A Novel Role for Calpain in the Endothelial Dysfunction Induced by Activation of Angiotensin II Type 1 Receptor Signaling

Rosario Scalia, Yulan Gong, Brett Berzins, Brin Freund, Danielle Feather, Gavin Landsberg, Gourav Mishra

**Rationale:** The cytosolic protease calpain has been recently implicated in the vascular remodeling of angiotensin II (Ang II) type 1 receptor (AT1R) signaling. The role of Ang II/AT1R/calpain signaling on endothelial function, an important and early determinant of vascular pathology, remains though totally unknown. Accordingly, we investigated the role of calpain in the endothelial dysfunction of Ang II.

**Objective:** To demonstrate a mechanistic role for calpain in the endothelial dysfunction induced by Ang II/AT1R signaling. To establish endothelial-expressed calpains as an important target of AT1R signaling.

**Methods and Results:** Subchronic administration of nonpressor doses of Ang II to rats and mice significantly increased vascular calpain activity via AT1R signaling. Intravital microscopy studies revealed that activation of vascular expressed calpains causes endothelial dysfunction with increased leukocyte–endothelium interactions and albumin permeability in the microcirculation. Western blot and immunohistochemistry studies confirmed that Ang II/AT1R signaling preferentially activates the constitutively expressed \( \mu \)-calpain isoform and demonstrated a calpain-dependent degradation of \( \alpha \)2 integrin, along with upregulation of nuclear factor \( \kappa \)B-regulated endothelial cell adhesion molecules. These physiological and biochemical parameters were nearly normalized following inhibition of AT1R or calpain in vivo. Antisense depletion studies in microvascular endothelial cells, along with knockout and transgenic mouse studies, further confirmed the role of \( \mu \)-calpain in the endothelial adhesiveness induced by Ang II.

**Conclusions:** This study uncovers a novel role for calpain in the endothelial dysfunction of Ang II/AT1R signaling and establishes the calpain system as a novel molecular target of the vascular protective action of renin–angiotensin system inhibition. Our results may have significant clinical implications in vascular disease. (*Circ Res.* 2011;108:00-00.)

**Key Words:** adhesion molecules \( \Box \) nuclear factor \( \kappa \)B \( \Box \) protease \( \Box \) polymorphonuclear leukocyte adhesion

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Calpain is a ubiquitously expressed, cytosolic, Ca\(^{2+}\)-activated, neutral cysteine protease that consists of a large 80-kDa catalytic subunit and a small 30-kDa regulatory subunit. Two calpain isoforms, \( \mu \) and m, are ubiquitously expressed and, thus, are found in the vascular wall, where they regulate important functions of endothelial and smooth muscle cells. Two recent studies have implicated calpain in the vascular pathology of Ang II by demonstrating a mechanistic role for calpain in smooth muscle cell homeostasis and vascular remodeling. Surprisingly, no study has addressed the role of calpain in the endothelial dysfunction of Ang II, albeit recent work has emphasized a key mechanistic role for the vascular endothelium in Ang II–induced pathology that affects the entire vascular wall. Indeed, because of its strategic location between the blood compartment, the vascular wall, and the parenchyma of organ tissues the endothelium plays a very early mechanistic role in the initiation and progression of vascular disease, and related organ damage.

Several lines of research have now extensively demonstrated that upregulation of Ang II signaling causes endothelial dysfunction mainly via activation of the AT1R, even in the absence of significant changes in systemic blood pressure. The dysfunctional endothelium initiates and maintains vascular pathology by becoming abnormally adhesive to circulating leukocytes and leaky to plasma proteins, 2 processes responsible for vascular and organ tissue damage. Thus, activation of the Ang II/AT1R signaling pathway has been shown to: (1) upregulate endothelial cell adhesion molecules (eCAMs) in vitro, as well as and in microcirculation of
experimental animal models; (2) induce leukocyte–endothelial interactions; and (3) increase vascular permeability.

Preclinical and clinical data demonstrate that pharmacological inhibition of the renin–angiotensin system (RAS) attenuates endothelial dysfunction and cardiovascular events independently of blood pressure lowering effects. Of note, AT1R blockers (ARBs) attenuate expression of endothelial cell adhesion molecules, leukocyte–endothelium interactions, and vascular permeability. Despite intensive research, the precise molecular mechanisms by which Ang II/AT1R disrupts critical components of endothelial function remain largely unknown, which obstructs full therapeutic exploration of RAS inhibition in vascular disease.

We hypothesized that stimulation of Ang II/AT1R signaling induces endothelial dysfunction in part via activation of the endothelial calpain system. Accordingly we studied the role of μ- and m-calpain in the leukocyte–endothelium interaction and vascular permeability induced by Ang II/AT1 signaling. Using pharmacological and genetic approaches, we demonstrate that Ang II upregulates endothelial calpain activity via AT1R signaling thus causing a distinct endothelial inflammatory phenotype characterized by increased leukocyte–endothelium interactions and albumin permeability. We also demonstrate that inhibition of calpain activity is an important molecular mechanism of the vascular protective action of ARBs.

Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org

Ang II Infusion Protocols

All experimental procedures were approved by Temple University IACUC and performed according to the NIH guidelines for the use of experimental animals. Suppressor concentrations of Ang II were systemically delivered to randomly selected groups of rats or mice using Alzet osmotic minipumps implanted subcutaneously (Model 2002; ALZA Scientific Products, Mountain View, CA). Pumps were filled with either saline or with Ang II at the concentration of 20 ng/kg per minute or 500 ng/kg per minute, for rat or mice, respectively. All pumps delivered saline or Ang II for 14 days. Previous studies in the species rat have demonstrated that infusion of 20 ng/kg per minute induces endothelial dysfunction with inflammation in the absence of significant changes in systemic blood pressure. Because considerably higher doses of Ang II are required to elicit vascular responses in C57BL/6J mice,13 we used the higher dose of 500 ng/kg per minute Ang II.

Results

Ang II Infusion Increases Calpain in the Vasculature via AT1R Signaling

To study the relationship between Ang II and calpain, we first measured calpain activity in extracts of vascularized sections of mesenteries isolated from rat and mice infused with supressor doses of Ang II for 14 consecutive days. Calpain activity in tissue extracts was measured using the fluorogenic calpain substrate Succ-LLVY-AMC. We found evidence of 2-fold increase in vascular calpain activity under these experimental conditions (Figure 1). Treatment of Ang II–infused animals with 10 mg/kg per day of the AT1R blocker losartan attenuated calpain activity to values found in control animals (Figure 1). Moreover, infusion of Ang II to mice deficient in AT1a receptor, the major mouse AT1R isoform and the closest murine homolog to the single human AT1, failed to increase calpain activity in the vasculature thus demonstrating the obligatory role of the AT1R in the process of calpain activation by Ang II (Figure 1B).

Comparable results were obtained following pharmacological inhibition of calpain in Ang II–infused animals (Figure 1). Thus, a 5-day treatment with either 140 μg/kg per day ZLLal or 1 mg/kg per day PD150,2 selective calpain inhibitors, each with different molecular structure and mechanism of action, normalized calpain activity in the vasculature.

We next measured calpain activity in the intact microcirculation of live rats infused with Ang II. For these studies, we coupled the fluorescent based assay of calpain activity described in Figure 1 with in vivo microscopy of mesenteric postcapillary venules, which are microvessels comprised only by a layer of endothelial cells, a basement membrane, and pericytes. The advantage of this approach over the biochemical one described above is that it allows for simultaneous quantification and spatial localization of active calpains within the vascular endothelium of the microcirculation of live rats infused with Ang II. For these studies, we coupled the fluorescent based assay of calpain activity described in Figure 1 with in vivo microscopy of mesenteric postcapillary venules, which are microvessels comprised only by a layer of endothelial cells, a basement membrane, and pericytes. The advantage of this approach over the biochemical one described above is that it allows for simultaneous quantification and spatial localization of active calpains within the vascular endothelium of the mesentery.
observed microcirculation in vivo. In fact, the results obtained with the mesenteric tissue homogenates described in Figure 1 could in theory have been contaminated with vascular smooth muscle cells which also express calpain.2 Fluorescent staining clearly demonstrated that following Ang II infusion the wall of postcapillary venules experiences increased calpain activity (Figure 2B). Quantification of the fluorescent staining revealed a 3-fold increase in calpain activity in postcapillary venules of Ang II–infused rats, which was attenuated to control levels following pharmacological inhibition of either AT1R or calpain (P<0.05; Figure 2, bar graph to the left). These results are consistent with the biochemical analysis for calpain activity reported in Figure 1 and they further confirm that upregulation of Ang II/AT1R signaling increases calpain activity in the endothelium of postcapillary venules.

Identification of the Calpain Isoform Implicated in the Endothelial Dysfunction of Ang II/AT1R Signaling

Two calpain isoforms, namely μ-calpain and m-calpain, are constitutively expressed in endothelial cells.1 To investigate the effect of Ang II/AT1R signaling on μ- and m-calpain activity in vivo, we measured expression levels of the N-terminal domain of the calpain large 80 kDa subunit. On activation, calpains undergo autoproteolysis with removal of the 9 to 14 amino acids from the N-terminal domain of the large 80 kDa subunit.17 Accordingly, m- and μ-calpain autoproteolysis/activation levels in freshly isolated vascular segments of mesenteric tissue from all groups of rats were assessed by immunoblot analysis using primary antibodies to their respective N-terminal domains. We found increased proteolytic activity of μ-calpain only in Ang II–infused rats, as demonstrated by an average 70% loss in N-terminal...
siRNA used in this study effectively reduced (MMECs), freshly isolated from donor control rats. The cultures of mesenteric microvascular endothelial cells which recognizes both unautolyzed and autolyzed (ie, active) antibody against the stable domain IV of the large subunit, \( \text{H9262} \) (si)RNA technology to knockdown the vascular endothelium. Thus, we used small interfering \( \text{H9262} \) confirm expression level (Online Figure I, A and B).

To study the functional implications of calpain activation induced by the Ang II/AT\(_1\)R, we measured leukocyte–endothelium interactions and albumin permeability by intravital microscopy. As shown in A of Figure 2 and quantified in Figure 5, the venular endothelium of Ang II–infused rats experienced increased adhesiveness to circulating leukocytes. Specifically, we found evidence of a 3-fold increase in leukocyte rolling and a 4-fold increase in both leukocyte adhesion and extravasation in mesenteric postcapillary venules of Ang II–infused rats compared to control rats receiving saline (Figure 5A through 5C). Leukocyte rolling, adhesion and extravasation were all reduced following treatment with the either the AT\(_1\)R blocker losartan, the calpain inhibitor ZLLal, or the NF-\( \kappa \)B inhibitor PDTC (Figure 5).

Ang II–Induced Calpain Activation Causes Endothelial Dysfunction With Increased Leukocyte–Endothelium Interactions and Disruption of the Endothelial Cell Barrier

Domain genetic studies in vitro were undertaken to further confirm \( \mu \)-calpain as the target of Ang II/AT\(_1\)R signaling in the vascular endothelium. Thus, we used small interfering (si)RNA technology to knockdown \( \mu \)-calpain in primary cultures of mesenteric microvascular endothelial cells (MMECs), freshly isolated from donor control rats. The siRNA used in this study effectively reduced \( \mu \)-calpain expression levels of \( \mu \)-calpain in all groups of rats as demonstrated by lack of difference in C-terminal domain expression (Figure 3, upper lanes of A and B). A 5-day treatment of Ang II–infused rats with either losartan or ZLLal significantly blocked \( \mu \)-calpain activity (Figure 3, upper lanes of A and B, respectively). Total \( \mu \)-calpain content was quantified using a primary antibody against the stable domain IV of the large subunit, which recognizes both unautolyzed and autolyzed (ie, active) \( \mu \)-calpain. No significant differences in total \( \mu \)-calpain expression were found in all experimental groups rats (Figure 3, middle lanes of A and B and bar graphs), which indicates that Ang II did not change significantly the transcriptional regulation of calpain, at least in the time frame of our experimental conditions. In contrast, the proteolytic activity of m-calpain was not increased by Ang II infusion as demonstrated by lack of changes in m-calpain N-terminal domain expression level (Online Figure I, A and B).

Parallel genetic studies in vitro were undertaken to further confirm \( \mu \)-calpain as the target of Ang II/AT\(_1\)R signaling in the vascular endothelium. Thus, we used small interfering (si)RNA technology to knockdown \( \mu \)-calpain in primary cultures of mesenteric microvascular endothelial cells (MMECs), freshly isolated from donor control rats. The siRNA used in this study effectively reduced \( \mu \)-calpain expression levels (Figure 4, immunoblot) without affecting expression levels of the m-calpain isoform (data not shown). Calpain activity in attached MEC was then measured using the fluorescent assay described above. Data shown in Figure 4 demonstrate that MMECs exposed to 10 nmol/L Ang II for 6 hours experience a 1.5 fold increase in calpain activity. The calpain-activating actions of Ang II were abolished by \( \mu \)-calpain siRNA treatment, as well as following inhibition of either the AT\(_1\)R or calpain with losartan and ZLLal, respectively (Figure 4). Taken together, the in vivo and in vitro data reported in Figures 1 through 4 strongly demonstrate that Ang II/AT\(_1\)R signaling increases \( \mu \)-calpain activity in the vascular endothelium. They also suggest that \( \mu \)-calpain may represent an important target of the pleiotropic actions of AT\(_1\)R blockers in cardiovascular disease.
Furthermore, Ang II failed to increase leukocyte adhesion in the mesenteric microcirculation of \( \mu \)-calpain deficient mice (Online Figure II).

Parallel ex vivo experiments with aortas and leukocytes isolated from wild-type C57BL/6 mice demonstrated an essential role for the vascular endothelium in the proadhesive action of Ang II (Online Figure III, A). Specifically, when Ang II–stimulated aortic segments were incubated with unstimulated leukocytes a significantly high number of leukocytes adhered to the aortic endothelium (\( P<0.05 \) versus control unstimulated aortas/unstimulated leukocytes). In contrast, no difference from control was observed following incubation of unstimulated aortas with Ang II–stimulated leukocytes (\( P>0.05 \)), even though we found evidence of calpain-dependent increase in CD11b expression in circulating leukocytes of Ang II–infused rats (Online Figure IV). The increased adhesiveness of Ang II–stimulated aortas was also prevented by incubation of the aortic tissue with losartan or ZLLal (Online Figure III, A).

Additional mechanistic studies were undertaken to dissect the contribution of endothelial-expressed and leukocyte-expressed calpains in these studies. The results obtained demonstrated that endothelial-expressed \( \mu \)-calpain and not leukocyte-expressed calpain was the target of Ang II under our experimental conditions. Specifically, Ang II failed to increase the adhesion of leukocytes isolated from wild-type mice to the aortic endothelium of \( \mu \)-calpain deficient mice as well as of mice overexpressing the endogenous inhibitor of calpain, calpastatin (Online Figure III, B). In contrast, increased leukocyte adhesion to Ang II was observed when aortas isolated from wild-type mice were incubated with leukocytes isolated from \( \mu \)-calpain deficient mice or calpastatin overexpressing mice (Online Figure III, B). These data are in agreement with data on leukocyte adhesion obtained by

\[ \text{Figure 4. Depletion of } \mu \text{-calpain with siRNA prevents upregulation of calpain activity in MMECs exposed to Ang II.} \]

Treatment of MMECs with siRNA to \( \mu \)-calpain effectively reduces calpain expression levels (immunoblot). Ang II fails to upregulate calpain activity in \( \mu \)-calpain deficient MMECs (bar graph), thus demonstrating that the endothelial expressed \( \mu \)-calpain isoform is the specific molecular target of Ang II/AT1R signaling under our experimental conditions. Ang II increases calpain activity via activation of the AT1R, as demonstrated by loss of increased calpain activity in MMECs treated with losartan. The calpain inhibitor ZLLal also prevented calpain activation in response to Ang II. Calpain activity in attached MMECs was measured using a standard fluorescent assay and the calpain selective substrate T-Succ-LLVY-AMC. Numbers at the base of the bars indicate the number of independent experiments.

\[ \text{Figure 5. Leukocyte–endothelium interactions in mesenteric postcapillary venules.} \]

Leukocyte rolling (A), adhesion (B), and extravasation (C) were studied in all experimental groups of rats by bright field intravital microscopy and expressed as the number of leukocytes/min, leukocytes/100-\( \mu \)m vessel length, and leukocytes/1000 \( \mu \)m² extravascular space, respectively. Ang II increases leukocyte endothelium interactions in the microcirculation, which were reduced similarly by losartan, ZLLal or PDTC treatment. Bars represent means±SEM, and numbers at the base of the bars represent the number of rats studied in each group.
intravital microscopy and they demonstrate that in the setting of increased Ang II/AT1R signaling, the vascular endothelium develops a widespread proadhesive phenotype affecting both large conduit vessels and the microcirculation.

We also used fluorescent intravital microscopy of postcapillary venules to measure albumin leakage, another established marker of endothelial function. Data reported in Figure 6 demonstrate that Ang II disrupts the physiological endothelial cell barrier as demonstrated by the increased albumin leakage found in Ang II–infused rats. This permissive action of Ang II on albumin permeability was abolished by treatment with Ang II–infused rats with either losartan or ZLLal (Figure 6).

Figure 6. Elevated Ang II/AT1R signaling increases albumin permeability. The average permeability index to Texas red–labeled albumin was calculated during intravital microscopy in single mesenteric postcapillary venules of all experimental groups of rats. A 2-week exposure of the mesenteric microcirculation to a low, subpressor dose of Ang II increases albumin permeability via AT1R signaling and in a calpain-dependent manner. Thus, both losartan and the calpain inhibitor ZLLal attenuated albumin permeability in Ang II–injected rats. Data are expressed as the means±SEM, and numbers at the base of the bars represent the number of rats studied in each group.

These functional results were observed in the absence of significant changes in mean arterial blood pressure, vessel diameter, venular shear rates, and total leukocyte blood counts (Online Table I).

Taken together, these data first demonstrate that endothelial expressed calpains are, at least in part, responsible for the endothelial inflammatory actions Ang II/AT1R signaling in the cardiovascular system.

Ang II/Calpain Signaling Upregulates Endothelial eCAMs via Downregulation of IκBα

To investigate the molecular mechanism(s) responsible for the dysfunctional endothelial phenotype induced by Ang II–mediated μ-calpain activation, we studied expression levels of the cell adhesion molecules intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin both in vivo and in vitro. Figure 7 illustrates ICAM-1 expression levels in all experimental groups of rats studied by immunohistochemistry. Thus, we found a 5-fold increase in ICAM-1 expression in the microcirculation of Ang II–infused rats compared to control rats given saline. Consistent with the functional data reported above, ICAM-1 expression was attenuated by treatment with losartan, ZLLal, or the NF-κB inhibitor PDTC (Figure 7, left bar graph). Similar results were observed for VCAM-1 and E-selectin (Online Figure V).

Flow cytometric studies in MMECs further confirmed the increased ICAM-1 expression levels in MECs exposed to 10 mmol/L Ang II for 6 hours, a phenomenon that was prevented by either inhibition of AT1R, calpain, or NF-κB with losartan, ZLLal, and PDTC, respectively (Figure 7, right bar graph).

Furthermore, The effect of Ang II on eCAMs expression were also found to be largely μ-calpain dependent as demonstrated
measured IκBα levels in all experimental groups of rats were studied by Western blot analysis in the cytosolic fractions of vascularized mesenteric tissue lysates (immunoblot). Bar graph shows densitometric analysis of IκBα expression levels in all experimental groups of rats. Reduced IκBα expression levels were found in the mesenteric vasculature of Ang II–infused rats. Treatment of Ang II–infused rats with either losartan or the calpain inhibitor ZLLal significantly restored IκBα expression. Equal loading was confirmed by β-actin staining. Bars represent mean±SEM, and numbers at the base of the bars represent the number of rats studied in each group.

Figure 8. IκBα expression in the vasculature of Ang II–infused rats. IκBα levels in all experimental groups of rats were studied by Western blot analysis in the cytosolic fractions of vascularized mesenteric tissue lysates (immunoblot). Bar graph shows densitometric analysis of IκBα expression levels in all experimental groups of rats. Reduced IκBα expression levels were found in the mesenteric vasculature of Ang II–infused rats. Treatment of Ang II–infused rats with either losartan or the calpain inhibitor ZLLal significantly restored IκBα expression. Equal loading was confirmed by β-actin staining. Bars represent mean±SEM, and numbers at the base of the bars represent the number of rats studied in each group.

by lack of ICAM-1 upregulation following siRNA depletion of μ-calpain (Figure 7, right bar graph). These data correlate with the antiadhesive effect of AT1R blockade and calpain inhibition on leukocyte–endothelium interactions reported above.

In the vascular endothelium, ICAM-1, VCAM-1, and E-selectin are largely regulated by NF-κB, an inflammatory nuclear transcription factor that it sequestered inactive in the cytoplasm by the inhibitor protein IκBα. Accordingly, we measured IκBα expression levels in vascular sections of the mesentery from all experimental groups of rats. Data reported in Figure 8 demonstrate that the microcirculation of Ang II–infused rats experiences loss of IκBα, which can be prevented by pharmacological inhibition of either AT1R or calpain with losartan or ZLLal, respectively. Taken together, these data demonstrate that Ang II/AT1R signaling causes IκBα degradation in part via increased calpain activity and they provide a novel mechanism by which Ang II/AT1R signaling induces transcription of inflammatory mediators in the dysfunctional endothelium.

Discussion

The present work, to our knowledge, is the first to uncover the role of the cytosolic protease calpain in the endothelial dysfunction associated with elevated Ang II signaling. We show that subpressor doses of Ang II selectively activate the endothelial expressed μ-calpain isoform, a process that induces a distinct endothelial inflammatory phenotype. Importantly, we show that μ-calpain is a molecular target of AT1R signaling, which may also help uncover new mechanism(s) of the pleiotropic actions of ARBs.

Disruption of the regulatory functions of the vascular endothelium results in multiple maladaptive modifications, including making the endothelium hyper adhesive to circulating leukocytes and leaky to macromolecules. Studies in research animals and humans have clearly linked Ang II/AT1R signaling to endothelial dysfunction, independently of its vasopressor actions. In agreement with our results, others have demonstrated that nanomolar concentrations of Ang II acutely increase leukocyte–endothelium interactions18 and vascular permeability19 in the rat microcirculation without affecting local or systemic hemodynamic. Leukocyte–endothelium interactions and vascular permeability are 2 fundamental functions of the endothelium that are also crucial to the homeostasis of organ tissues, including the vascular wall itself. In fact, leukocyte trafficking is mechanistically important in vascular pathology. Extravasation of proteins into the adjacent interstitial compartment modifies the composition of the interstitium, which disrupts traffic of fluid and vital substrates to the cellular mass, and the removal of waste products.20 Accordingly, full understanding of the molecular mechanisms by which Ang II increases leukocyte–endothelium interaction and vascular permeability becomes crucial for the management of vascular disease and related organ damage. With most data indirectly derived from studies using pharmacological inhibitors of the RAS, our understanding of the primary cellular pathways(s) responsible for the endothelial inflammatory action of Ang II remains largely elusive, which hinders development of new therapeutic strategies. Our present data are the first to identify in the calpain system a direct molecular effector of the endothelial Ang II/AT1R signaling pathway. This novel working hypothesis is supported by data in the scientific literature. Calpains are cytosolic proteases that require calcium for their activation and function.17 Of interest, published data have demonstrated that in cultured endothelial cells Ang II increases intracellular calcium concentrations via activation of AT1R.21 The detailed signaling pathway(s) by which activation of the AT1R leads to increased endothelial calpain activity with subsequent leukocyte adhesion remains unknown. Data in the literature have highlighted a role for the calcitonin gene-related peptide (CGRP) in the leukocyte adhesion associated with activation of the AT1R,22 which may suggest a role for CGRP in these studies. We found that Ang II selectively activates the μ-calpain isoform, but not the m-calpain isoform. This result is in agreement with recent studies demonstrating a role for μ-calpain in the homeostasis of the vascular wall2,3,22 and it compatible with the knowledge that only the Ca2+ concentrations required by autolysed μ-calpain are in the physiological range, whereas the Ca2+ concentrations required by m-calpain are much higher than those found in living cells.17 Nonetheless, the possibility that with chronic disease other vascular expressed calpain isoform may also become deregulated should not be entirely excluded based on in vitro studies demonstrating a role for m-calpain in the microvas-
cular endothelial toxicity induced by inflammatory media-
tor. Furthermore, evidence in the literature suggests the possibility of sequential activation of calpain isoforms.

Specific adhesion molecules expressed on the vascular endothelium and circulating leukocytes regulate leukocyte–endothelium interactions in the cardiovascular system. In this study, we focused on the role of NF-κB–regulated cell adhesion molecules such as ICAM-1, VCAM-1 and E-selectin. Despite intensive research, the molecular mechanisms by which Ang II/AT1R signaling upregulates NF-κB–controlled eCAMs is not fully understood. In our in vitro and in vivo results first demonstrate a mechanistic role for μ-calpain in the process of eCAMs upregulation by Ang II. Interestingly, others have implicated calpains in the upregulation of eCAMs expression via NF-κB.

We also found evidence of increased CD11b expression levels on the cell surface of blood resident leukocytes of Ang II–infused rats, although isolated leukocytes stimulated with Ang II failed to adhere to the endothelium of control aortas in vitro. This result indicates that activation of endothelial expressed CAMs is necessary for the vascular inflammatory action of Ang II, which is consistent with published information demonstrating that Ang II–induced leukocyte–endothelium interactions can be largely abrogated by eCAMs blockade alone. Although Ang II–induced upregulation of CD11b was also attenuated by calpain inhibition in our study, the overall impact of calpain inhibition on leukocyte function remains still controversial. Studies have reported increased leukocyte adhesion, polarization, and chemotaxis following inhibition of basal calpain activity in resting leukocytes. In contrast, more recent studies demonstrate that inhibition of calpain activity reduces the adhesiveness and migratory properties of leukocytes primed by clinically relevant inflammatory stimuli.

A recent study also supports a role for μ-calpain in neutrophil-induced ICAM clustering, a process important in leukocyte extravasation. Overall, emerging views in the field consider activation of leukocyte-expressed calpains necessary for leukocyte expansion, a process central in leukocyte extravasation. Relevant to the physiology of leukocyte–endothelium interactions, endothelial expressed calpains have been shown to influence the adhesive and migratory behavior of circulating leukocytes. Conversely, adhesion of leukocytes initiates calcium signaling events that lead to activation of the endothelial calpain system, which in terms favors leukocyte transmigration in inflammation. Further studies are clearly needed to understand how inhibition of the calpain system impacts the adhesive behavior of resting and primed leukocytes, both in the presence and absence of the vascular endothelium.

Leukocytes interact with the endothelium in a process that allows blood resident leukocytes to cross the barrier created by endothelial cells. This cellular migration can result in modifications of the vascular permeability that permit the nonphysiological transfer of macromolecules from the blood compartment to the vascular wall and peripheral tissue. Sumagin et al have recently shown that vascular permeability alterations can be induced by endothelial expressed CAMs, such as ICAM-1, following ligation by rolling or adhering leukocytes. Interestingly, we also found that inhibition of μ-calpain prevents albumin permeability in microvascular networks inflamed by Ang II, which suggest a key role for the endothelial calpain system in this process. In the present study, we did not investigate whether the hyperpermeability of Ang II was secondary to adhering leukocytes or primarily driven by eCAMs-induced signaling. Because studies in this direction can provide additional therapeutic targets, further work should be undertaken to understand the primary contribution to vascular permeability of calpain-mediated eCAMs upregulation in disease states associated with elevated Ang II signaling.

The molecular mechanism by which Ang II upregulates eCAMs appears to be related to activation of the nuclear transcription factor NF-κB. In vitro studies have demonstrated increased activation of NF-κB in response to Ang II in several cell types, including VSMCs and endothelial, glomerular, tubular, and mononuclear cells. In vivo, increased NF-κB activity and expression have been shown in the vasculature, heart, and kidneys of double transgenic rats and Ang II–infused wild-type rats. Nonetheless, the precise signaling pathway by which Ang II upregulates NF-κB remains largely unknown. Interestingly, calpain is known to degrade the NF-κB–inhibitory subunit IκBα, thus leading to NF-κB activation. Here, we report evidence of calpain-dependent degradation of IκBα in response to activation of Ang II/AT1R signaling in the rat vasculature, which uncovers a novel molecular mechanism by which Ang II upregulates NF-κB activity and actions in the vascular wall.

Data in the literature have consistently shown that Ang II–induced vascular inflammation is largely mediated via stimulation of the AT1R. Relevant to the present study, the AT1R blockers, such as losartan, attenuate (1) leukocyte–endothelium interactions in Ang II–infamed microvascular networks; (2) vascular permeability in experimental animal models; and (3) albuminuria in patients with cardiovascular disease. However, the precise cellular targets of the endothelial pleiotropic actions of ARBs remains largely unknown. Our results strongly demonstrate a role for the endothelial calpain system in the beneficial vascular actions of ARBs. In fact, losartan prevented calpain activation both in vivo and in vitro, a result that was also confirmed in AT1R deficient mice. Furthermore, pharmacological blockade of calpain achieved a degree of endothelial protection which was comparable to that observed with losartan treatment in vivo. Thus, both losartan and the calpain inhibitor ZL771L stabilized IκBα levels and prevented leukocyte–endothelium interactions and albumin permeability in the face of increased Ang II levels.

In summary, the present studies demonstrate that μ-calpain contributes to the development of Ang II–induced endothelial dysfunction. Specifically, inhibition of calpain activation attenuates leukocyte–endothelium interactions, albumin permeability, endothelial CAMs expression, and IκBα degradation in Ang II–infused rats. Finally, these protective actions of calpain inhibition are related to inhibition of AT1R signaling, which demonstrate a mechanistic role for calpain in the pleiotropic actions of ARBs. Our studies may have important clinical implications in that they provide evidence for a novel molecular target in the treatment of Ang II–mediated vascular disease.
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Disclosures

None.

References

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**Novelty and Significance**

**What Is Known**

- The dysfunctional endothelium upregulates CAMs, thus becoming adhesive to circulating leukocytes, a process causative of vascular pathology.
- Ang II/AT1R signaling upregulates CAMs in the vascular endothelium.
- The cytosolic protease calpain modulates the expression of CAMs and of the NF-κB inhibitor IκBα.

**What New Information Does This Article Contribute**

- Ang II/AT1R signaling increases the activity of μ-calpain in the vascular endothelium.
- μ-Calpain upregulates NF-κB activity and leukocyte–endothelium interaction in microvascular networks inflamed by Ang II.
- Inhibition of calpain activity prevents leukocyte–endothelium interactions induced by Ang II/AT1R signaling.

The precise mechanism by which Ang II/AT1R signaling induces leukocyte–endothelium interactions remains elusive. Here, we show that μ-calpain expressed in the vascular endothelium mediates leukocyte–endothelium interactions caused by activation of Ang II/AT1R signaling, which occurs in vascular disease. Our data further show that μ-calpain regulates IκBα expression in the vasculature in response to Ang II. By uncovering the role of μ-calpain in the regulation of IκBα we provide a novel mechanism that connects Ang II/AT1R signaling with NF-κB activity in vascular pathology. Thus, calpain may potentially be a therapeutic target for preventing NF-κB-induced endothelial dysfunction and vascular inflammation. Because the protective actions of calpain inhibition are related to inhibition of AT1R signaling, our study also demonstrates a mechanistic role for calpain in the pleiotropic actions of ARBs.
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Supplemental Methods

Animals. Male Sprague-Dawley rats (ACE, Boyertown, PA) of 250 g average body weight were randomly divided into one of five groups: 1) control rats injected with vehicle; 2) rats infused with a low, subpressor concentration of AngII; 3) rats infused with AngII and treated with the AngII receptor-1 blocker losartan; 4) rats infused with AngII and treated with the calpain inhibitor ZLLal; 5) rats infused with AngII and treated with the NF-κB inhibitor pyrrolidine dithiocarbamate. Wild-type C57BL/6J mice (Stock #002682, Jackson Laboratory, Ann Arbor MI), angiotensin II Type 1 receptor deficient (−) mice (Stock #000664, Jackson Laboratory, Ann Arbor MI), mice deficient in μ-calpain (Capn1−/−; stock number EM:02362; European Mouse Mutant Archive (http://www.emmanet.org/strains.php), and calpastatin transgenic mice (CalpTG; stock # EM:01922 European Mouse Mutant Archive (http://www.emmanet.org/strains.php)) were also used to further confirm the mechanistic contribution of the AT1r and μ-calpain in these studies. All mice used had an average body weight of 26±2 g.

Pharmacological inhibition of the AT1 receptor, or calpain activity, and NF-κB activity in vivo. Inhibition of the AT1r was achieved by treatment of in AngII infused animals with 10-mg/kg/day losartan. Losartan was administered in the drinking water. This concentration of losartan was based on previous studies demonstrating vascular protective actions in rats and mice.3−2. Calpain activity was blocked with ZLLal (benzoyloxy carbonyl- leucyl-leucyl- leucinal; Biomol Research Laboratories, Plymouth Meeting, PA), a selective calpain inhibitor with high selectivity for calpain over other proteosomal enzymes3 and with demonstrated efficacy in preventing activation of μ-calpain in the vascular endothelium of the live animal.4 The dipeptide, ZLLal, blocks calpain activity by binding to its catalytic site. Based on previous studies, ZLLal was used at the dose of 140 μg/Kg/day and dissolved in a final ethanol concentration of 0.01%5. To increase the specificity of our results in vivo, in a parallel set of experiments rats were treated with 1-mg/kg PD 150606 (PD150; Calbiochem, USA) a cell-permeable, selective non-peptide calpain inhibitor directed towards the calcium-binding sites of calpain.6 NF-κB was inhibited with pyrrolidine dithiocarbamate (PDTC), a widely used NF-κB blocker with demonstrated vascular protective actions in the setting of elevated AngII signaling.7 PDTC was administered as the dose of 50 mg/kg/day. All calpain inhibitors and PDTC were administered intraperitoneal once a day. In control experiments, intraperitoneal injection of saline (0.9% NaCl) alone or saline containing 0.01% ethanol to the animals did not affect microvascular parameters (data not shown). All treatments were administered for 5 consecutive days, beginning at day 9 on AngII infusion.

Intravital Microscopy of the rat mesenteric microcirculation. A. Preparing of the mesenteric window. Following anesthesia with 80-mg/kg/ip pentobarbital, rats were prepared for IVM studies as previously described.3 Four distal loops of ileal tissue, exteriorized through a midline incision laparotomy, were superfused with 37°C Krebs-Henseleit buffer in an intravital microscopy Plexiglas chamber attached on the stage of an Eclipse FN1 Physiosation Microscope (Nikon Corp., Japan). Three to four relatively straight, unbranched segments of postcapillary venules with lengths of >100 μm and diameters between 25 and 40 μm were randomly studied in each rat. Observation of the mesenteric microcirculation was made with 20X salt water-immersion lens. Images were projected by high-resolution, intensified video cameras (XR Mega-10 EX ICCD; Stanford Photonics INC) onto a high-resolution, color video monitor (Multiscan 200-sf, Sony), and the image recorded on A WIN XP Imaging Workstation. All data were analyzed using computerized imaging software (Micro-Manager). Leukocyte rolling, adhesion and transmigration were studied in mesenteric post-capillary 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 100-μm length of endothelium for at least 30 seconds. The number of leukocytes extravasated within 5-mm perivascular area were counted and normalized with respect to the area.

B. Venular diameter (Dven) measurement and calculation of shear rates. Dven was measured on-line using a video-caliper and the software Image Pro Plus v5.1. Red blood cell velocity and venular wall shear rates. Centerline red blood cell velocity was measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute, College Station, TX). Venular wall shear rate, (sec-1), was calculated by using the formula: g = 4.9*Vmean/Dven where Vmean = Vrbc/1.68.

C. Evaluation of vascular density. The microvascular density of the peritoneum could in theory change following a 14-day infusion of AngII, which would affect vascular permeability. Thus, we evaluated microvascular density according to a method previously reported.8 To avoid selection bias, we performed quantification in random segment of the visceral peritoneum of three predefined ileal loops (i.e, the most three distal loops of ileum). Images were taken with a 10x
objective positioned at random in the mesenteric window. The microscopic stage was driven by a stepping motor control MS-4000 XYZ Automated Stage (Applied Scientific Instrumentation, Eugene, OR), operated by a computer through a USB interface. With the aid of the MS-4000 XYZ, the microscopic stage was driven through a meander consisting of 6 steps of 1 mm in the X direction and 6 steps of 1 mm in the Y direction. At each of these 36 positions, the microscopic image was recorded in 10-µm intervals above, through and below the plane of focus (z-plane). Multiple z-planes were used for 2D deconvolution analysis, which removes the out-of-focus light that is prevalent in epifluorescent microscopy, thus allowing for a clear definition of the vascular structures within the mesentery. The vessel length per area was determined for each microscopic image, and the average was calculated. Capillaries, defined as vessels with a luminal diameter <5 µm\(^5\), were analyzed separately, and their relative contribution to total vessel length was also calculated. The vessel length per area, for each microscopic image, was calculated with the use of the software Image Pro Plus v5.1.

**D. Measurement of vascular permeability.** Permeability index of post-capillary venules in aging rats was determined by intravital fluorescent microscopy using a previously described technique\(^{11}\) and according to principles outlined by Bekker and coworkers\(^{12}\). The intravital microscopy setup described above was used for these studies. The preparation was epilluminated with an intensified 100-W super high-pressure mercury lamp, and fluorescence was assessed using dichroic filter cubes [TR: excitation 560, emission 630 nm]. Neutral density filters were placed in the epifluorescence light path in the in vivo experiments. Texas Red-labeled albumin (TR-BSA; Molecular Probes) was used as a tracer for microvascular permeability to micromolecules. TR-BSA was administered intravenously at a dose of 25-mg/kg body wt. For the evaluation of macromolecular leakage, venular segments with a diameter of 25 to 40 µm and an unbranched length of approximately 150 µm without any other vessels in the immediate vicinity were selected for study. When traumatic leaks were observed, the experiment was discontinued. Otherwise, epifluorescence recordings were made every 10 min for 120 min, after a 30 min stabilization period. A total of 4-5 randomly selected venules were studied in each rat. On the digitized image of the venule under study, the fluorescence intensity in three 10×50-µm intravascular regions (Iv) and the intensity in three 10×50-µm perivascular interstitium regions located 10 µm from the edge of the venule (It), were measured with computer-assisted digital image analyses. The average gray scale value, ranging from 0 for black to 256 for white, was calculated for each area using the software Image Pro Plus v5.1. The same three tissue fields were subsequently recorded for extravasation of macromolecules as a function of time. To minimize light exposure to the tissue, each measure of albumin leakage was performed during a brief (≈5-15 sec) exposure to epi-illumination, controlled with a computer-operated shutter (Uniblitz VMM-D1, Vincent Associates, Rochester, NY). The background intensity (I0; present before injection of TR-BSA) was subtracted from both It and Iv. As the molecule leaves the circulation, the intraluminal gray scale value falls and the perivascular gray scale value rises. Macromolecular leakage was defined as the permeability index (PI) calculated as (It - I0)/(Iv - I0). To allow for a more accurate determination of Iv we follow a previously published two-step approach\(^{13}\). First, intravascular TR-BSA intensity was normalized using an impermeable arteriole, because TR-BSA leakage from the venule into the tissue (in the plane of the microscope objective) can cause an overestimation of the plasma concentration of TR-BSA (given the 2-dimensional limitation in determining the location of the fluorescence). Second, because the intravascular intensity of TR-BSA is influenced by the diameter of the vessel, a calibration between the average observed diameter and fluorescence intensity was also performed, according to a previously described technique\(^{13}\).

**Adhesion of isolated mouse leukocytes to the mouse aorta in vitro.** For these studies, we used a well-established in vitro adhesion assay also described by our laboratory\(^{14}\). Briefly, the thoracic aorta and blood resident leukocytes were isolated from anesthetized donor control C57BLK mice, Capn1-/- mice, and CalpTG mice. Following midline thoracotomy, the aorta was quickly removed and placed in cold, oxygenated phosphate-buffered saline (PBS). After carefully removing the adventitia, aortas were cut to 4-mm segments with the aid of a dissecting microscope (PZMIV, WPI, Sarasota, FL). The segments were then carefully opened longitudinally and placed into culture dishes containing 1 ml K-H solution with their luminal surface facing up. Whole blood was obtained from anesthetized mice through a cannula inserted in the carotid artery. Leukocytes were isolated from whole blood by gradient centrifugation accordingly to previously described methods, also used by the PI laboratory\(^{15}\). Isolated leukocytes were then fluorescently labeled using PKH26GL staining kit (SIGMA), as previously described\(^{14}\). Briefly, a suspension of leukocytes was incubated with 4µM PKH26GL for a labeling period of 5min. The labeling was stopped by adding PBS containing 10% fetal calf serum. Aortic segments and isolated leukocytes were then separately incubated with 100 nM AngII for 120 min at 37°C in the presence or absence of either 1 µM losartan or 10 µM ZLLal to block the AT1r and calpain, respectively. At the end of the incubation period aortic sections and leukocytes were gently washed with K-H buffer to remove residual AngII, losartan and ZLLal from the incubation media. Leukocytes were then incubated with the aortic segments at a
concentration \(10^5\) cells/aortic segment in incubation wells for 60 min at room temperature in an orbital shaker platform. The aortic segments were then removed, gently washed in fresh K-H buffer, and placed lumen side up on microscope slides. The aortic segments were covered with a drop of immersion oil followed by a glass cover slip. The number of leukocyte adhering to the endothelial surface in four separate microscopic fields was counted under epifluorescent microscopy at a magnification of 200x. Results are expressed as total number of cells/microscopic field.

**Hematologic parameters.** Tail vein blood was used to obtain peripheral leukocyte counts using a Neubauer chamber according to standard hematology procedures.

**Immunohistochemical quantification of eCAMs.** Quantification of ICAM-1, VCAM-1 and E-selectin expression levels in the vascular endothelium of selected experimental groups of rats was performed by immunohistochemistry as previously described\(^{16}\). After completion of the intravital microscopy, the superior mesenteric artery and vein were cannulated for perfusion of the small bowel. The ileum was first washed free of blood by perfusion with Krebs-Henseleit buffer warmed to 37°C and bubbled with 95% O\(_2\) and 5% CO\(_2\). Once the venous perfusate was free of red blood cells, perfusion was initiated with iced 4% paraformaldehyde mixed in phosphate-buffered 0.9% NaCl for 5 minutes. Rats were then euthanized by intravenous injection of an overdose of sodium pentobarbital. A 3- to 4-cm segment of ileum was isolated from the perfused intestine and fixed in 4% paraformaldehyde for 90 minutes at 4°C. Tissue sections were embedded in plastic (Immunobed, Polysciences Inc), and 4-µm-thick sections were cut and transferred to Vectabond-coated slides (Vector Laboratories), as previously described by our laboratory\(^{17}\). Immunohistochemical localization of eCAMs was accomplished by using the avidin/biotin immunoperoxidase technique (Vectastain ABC reagent, Vector Laboratories) and monoclonal antibody monoclonal antibodies against ICAM-1 (BD Transduction Laboratories, San Diego, CA), VCAM-1 (BD Transduction Laboratories, San Diego, CA), and E-selectin (RME-1\(^{18-20}\), a generous gift from Dr. Andrew C. Issekutz, Dalhousie University, Halifax, NS. Canada). Three to four rats were studied in each group, and 50 venules were analyzed per tissue section, with 20 sections examined per rat. The percentage of eCAMs positive staining was thus determined on 500 venules per rat.

**Flow cytometry evaluation of ICAM-1 and CD11b.**

**A. ICAM-1 expression levels** in \(2 \times 10^6\) isolated MMEC was performed by FACS analysis using an anti-rat R-PE-conjugated monoclonal antibody (MR106, BD Biosciences USA) or control isotype IgG according to standard procedure also reported by the PI team\(^{21}\). Briefly, antibodies were added for 40 min at 4°C followed by extensive washing with PBS.

**B. Expression levels of CD11b** were studied in circulating leukocytes from control rats infused vehicle, rats infused with subpressor doses of AngII and, AngII infused rats also given 140 µg/kg calpain inhibitor ZLLal. Blood samples (1ml) were obtained by cardiac puncture from anesthetized rats. Duplicate blood samples (100 µl) were incubated with either 10 µl of an anti-rat CD11b-FITC mAb for 20 min on ice (BD Transduction Laboratories, San Diego, CA), 10 µl of fluorescein isothiocyanate-labeled mouse anti-rat immunoglobulin G Ab, or with no Ab. Erythrocytes were then lysed by exposure to 1 mL of 1:10 dilution of lysing buffer (Erythrolyse, Serotec) for 15 min and centrifuged at 1000 rpm for 5 min. The supernatant was aspirated, and leukocytes were resuspended in 2 mL of CELLwash (BD Biosciences) before centrifugation again for 5 min at 1000 rpm followed by aspiration and resuspension of the leukocytes in 500 µL of CELLwash. FACS analysis was then performed on a FACSCAN (Becton Dickinson, USA) equipped with the CELLQuest software. Analyses were performed using the appropriate FL1/FL2 channel histograms from which the geometric mean fluorescence intensity channel (MFC) was calculated for each sample. In the case of leukocytes, cells were displayed on a density plot according to their forward- and side-scatter characteristics. Populations of neutrophils and lymphocytes were then gated (few monocytes were present), and the fluorescent intensity of the gated neutrophils was analyzed. Therefore, subsequent data utilizing FACS include neutrophils only because this reflects the cell type largely detected using intravital microscopy.

**Fluorescent analysis of calpain activity.**

**A. In tissue extracts.** Highly vascularized mesenteric segments were isolated, snap frozen in liquid nitrogen, and homogenized in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10 mM EGTA, and 250 mM sucrose with a Polytron homogenizer (Biospec Products, Inc., Bartlesville, OK) at 28,000 rpm for 10 seconds. The homogenates were centrifuged at 3,500 rpm for 10 minutes, and the supernatant was collected and further centrifuged at 14,000 rpm for 20 minutes. The final supernatant was saved and assayed for protein content using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Total protein (50 µg) was incubated with 30 µM of the fluorogenic calpain substrate Suc-c-LLVY-AMC...
(N-succinyl-l-leucyl-l-valyl-l-tyrosine-7-amino-4-methylcoumarin; Bachem, Torrance, CA) in PBS with 1 mM Ca+2 for 60 minutes at 37°C. Mean fluorescent signals (excitation λ 360 nm and emission λ 460 nm) were measured using a microplate fluorescence reader (Bio-Tek-FLx800, Winooski, VT). Calpain activity was calculated as described above (ΔI=I_f-I_0).

B. In vivo. To localize active calpain in the rat mesenteric post-capillary venules, 500 nM of the fluorogenic calpain substrate 7-amino-4-chloromethylcoumarin, t-BOC-L-leucyl-L-methionineamide (t-BOC-Leu-Met-CMAC; Invitrogen, Carlsbad, CA) dissolved in K-H buffer was superfused on the mesenteric window for 60 minutes during intravitral microscopy. Previous studies have validated the specificity of t-BOC-Leu-Met-CMAC for calpains by using specific calpain inhibitors, modulation of intracellular pH and calcium, confirmation that substrate levels exceed the Km for calpains, and identification of the CMAC-GSH conjugate as the major intracellular fluorescent product generated by hydrolysis of the probe. In preliminary studies, t-BOC-Leu-Met-CMAC was superfused on the mesentery at varied concentrations ranging from 10 nM to 1 µM to obtain the optimal concentration of t-BOC-Leu-Met-CMAC that did not elicit alterations of the preparation (e.g., leukocyte rolling). No significant elevation of leukocyte rolling was observed with concentrations below 35 µM. Fluoro-micrographs of t-BOC-Leu-Met-CMAC complex in the tissue were captured using a 360 nm excitation filter, a 390 nm dichroic reflector and a 410 nm long pass emission filter, as previously described. Fluoro-micrographs were captured at intervals of 10 minutes for 1 hour using the intravitral microscopy setup described above. Fluorescence intensity along 100 µm of vessel wall was then quantified by densitometry in ten 10-µm-diameter circular regions of interest (ROI) along each side of the venule using Image J software (National Institutes of Health, Bethesda, MD). Twenty ROIs were averaged for single fluorescence intensity values at baseline (I0) and at 60 minutes after t-BOC-Leu-Met-CMAC superfusion (I_f), and calpain activity was calculated as the delta change in fluorescence intensity (ΔI=I_f-I_0).

C. In Attached MMEC in culture. A modified protocol was used to measure calpain activity in attached mesenteric microvascular endothelial cell (MMEC). Following treatment, MMEC were washed with PBS and then incubated in PBS containing 60 µM of the fluorogenic calpain substrate T-BOC-LEU-MET-CMAC plus 1 mM Ca+2 for 30 minutes at 37°C, as described previously. Mean fluorescence signals from MMEC grown to confluence in a 24-well plate were measured using the microplate fluorescence reader described above. Specificity of the T-BOC-LEU-MET-CMAC for calpain was confirmed by both pharmacological inhibition and siRNA depletion of calpain activity.

Western blot analysis of μ-calpain, m-calpain isoforms expression levels. Activity of the two calpain isoforms expressed in the vascular endothelium was studied by western blot analysis, using monoclonal antibodies against either the activation-cleaved N-terminus domain (RP1 calpain-1 or RP2-calpain-2) or the stable Domain IV (RP3 calpain-1 and RP3 calpain-2; Triple Point Biologics, Portland OR) of the large subunit of m- and μ-calpain, respectively. Calpains autolize the amino-terminal end of domain I upon activation. The resulting loss of amino-terminal domain antibody detection can be used as a measure of μ- and m-calpain activation. Antibodies against the stable domain IV of μ- and m-calpain were used to measure total μ- and m-calpain expression levels.

Isolation of Mesenteric microvascular endothelial cells (MMEC) and knockdown of μ-calpain. MMEC were isolated from densely vascularized segments of mesenteries as previously described by our laboratory. Briefly MMEC were isolated using magnetic microbeads (Miltenyi Biotech, Auburn CA) ligated with anti-rat PECAM-1 antibody, and phenotyped by FACS analysis of PECAM-1 and by functional uptake of acetylated LDL. Rat MMEC were cultured at 37°C with 5% CO2 in Dulbecco’s Modified Eagle’s medium supplemented with 150 µg/ml EC growth supplement (Sigma, ST Louis, MO) on gelatin-coated tissue culture dishes. To minimize changes in the Ang-II responsive phenotype of MMEC we only used primary passage MMEC cultures. A small interfering RNA (siRNA) against μ-calpain (Santa Cruz Biotechnology, Santa Cruz, CA) was used to selectively inhibit μ-calpain activity by depleting its cytosolic expression, according to a previously described protocol.
Supplemental Figures and Table

Online Table I. Hematologic and hemodynamic values all experimental groups of rats

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>MABP (mmHg)</th>
<th>Venular Diameter (µm)</th>
<th>Venular Shear Rates (sec⁻¹)</th>
<th>WBC (10³/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (n=6)</td>
<td>112±9</td>
<td>30.1±3.2</td>
<td>710±59</td>
<td>8.6±0.7</td>
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<tr>
<td>AngII (n=7)</td>
<td>122±12</td>
<td>28.5±1.5</td>
<td>685±66</td>
<td>8.2±0.4</td>
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<tr>
<td>AngII + Losartan (n=7)</td>
<td>108±14</td>
<td>30.7±1.2</td>
<td>655±67</td>
<td>8.3±0.1</td>
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<tr>
<td>AngII + ZLLal (n=7)</td>
<td>115±12</td>
<td>29.5±0.8</td>
<td>700±43</td>
<td>9.0±0.1</td>
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<tr>
<td>AngII + PDTC (n=4)</td>
<td>111±8</td>
<td>27.0±1.4</td>
<td>679±52</td>
<td>8.9±0.5</td>
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</table>

Online Figure I. AngII/AT1 signaling does not upregulate the m-calpain isoform in the vasculature. m-Calpain activity was assessed by immunoblot analysis using a primary antibody that selectively recognizes the N-terminus domain of the large 80 kDa subunit of m-calpain. Upon activation, the N-terminus domain is cleaved from the calpain large subunit, which results in loss of antibody recognition. Lack of significant changes in N-terminus domain expression levels (panels A and B) demonstrates that AngII does not cleave m-calpain under our experimental conditions. Similarly, no changes in C-terminus domain expression levels were observed in all groups of rats as detected using a primary antibody against the stable C-terminus domain of the large subunit of m-calpain (data not shown). Densitometric analysis of the N-terminus domains expression levels in all groups of rats is shown in the bar graphs. Numbers at the base of the bars indicate the number of rats studied in each group.
Online Figure II. Mice deficient in \( \mu \)-calpain are protected against leukocyte adhesion induced by AngII. Leukocyte adhesion in live mice was studied by intravital microscopy of peri-intestinal venules. Leukocyte adhesion was induced by superfusion of the exposed mesenteric tissue with 100 nM AngII for 60 minutes during intravital microscopy experiments. AngII increased leukocyte adhesion in C57BLK mice but not in mice deficient for \( \mu \)-calpain, which demonstrates a key role for \( \mu \)-calpain in the inflammatory action of AngII. Bars represent mean \( \pm \) SEM, and numbers at the base of the bars represent the number of mice studied in each group.

Online Figure III. The obligatory role of the vascular endothelium and of endothelial expressed \( \mu \)-calpain in the mechanism of AngII-induced leukocyte-endothelium interactions. Circulating leukocytes and thoracic aortas were isolated from wild-type, \( \mu \)-calpain\(^{+/-}\), and calpastatin overexpressing donor mice. Leukocytes were fluorescently labeled as reported above. Isolated leukocytes and 4 mm length aortic segments were then separately exposed to 100 nM AngII for 120 minutes before being incubated together for 60 min in an orbital shaker to allow adhesion of leukocytes to the aortic endothelium. Panel A. When AngII-stimulated aortic segments were incubated with unstimulated leukocytes a significantly higher number of leukocytes adhered to the aortic endothelium. In contrast, no difference from control (i.e., unstimulated aortic segment/unstimulated leukocytes) was observed following incubation of AngII-stimulated leukocytes with unstimulated aortas. The increased adhesiveness to leukocytes of AngII-stimulated aortas was also prevented by treatment of the aortic segments with losartan or ZLLal. Panel B. AngII failed to increase the adhesion of leukocytes isolated from wild-type mice to the aortic endothelium of \( \mu \)-calpain deficient mice and of mice overexpressing the endogenous calpain inhibitor, calpastatin. In contrast, higher than control leukocyte adhesion was observed when leukocytes isolated from \( \mu \)-calpain deficient mice or calpastatin overexpressing mice were incubated with AngII-stimulated aortic segments from wild-type mice. Bars represent mean \( \pm \) SEM, and numbers at the base of the bars indicate the number of rats studied in each group.
studied in each group. NF markedly increased in AngII infused rats. Losartan treatment, calpain inhibition (ZLLal), or very low level of VCAM of rats are illustrated. Percentage of venules staining positive for VCAM in all experimental groups of rats was studied by immunohistochemistry in all experimental groups of rats. Treatment of rats with the calpain inhibitor ZLLal. AngII upregulates CD11b in circulating leukocytes, a process that is partially attenuated by systemic treatment of rats with the calpain inhibitor ZLLal. MFC indicates mean fluorescence channels values for CD11b staining measured by flow cytometry. A representative flow cytometry histogram is shown in the upper panel. Numbers at the base of the bars indicate the number of rats studied in each group.

Online Figure IV. Expression levels of CD11b in circulating leukocytes of AngII infused rats. Bar graph shows summary of CD11b expression levels in leukocytes from 1) control rats infused with saline; 2) rats infused with AngII; and 3) AngII infused rats treated with the calpain inhibitor ZLLal. AngII upregulates CD11b in circulating leukocytes, a process that is partially attenuated by systemic treatment of rats with the calpain inhibitor ZLLal. MFC indicates mean fluorescence channels values for CD11b staining measured by flow cytometry. A representative flow cytometry histogram is shown in the upper panel. Numbers at the base of the bars indicate the number of rats studied in each group.

Online Figure V. AngII increases expression levels of VCAM-1 (Panel A) and E-selectin (Panel B) in the vascular endothelium. VCAM-1 and E-selectin expression in microvessels (V) of the mesentery was studied by immunohistochemistry in all experimental groups of rats. Percentage of venules staining positive for VCAM-1 and E-selectin in all experimental groups of rats are illustrated in the right and left bar graphs, respectively. Control microvessels had a very low level of VCAM-1 and E-selectin expression. VCAM-1 and E-selectin expression was markedly increased in AngII infused rats. Losartan treatment, calpain inhibition (ZLLal), or NF-κB inhibition (PDTC) significantly attenuated VCAM-1 and E-selectin expression. Bars represent mean ± SEM, and numbers at the base of the bars indicate the number of rats studied in each group.
Additional References
