouse, labeled with AlexaFluor 488) were purchased from Molecular Probes Europe (Leiden, Netherlands). Nitrocellulose membrane was obtained from Sartorius (Göttingen, Germany) or from Amersham Pharmacia Biotech (Freiburg, Germany). BCA protein assay Reagents A and B were obtained from Pierce (St. Augustin, Germany), and collagen for the...
coating of glass plates was obtained from Biochrom (Berlin, Germany). Silicon sensor chips for capacitance measurements were obtained from Micronas (Freiburg, Germany). Calpastatin peptide-penetratin conjugate (CPP) was synthesized as 27-mer and 16-mer disulfide-linked peptide as described previously (25). All other reagents used were of analytical grade quality and were supplied by Merck (Darmstadt, Germany) or Sigma (Taufkirchen, Germany).

Methods

The care of experimental animals and all experimental procedures were in accordance with German animal protection laws and were officially approved by the institutional review board and state authorities (Government of Upper Bavaria).

Cell culture. HUVEC were isolated from fresh human umbilical veins as described previously (26). The cells were maintained in medium 199 (M199) supplemented with 20% newborn calf serum and 50% endothelial cell growth medium (Promocell, Heidelberg, Germany). All cells used for the experiments were in the first or second passage.

For inhibition of calpain with CI in cell experiments, the inhibitor was dissolved in DMSO and used in concentrations of 12 and 48 µM. Control cells were incubated with DMSO at 0.1 and 0.4%, accordingly. Both concentrations of the inhibitor have been shown to inhibit calpain activity in whole cell experiments (7, 17). To test whether the penetratin moiety in the calpastatin peptide-penetratin conjugate per se modulates calpain activity in cells, we incubated LCLC 103H cells with Ac-cys-penetratin in concentrations up to 10 µM. No change in ionomycin-induced cleavage of the synthetic calpain substrate Suc-LLVY could be detected with any Ac-cys-penetratin concentration (Fig. 1A). Furthermore, 1 µM Ac-cys-penetratin did not alter calpain activity in HUVEC stimulated by 2 µM ionomycin, whereas 1 µM CPP significantly reduced activity (Fig. 1B).

Retraction experiments of HUVEC. PHASE-CONTRAST MICROCOPY. HUVEC were grown to confluence on plastic culture dishes (35 mm) in supplemented M199 (see Cell culture). Confluent cells were washed once, incubated for 10 min in HEPES buffer, and then put on a microscope (Axiovert 100; Zeiss, Jena, Germany) with a heated stage (37°C). CPP (1 µM) was added to some dishes immediately before the measurements were obtained. A phase-contrast picture of the cells was taken every 10 s for 15 min. Every picture was divided into quarters, and the mean brightness value was evaluated (TILL Vision Software; TILL Photonics, Lochham, Germany). When cells rounded, they developed a bright ring along their borders in the phase contrast. Thus the mean brightness of the images rose during cell retraction. The difference between control and inhibitor-treated cell monolayers was assessed by comparing the mean values of brightness at the end point of the experiments (15th minute).

CAPACITANCE MEASUREMENTS. To evaluate changes in the electrical capacitance of confluent monolayers of HUVEC treated with or without CI (12 µM) or CPP (1 µM), we grew the cells on collagen-coated silicon sensor chips with interdigitated electrode structures (IDES) in supplemented M199. A Teflon container was attached on top of the chips, leaving a circular cell culture area of 6.2 mm in diameter. Measurements were carried out in HEPES buffer. The electrode material was palladium; single-electrode width and distance were 50 µm. The total area of the IDES overgrown with cells was 2 × 1 mm². Capacitance measurements were performed at a frequency of 10 kHz. Further details of monitoring of cell morphology with capacitance measurements were described previously (6).

Inhibitor treatment and preparation of HUVEC lysates. To evaluate a basal autolytic activation of μ-calpain in HUVEC, we washed untreated cells with PBS+ and then lysed cells in ice-cold buffer [pH 7.4; 20 mM Tris, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.25 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µg/ml pepstatin, and 10 µg/ml leupeptin]. The membrane fraction was isolated and analyzed by Western blotting.

AJP-Heart Circ Physiol • VOL 290 • MAY 2006 • www.ajpheart.org

Fig. 1. Effect of penetratin on intracellular calpain activity. A: LCLC 103H cells were incubated with various concentrations of Ac-cys-penetratin and subsequently stimulated with 2 µM ionomycin for 30 min. Cleavage of Suc-LLVY-amc (a calpain substrate) was measured fluorometrically (% activity of stimulated control) B: human umbilical vein endothelial cells (HUVEC) were incubated with 1 µM Ac-cys-penetratin or 1 µM calpastatin peptide-penetratin (CPP) for 30 min before stimulation with 2 µM ionomycin. Calpain activity was measured using the Suc-LLVY-amc peptide substrate for fluorometric detection. The mean fluorescence determined for cultures stimulated just with ionomycin was set as 100% activity. The mean relative response (SD) of 4 cultures per group is plotted. Note the residual activity of calpain in the unstimulated cells.

To assess Syk in the membrane fraction of HUVEC, we washed confluent cells twice and then incubated cells in HEPES buffer with vehicle (DMSO 0.4%) or CI (48 µM) for 1 h. This higher concentration was employed to speed the onset of inhibition, because the rate of access to HUVEC in culture depends alone on the concentration gradient. Rapid inhibition, in turn, is important for detecting the notoriously transient changes in Rho activity. For determining effects on Rho, inhibitor treatment was conducted for 2 min. The cells were then lysed immediately in ice-cold buffer (pH 7.4; 20 mM HEPES, 10 mM KCl, 10 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 1 tablet Complete Mini inhibitor cocktail/10 ml buffer). Cell debris was removed by centrifugation (4°C, 1,000 g, 10 min). Equal amounts of protein (BCA assay) from vehicle- and inhibitor-treated cell lysates were used for the isolation of the membrane fraction (centrifugation at 4°C, 100,000 g, 1.5 h). The pellet (membrane fraction) was solubilized in sample buffer and boiled for 10 min before blotting.
Active RhoA in HUVEC was determined by pull-down assay. Lysis buffer (pH 7.5) contained 25 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% Igepal, 10% glycerol, 10 µg/ml aprotime, and 10 µg/ml leupeptin. Lysate (30 µl) was incubated with 30 µl of agarose-bound Rhotekin-GST substrate, briefly centrifuged (10,000 g, 30 s, 4°C), and washed three times with 500 µl of lysis buffer. The residue was suspended in 40 µl of lysis buffer together with 20 µl of sample buffer (250 mM Tris, 8% SDS, 40% glycerol, 0.02% bromphenol blue, and 400 mM mercaptoethanol) and 2 µl of 1 M DTT and then boiled for 5 min before blotting.

Western blot analyses. Proteins were separated by SDS-PAGE (10% polyacrylamide gels), transferred onto nitrocellulose in a semi-dry blotting apparatus (polyvinylidene difluoride and tank blotting for RhoA) and afterwards stained with amido black or Ponceau S to control equal loading. After removal of the dye, the membrane was blocked for 2 h in skim milk powder or blocking buffer (pH 7.5; 200 mM NaCl, 50 mM Tris, 3% BSA, 0.05% Tween 20, and 10% horse serum) and incubated with Syk antibody (1:500) in skim milk powder, µ-calpain antibody (1:2,000) in blocking buffer, or Rho antibody (activity assay kit; Upstate) at 4°C overnight. The membrane was washed (4 times for 15 min) before incubation for 2 h with the peroxidase-conjugated secondary antibody (1:10,000) in skim milk powder or blocking buffer. After renewed washing, immunoreactive protein was visualized by chemiluminescence and quantified (Gel Doc 1000, Molecular Analyst software; Bio-Rad, Munich, Germany).

Immunofluorescence. HUVEC were disseminated on glass plates in supplemented M199. After 3 h, the adherent cells were washed twice with HEPES buffer and incubated for 30 min in the presence of calpain inhibitors (12 µM CI, 1 µM CPP) or vehicle (0.1% DMSO). Cells fixed in formalin (3.7%) were permeabilized with 0.1% Triton-X-100, blocked with 1% BSA, and then stained for actin (with phalloidin) and Syk, Rac1, or RhoA (primary antibodies: 1:200 in 1% BSA, 45 min; secondary antibodies: 1:400 in 1% BSA, 30 min). Immunofluorescence was viewed in a confocal microscope (LSM 410 invert; Zeiss).

Perfusion model for heart homogenates and transudate measurements. Guinea pigs (Pirbrite-White) of 250- to 280-g body weight were killed by neck dislocation using a specially designed instrument. Perfusion model for heart homogenates and transudate measurements. Guinea pigs (Pirbrite-White) of 250- to 280-g body weight were killed by neck dislocation using a specially designed instrument. After cannulated the pulmonary artery and ligated all veins (pulmonary, coronary), we removed the heart. Hearts were rapidly excised and perfused with a modified Krebs–Henseleit; Zeiss).

RESULTS

Retraction of HUVEC

Treatment of confluent HUVEC with CPP (1 µM) or CI (12 µM) led to a rapid retraction and rounding of the cells as evaluated by both phase-contrast microscopy and electrical capacitance measurements. In phase-contrast micrographs, brightening corresponds to retraction and rounding of the cells. Figure 2A shows exemplary micrographs of confluent HUVEC monolayers treated with CPP (1 µM). After 15 min, the cells exhibited a larger white rim than at the beginning of the measurement. Calculation of image brightness during the incubation with the calpain inhibitor revealed a significant increase of this parameter compared with controls (Fig. 2B). The increase in brightness started within 2–3 min, reached a steady state by 5–6 min, and lasted up to the end of the experiment (Fig. 2B). Likewise, measurement of the electrical capacitance showed a loss of vascular endothelial integrity after treatment of confluent HUVEC with CI (12 µM). As depicted in Fig. 2C, the generation of cell free area, caused by retraction of the cells, led to a steep increase in capacitance within 2–5 min after addition of either calpain inhibitor. Capacitance fell slowly again over a period of 50 min. A change of buffer (without CI) induced a transitory decrease of capacitance but did not really alter the slow return to basal values (Fig. 2C). Addition of solvent alone had no effect. Histamine and thrombin, two well-characterized mediators of permeability, elicited similar effects on capacitance, as did CI (Fig. 2C, right, and data not shown). An earlier withdrawal of CPP (after 10 min) accelerated the recovery of chip coverage (Fig. 2C, right).

Assessment of Basal Autolytic Activation of µ-Calpain

According to Western blots, a band of autolytically degraded µ-calpain was detected at 76–78 kDa in the membrane fraction of control heart homogenates (guinea pig) and untreated HUVEC lysates (Fig. 3A). Calpain of this size is known to be enzymatically active.

Localization of Syk in HUVEC

Incubation of HUVEC with CI (48 µM) for 1 h led to a significant increase of the nonreceptor tyrosine kinase Syk (72 kDa) in the membrane fraction of the cells, as determined by Western blotting [205% (161); P < 0.01, Mann-Whitney rank sum test, n = 5]. A representative blot is depicted in Fig. 3B, in which platelet lysate served as positive control for Syk. Immunofluorescence microscopy of HUVEC double-stained
for actin (blue) and Syk (red) likewise revealed a translocation to the membrane of the cells after incubation with CI (12 μM) or CPP (1 μM) for 30 min (Fig. 3C). As judged by immunofluorescence, Rac1 and RhoA also tended to be translocated to the membrane in the presence of CI (12 μM) or CPP (1 μM), but these effects were not as distinct. A representative confocal image for Rac is shown in Fig. 4A. According to the pull-down assay in Western blots, there was a 20% activation of RhoA in HUVEC exposed to 48 μM CI for 2 min (P < 0.05, n = 4; Fig. 4B).

Transudate Formation by Isolated Hearts

Transudate, a direct measure of net fluid filtration in the coronary system, appeared on the epicardial ventricular surface of the isolated perfused hearts at a rate of ~90 μl/min. Under the chosen conditions of constant volume perfusion (5 ml/min) and short ischemic challenge (15 min), transudate flow of isolated guinea pig hearts did not change substantially during reperfusion (Fig. 5). However, perfusion of isolated guinea pig hearts with the cell-permeable inhibitors CI (12 μM; Fig. 5A) or CPP conjugate (0.5 μM; Fig. 5B) unexpectedly led to a significant increase in transudate formation during reperfusion. Surprisingly, control hearts that received perfusion with one or the other calpain inhibitor for two 10-min intervals without an intervening ischemic challenge also developed a coronary leak (Fig. 5). In all cases, leak remained elevated long after the cessation of infusion of inhibitor. Treatment of ischemic hearts with the unconjugated 27-aa calpastatin peptide alone (3 μM) did not increase coronary vascular permeability after ischemic challenge (Fig. 5B), and no change of transudate flow was seen in control hearts perfused only with the modified Krebs-Henseleit solution for 60 min (results not shown). Furthermore, application of a 27-mer–7-mer conjugated peptide of calpastatin peptide plus the COOH-terminal, 7-aa remainder of the

Fig. 2. Retraction of HUVEC. A: phase-contrast photomicrographs of confluent HUVEC treated with CPP (1 μM) taken at the beginning (left) and end (right) of an experiment. After 15 min, cells had retracted and the white rims had increased. The images are representative of 5 experiments. B: light transmission through a monolayer of HUVEC incubated with or without CPP (1 μM). The picture is representative of 3 experiments. Mean brightness increased with CPP treatment at the end of the experiments by 21.1 (6.4) arbitrary units. *P < 0.01, CPP vs. control (Mann-Whitney rank sum test, n = 5). C: changes in the cell monolayer integrity are depicted as changes in capacitance (∆Cp) vs. time. An increase in capacitance reflects an increased cell-free area on the chip and, thus, retraction within the cell monolayer. The tracings of 4 independent cultures treated with calpain inhibitor I (CI) are shown at left. Addition of solvent alone (sham stimulation) had no effect on Cp (bottom tracing). Representative tracings for addition of CPP, histamine, and early washout of CPP are shown at right.
penetratin molecule (1 μM) had no influence on coronary leak (preliminary experiment).

Although buffer-perfused hearts are inherently prone to edema, the rise in transudate formation due to treatment with inhibitors was accompanied by further edema formation: the mean ratio of wet weight to dry weight was significantly increased from 8.51 (0.17) (ischemia/0.1% DMSO; n = 3) to 9.48 (0.36) (ischemia/CI; n = 7) and 9.31 (0.35) (control/CI; n = 5), both P < 0.05.

**Development of Coronary Perfusion Pressure in Isolated Hearts**

Coronary perfusion pressure, a direct marker of coronary resistance under constant flow conditions, did not change significantly because of ischemia alone. Infusion of CI with or without ischemia led to a sustained increase of coronary perfusion pressure (Fig. 6A). With CPP, the increase of perfusion pressure was only transient in the first 10 min of reperfusion (Fig. 6B). Endothelin-1, a well-known vasoconstrictor, expectedly caused a marked increase in perfusion pressure, but without changing transudate formation (Fig. 6C).

**DISCUSSION**

Commonly used calpain inhibitors like CI normally penetrate cell membranes easily but lack full calpain specificity. On the other hand, calpastatin peptide (27 aa), which is derived from the endogenous calpain inhibitor and binds near the catalytic center of calpains (16, 20), is highly specific but poorly permeable. In our study we were able to use a combination of this calpastatin peptide and penetratin, the 16-aa fragment of the homeodomain of the *Drosophila* transcription factor Antennapedia. Penetratin has been shown to effectively transport peptides of <100 aa into the cell (22). Specificity and cell permeability of the calpastatin peptide-penetratin conjugate have been tested (11). Penetratin per se does not inhibit intracellular calpain activity (Fig. 1, A and B). In isolated hearts, the conjugate showed potent activity at a concentration of 0.5 μM, whereas calpastatin peptide alone had no effect at all, even at a concentration of 3 μM. Although inhibitory
activity of the nonconjugated calpastatin peptide against \( \mu \)- and \( m \)-calpain has been described at low nanomolar concentrations in inhibition assays, the amounts necessary in whole cell experiments were indicated to be \( 50 \) \( \mu \)M (7, 20). Thus CPP, combining full calpain specificity with excellent cell permeability, opens up new possibilities to ascertain the functions of calpains.

Fig. 5. Transudate formation of isolated guinea pig hearts. Measurements of non- and posts ischemic hearts treated with or without CI (A), CPP (B), or calpastatin peptide (CP) alone (B). Perfusion of hearts with one or the other inhibitor led to a significant increase in transudate formation, largely independent of an additional, 15-min ischemic challenge. Basal transudate averaged 0.09 ml/min in all groups. In A, \( *, \) ischemia/DMSO 0.1% \( (n = 3); \odot, \) ischemia/CI (12 \( \mu \)M; \( n = 7); \blacktriangleleft, \) control/CI (12 \( \mu \)M; \( n = 4). In B, \( *, \) ischemia \( (n = 13); \odot, \) ischemia/CPP (0.5 \( \mu \)M; \( n = 3); \blacktriangleleft, \) control/CPP (0.5 \( \mu \)M; \( n = 1); \blacktriangleleft, \) ischemia/CP (3 \( \mu \)M; \( n = 4). *P < 0.05.

Fig. 6. Coronary perfusion pressure of isolated guinea pig hearts. Perfusion pressure was measure in isolated hearts treated with or without CI (A) or CPP (B). Perfusion pressure increased significantly for control + CI vs. baseline \( (#P < 0.05), \) ischemia + CI/CPP vs. baseline \( (*P < 0.05), \) control/ischemia + CI vs. ischemia + DMSO \( (\#P < 0.05), \) and ischemia + CPP vs. ischemia \( (\&P < 0.05). \) C: perfusion pressure and transudate flow of time control hearts (15th– 60th minute), subsequently treated with endothelin-1 (500 pM) for 20 min (65th– 85th minute).
Total abolition of \( \mu \)- and m-calpain activity by knockout of the small subunit gene in mice leads to lethal defects in vasculogenesis with characteristic rounding of the endothelial cells (1). In addition, inhibiting calpains reduces spreading, formation of focal adhesions and actin filament networks of endothelial cells (18). Thus the constitutively high levels of \( \mu \)- and m-calpain in endothelial cells (9) presumably fulfill an important basal function.

Confluent HUVEC monolayers are widely used as an in vitro model for vascular permeability. Our approach was slightly different: by measuring the increase in image brightness that occurs in phase-contrast microscopy when the cells in the monolayer change their morphology, it was possible to produce a sequence of pictures that allowed us to watch the cells retract and round up in situ. The addition of CPP (1 \( \mu \)M) led to a rise in light transmission (an integrative signal of morphological change) that occurred within 6 min and lasted up to the end of the experiment. To verify the results obtained by microscopy, we also performed electrical capacitance measurements of HUVEC monolayers grown on semiconductor chips. This has been described previously as a suitable method to evaluate changes in endothelial morphology caused by thrombin (14). Treatment of confluent HUVEC with CI or CPP led, within 2–5 min, to a decrease in monolayer integrity, expressed as a steep rise in capacitance that subsided only slowly. Thus calpain inhibition seems to induce rounding of HUVEC, as also has been described for endothelial cells in the atrial walls of Capn4\(^{-/-}\) embryos (1). These results indirectly support the existence of a basal calpain activity in endothelial cells. This is further evidenced by the fact that an autolytically activated form of membrane-associated \( \mu \)-calpain can be found in Western blots of both control hearts and untreated HUVEC lysates (Fig. 3). Furthermore, we also found a basal calpain activity of unstimulated HUVEC in a peptide cleavage assay (Fig. 1B).

The hypothesis that calpains regulate the physiological barrier function of endothelial cells was substantiated in situ. The isolated, buffer-perfused guinea pig heart is well suited for assessing changes in coronary vascular permeability (13, 23). Net filtration of fluid across the endothelium of the intact coronary bed is visualized directly by the formation of transudate on the epicardial surface and depends mainly on coronary vascular integrity. Thus hearts perfused only with buffer show no change in transudate formation. On the other hand, supplementing perfusate with either one of the calpain inhibitors, CI or CPP, caused a dramatic increase in postischemic transudate flow, quite the opposite of what we had expected on the basis of literature concerning cardioprotective actions of calpain inhibition (30, 32). Even more surprising came the observation that calpain inhibitors increased coronary leak in control hearts not even subjected to ischemia, the time course of leak development (no instantaneous onset) being in good accordance with a process evolving from enzymatic inhibition. This suggests a basal calpain activity in the vascular cells already under normal conditions, important for maintaining coronary vascular integrity. Such a role may perhaps be accentuated during reperfusion, as suggested by the tendency toward a greater development of leak in postischemic hearts treated with calpain inhibitors than in nonischemic controls (cf. Fig. 5).

Our results oppose the hypothesis that calpains participate directly in the development of edema seen in reperfused hearts; edema was, in fact, enhanced by calpain inhibition. Postischemic activation of calpains should even serve to ameliorate vascular leak. Indeed, there was no increase in coronary transudate formation whatsoever in our postischemic controls. However, calpain inhibition also has been shown to reduce adhesion of polymorphonuclear leukocytes to the endothelium after ischemia-reperfusion in rat hearts, thereby attenuating myocardial reperfusion injury (15). Accordingly, the outcome of calpain inhibition during ischemia-reperfusion (15, 30, 32) seems to depend on the precise experimental conditions. It also may depend on the particular inhibitor employed.

Numerous calpain substrates relevant for signal transduction can be found in the literature (12). The nonreceptor tyrosine kinase Syk phosphorylates a variety of cytoskeletal proteins (e.g., paxillin, \( \alpha \)-tubulin, cortactin) (8) and is linked to signaling cascades of integrins such as \( \alpha_{\text{IIb}}\beta_3 \) in platelets (10). Furthermore, \( \beta_3 \)-integrin-dependent morphological changes and respiratory burst in neutrophils are essentially linked to Syk (8). In our experiments, treatment of HUVEC with calpain inhibitors (CI, CPP) led to a translocation of the kinase to the cell membrane. This might, on one hand, indicate an impaired degradation of Syk and, hence, an accumulation of the kinase at the membrane. On the other hand, inhibition of calpain might prevent the detachment of Syk from its place of action at the membrane, prolonging activity of the kinase. Indeed, calpain is known to become activated upon \( \beta_3 \)-integrin interaction with the extracellular matrix and to be responsible for proteolysis of the cytoplasmic tail of the \( \beta_3 \)-integrin at sites that are important for Syk binding (3, 5, 31). Irrespectively, both alternatives suggest a potential regulation of Syk by calpain, possibly by way of spatial and temporal limitation of the tyrosine kinase’s activity. Translocation of RhoA and Rac1 to the cell membrane signifies activation of these small G proteins, and a rapid activation of RhoA was seen in HUVEC treated with calpain inhibitor. Accordingly, inhibition of calpains would lead to sustained signal transduction via Syk and complementary effects on RhoA and Rac1 (17, 27) that might result in the observed morphological changes of HUVEC and, furthermore, in the abolition of the endothelial barrier function seen in the intact coronary system.

The hypothesis that endothelial morphology and not vascular tone is of importance in the observed increase of endothelial permeability is further strengthened by the finding that transudate formation changes to a much higher degree (~5-fold) compared with perfusion pressure, one of the possible determinants of net filtration (only 1.6-fold). Perfusion pressure does not seem to play a prominent role for transudate formation in our model, because even severe pressure increases by endothelin-1 infusion did not cause any leak formation (Fig. 6C).

Together, our results suggest a physiological importance of constitutive calpain activity, already at normal intracellular Ca\(^{2+}\) levels, with a particularly prominent role in determining endothelial barrier function and endothelial cell morphology. Whether inhibition of these proteases is a suitable therapeutical target with respect to reperfusion damage seems questionable.

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REFERENCES


19. Kupatt C, Habazettl H, Goedecke A, Wolf DA, Zailer S, Boekstegers P, and Becker BF. This work was supported by SFB 469 of the German Research Council.


