Homocysteine-mediated activation and mitochondrial translocation of calpain regulates MMP-9 in MVEC

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Moshal, Karni S., Mahavir Singh, Utpal Sen, Dorothea Susanne E. Rosenberger, Brooke Henderson, Neetu Tyagi, Hong Zhang, and Suresh C. Tyagi. Homocysteine-mediated activation and mitochondrial translocation of calpain regulates MMP-9 in MVEC. Am J Physiol Heart Circ Physiol 291: H2825–H2835, 2006. First published July 28, 2006; doi:10.1152/ajpheart.00377.2006.—Hyperhomocysteinemia (HHcy) is associated with atherosclerosis, stroke, and dementia. Hcy causes extracellular matrix remodeling by the activation of matrix metalloproteinase-9 (MMP-9), in part, by inducing redox signaling and modulating the intracellular calcium dynamics. Calpains are the calcium-dependent cysteine proteases that are implicated in mitochondrial damage via oxidative burst. Mitochondrial abnormalities have been identified in HHcy. The mechanism of Hcy-induced extracellular matrix remodeling by MMP-9 activation via mitochondrial pathway is largely unknown. We report a novel role of calpains in mitochondrial-mediated MMP-9 activation by Hcy in cultured rat heart microvascular endothelial cells. Our observations suggested that calpain regulates Hcy-induced MMP-9 expression and activity. We showed that Hcy activates calpain-1, but not calpain-2, in a calcium-dependent manner. Interestingly, the enhanced calpain activity was not mirrored by the decreased levels of its endogenous inhibitor calpastatin. We presented evidence that Hcy induces the translocation of active calpain from cytosol to mitochondria, leading to MMP-9 activation, in part, by causing intramitochondrial oxidative burst. Furthermore, studies with pharmacological inhibitors of calpain (calpeptin and calpain-1 inhibitor), ERK (PD-98059) and the mitochondrial uncoupler FCCP suggested that calpain and ERK-1/2 are the major events within the Hcy/MMP-9 signal axis and that intramitochondrial oxidative stress regulates MMP-9 via ERK-1/2 signal cascade. Taken together, these findings determine the novel role of mitochondrial translocation of calpain-1 in MMP-9 activation during HHcy, in part, by increasing mitochondrial oxidative stress.

cysteine proteases; thioredoxin; Nicotinamide adenine dinucleotide phosphate-oxidase-4; mitochondrial redox signaling; cardiovascular remodeling; calcium; extracellular regulated kinase 1/2; mitogen-activating protein kinase; calpastatin; antiproteolytic therapy; microvascular endothelial cells; matrix metalloproteinase-9

A GROWING BODY OF LITERATURE indicates that elevated levels of homocysteine [hyperhomocysteinemia (HHcy)] are an independent risk factor for coronary, cerebrovascular, and peripheral atherosclerotic diseases (5, 11, 12). Matrix metalloproteinases (MMPs) are the members of Zn-containing endopeptidases that share structural domains but differ in the substrate specificity, cellular sources, and inducibility that are responsible for matrix turnover. It is well known that Hcy-induced vascular dysfunction is caused by extracellular matrix remodeling. Hcy induces extracellular matrix remodeling by MMP-9 activation, in part, by inducing the redox signaling and modulating the intracellular calcium homeostasis (13, 14, 19, 20).

Calpains are the family of calcium-dependent cysteine proteases that have previously been implicated in vasculopathies such as myocardial reperfusion injury (15), circulatory shock (18), and cerebral/ischemia injury (25). Several cellular events cause calpain activation, such as increase in intracellular calcium, autocatalysis of procalpains, and decreased levels of its endogenous inhibitor calpastatin. It is known that membrane translocation activates calpain.

Popp et al. (23) suggested that the calpain/calpastatin system regulates MMP-2 and -9 expression levels in leukemic cells. Since Hcy is also known to modulate intracellular calcium levels (17, 21), we speculate the role of calpains in MMP-9 regulation. To date, the mechanism of MMP-9 regulation by calpain is largely unknown.

Augilar et al. (1) showed calpain-like activity in the isolated rat liver mitochondria and that calpain inhibition blocked the calcium-induced mitochondrial damage. Mitochondrial abnormalities have been identified in HHcy (3, 33). It is not determined whether Hcy-induced mitochondrial damage parallels the calpain activation. Attention has been focused on the mitochondrial abnormalities leading to vascular complications by modulating the intramitochondrial redox state. Previous studies from our laboratory showed that Hcy induces oxidative stress and activates MMP-9 in an ERK-1/2-dependent manner (21).

The present study was undertaken to determine the role of calpains in mitochondrial regulation of MMP-9 in cultured rat heart microvascular endothelial cells (MVEC) during HHcy. Herein we demonstrate that Hcy activates calpain-1 and translocates it to mitochondria that cause modulation in the mitochondrial redox state, leading to MMP-9 activation. We presented evidence that calpain and ERK-1/2 (MAP kinase) are the key events within a single pathway that regulates Hcy-induced MMP-9 activation and, moreover, that the ERK-1/2 pathway lies downstream of calpain.

MATERIALS AND METHODS

Materials

Calpain inhibitors (calpeptin and calpain-1 inhibitor), PD-98059, α,α-homocysteine, BAPTA-AM, FCCP, 2',7'-Dichlorofluorescein diacetate (DCFH-DA), gelatin, and all other buffer reagents were

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obtained from Sigma. Antibodies–polyclonal antibodies against rabbit polyclonal antibodies for calpastatin, NADPH oxidase-4 (NOX-4), thioredoxin (TRX)—were from Santa Cruz Biotechnology. Anti-GAPDH monoclonal antibody is from Biodign. Phospho-ERK-1/2 and total ERK-1/2 polyclonal antibodies were from Cell Signaling Technology. Primary antibodies for calpain-1 and calpain-2 domain IV were obtained from Abcam. Antibodies for calpain-1 and calpain-2 NH2-terminal domain were from Triple Point Biologics, TIM44 was obtained from BD Biosciences. The synthetic calpain substrate AMC was from Calbiochem, and t-BOC-Leu-Met and MitoTracker Red were from Molecular Probes (Eugene, OR).

Cell Culture

Hearts were removed from 8-wk-old Wistar rats and processed for isolation of heart microvascular endothelial cells followed by their characterization using CD31 labeling, as mentioned elsewhere (31), with slight modification. The procedure involving the animals was approved by the Institutional Animal Care and Use Committee. The cells were grown on fibronectin-coated 75-cm² flasks in MCDB-31 medium with 20% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), basic endothelial growth factor (3 ng/ml), and heparin (5 U/ml) at 37°C under 5% CO2-95% air. Cells between passages 8 and 10 were grown to near confluence and serum starved overnight before treatment.

MMP-9 Activity by In-Gel Zymography

MVEC were cultured in six-well plates and treated with or without calpain inhibitors, i.e., calpeptin (5 μM) and calpain-1 inhibitor (1 μM) for 30 min followed by Hcy (120 μM) treatment for 1 h. MMP-9 activity in the different treatment groups was measured by in-gel gelatin zymography (13). Briefly, after the treatment, the supernatant from the cultured cells was collected and concentrated by using minicon filters (Millipore) with 15-kDa cutoff. The conditioned media were assayed for protein concentration using Bradford assay, and 20 μg of the protein were electrophoresed under nonreducing conditions on 7.5% SDS-polyacrylamide gel containing 2 mg/ml gelatin. The gels were rinsed in renaturation buffer containing 2.5% Triton X-100, followed by overnight incubation in activation buffer [in mM: 50 Tris·HCl (pH 7.4), and 5 CaCl2] at 37°C. The gels were stained in Coomassie blue R-250 and then destained appropriately with 10% acetic acid. The clear digested regions representing MMP-9 activity, as assessed by running prestained molecular weight markers, was quantitated densitometrically using Un-Scanit software (Scientific Software Services).

Reverse Transcription-Polymerase Chain Reaction

MVEC were cultured in six-well plates and treated with or without different doses of Hcy (30, 60, or 120 μM) for 1 h. Total RNA was isolated from cells using an RNeasy kit. The concentration of total RNA was quantified by measuring the absorbance at 260 nm. Samples with a peak area ratio of 28S-to-18S rRNA >2.0 were used. Four micrograms of RNA were reverse transcribed to cDNA following the manufacturer’s instruction using the SuperScript III First-Strand Synthesis for RT-PCR kit (Invitrogen Life Technologies) containing an oligo(dT) primer in a final reaction volume of 20 μl. The amplification of calpain-1, -2, and calpastatin was carried out exactly as mentioned in the literature (18). MMP-9 and GAPDH were amplified as mentioned in the literature (19). Aliquots of each sample (15 μl) PCR product together with negative controls were subjected to electrophoresis on 0.8% agarose gel and stained with ethidium bromide. The density of bands for the RNA samples on the digitalized images was measured, and the differences in RNA loading were corrected by dividing the density of the individual mRNA band by that of the GAPDH mRNA band.

Calpain Activity Assays

To determine the calpain activity in the whole cells, a synthetic calpain substrate, succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin (AMC) was used. This assay was adapted from literature reports (20) with slight modification. Briefly, cells were grown in six-well plates and incubated in the presence or absence of BAPTA-AM and calpain-1 inhibitor (c1 inhibitor) for 30 min, followed by Hcy treatment for 1 h. The cells were trypsinized, harvested, and resuspended in HEPES buffer containing a cuvette warmed to 37°C. The cells were stained with 40 μM AMC substrate, and the fluorescence of hydrolyzed AMC was measured for 5 min in spectramax M2 (Molecular Devices) with excitation at 360 nm and emission at 460 nm. The end point data were taken as a measure of calpain activity and plotted as a %fold increase over the basal proteolytic activity.

Western Blot Analysis

Calpain activity and calpain and calpastatin levels. Cells were pretreated with calpain inhibitors (calpeptin and calpain-1 inhibitor) for 30 min followed by Hcy treatment for 1 h and lysed with lysis buffer [in mM: 50 Tris·HCl (pH 7.4), 150 NaCl, 1% Triton X-100, and protease inhibitor cocktail]. The cell lysates were assayed for protein concentration using Bradford assay (21), and 20 μg of the protein were fractionated by SDS-polyacrylamide gel electrophoresis and immunoblotted with the primary antibody against NH2-terminal domain and domain IV of the large subunit of calpain-1 and -2 followed by the immunodetection with ECL plus detection kit (Amersham Biotech) using horseradish peroxidase-conjugated secondary antibody. Activated calpains autolytically cleaves their NH2-terminal ends, resulting in loss of NH2-terminal antibody recognition, whereas the antibody against domain IV recognizes both the autolysed and unautolysed form of calpains. Calpain-1, -2, and calpastatin levels were assessed by immunoblotting with specific antibodies.

ERK-1/2 activation. Cells were pretreated with or without calpain inhibitors (calpeptin and calpain-1 inhibitor) and FCCP (0.2 mM), followed by Hcy treatment for 1 h. Cell extract was prepared as mentioned above. Thirty to forty micrograms of the protein were fractionated by SDS-polyacrylamide gel electrophoresis and immunoblotted with the primary antibody against phospho-ERK-1/2 and total ERK-1/2 and immunodetected with ECL plus kit by using horseradish peroxidase-conjugated secondary antibody.

Subcellular Fractionation

Mitochondria and cytosolic fractions were prepared from heart MVEC by using differential centrifugation protocol adapted from the literature reports (22) with slight modifications. Briefly, cells were harvested from T-75 cm² flask and homogenized in 0.2 ml of ice-cold sucrose buffer [in mM: 250 sucrose, 10 Tris·Cl (pH 7.4), and 1 magnesium acetate] with protease inhibitor cocktail with a 2-ml dounce homogenizer. Homogenate was centrifuged at 700 g for 10 min at 4°C; the pellet represented the nuclear fraction was discarded. The postnuclear fraction was centrifuged at 10,000 g for 15 min at 4°C. The supernatant represents the cytosolic fraction. The mitochondrial pellet was washed twice with isolation buffer and centrifuged again at 10,000 g for 15 min at 4°C. The protein content was determined, and 50 μg of the protein were electrophoresed and analyzed for mitochondrial translocation of calpain by Western blot with primary antibody against NH2-terminal domain and domain IV of the large subunit of calpain-1, followed by immunodetection with ECL plus detection kit using horseradish peroxidase-conjugated secondary antibody. The fractions were analyzed via Western blot for prohibitin (mitochondrial marker) and GAPDH (cytosolic marker). To obtain the mitochondrial membrane fraction, isolated mitochondria were treated with digitoxin (75 μg/mg protein) and incubated on ice for 15 min. After incubation, the mitochondrial suspension was diluted in 8 vol of sucrose buffer and centrifuged at 10,000 g for 15
Reactive Oxygen Species Measurements

Intramitochondrial reactive oxygen species production. DCFH-DA was used to access intramitochondrial reactive oxygen species (ROS) because DCFH-DA is not positively charged and its accumulation is independent of mitochondrial membrane potential. DCFH-DA is membrane permeable and can be loaded and deacetylated inside the mitochondria by esterase to form highly fluorescent dichlorofluorescein (DCF). The method is adapted from the literature (23) with slight modification. Briefly, isolated mitochondria from different treatment groups were incubated with 10 μM DCFH-DA for 45 min at room temperature, and the time-based fluorescence scan for DCF was performed in the presence or absence of calpain-1 inhibitor at the indicated times with the use of a spectrophotometer (Fluorolog-2) set at excitation of 488 nm and emission of 525 nm. The CaCl₂ was added at the end of each experiment to determine the mitochondrial integrity.

Intracellular ROS measurements. Intracellular ROS production was determined by confocal imaging microscopy. A 10 mM stock solution of DCFH-DA was prepared in ethanol. The procedure was adapted from the literature (16). Briefly, MVEC were cultured onto the coverslips and were serum starved before treatment. The cells were treated with or without calpain-1 inhibitor (1 μM) and FCCP (0.2 mM), a mitochondrial uncoupler, for 30 min, followed by Hcy treatment for 1 h. The cells were washed with PBS and then loaded with 10 μM DCFH-DA for 45 min at room temperature in the dark. The confocal fluorescence images were obtained by laser confocal microscope (FluoView 1000).

To enable the comparison of changes in fluorescence intensity and punctuate staining pattern, the images were taken under an identical set of conditions for all treatment groups.

Statistics

Group data are presented as means ± SE. Statistical analysis was performed using unpaired Student’s t-test between control, Hcy, and other pharmacological treatment groups. Statistical significance was accepted at P < 0.05.

RESULTS

Calpain is required for Hcy-induced MMP-9 expression and activity. Our central hypothesis is the involvement of calpains in the induction of MMP-9 activation by Hcy. Therefore, we determined whether inhibition of calpain would affect the Hcy-induced activation of MMP-9. In our experimental conditions, calpeptin (general calpain inhibitor) and calpain-1 inhibitor significantly reduced the Hcy-induced MMP-9 mRNA expression levels and activity (Fig. 1). Calpain-2 inhibitor showed no significant decrease in Hcy-induced MMP-9 mRNA expression levels and activity (data not shown). The GAPDH signal used as an input did not show any change in protein expression (fold change over control). Fold change of density level. Values of MMP-9 activity and MMP-9 mRNA expression (fold change over control). Fold change of activity and expression corresponds to fold change of density level. Values are averages ± SE from 4 different experiments. *P < 0.05 compared with Hcy treatment; # P < 0.05 compared with control.
Cultured MVEC. Activated calpains autolytically cleave their NH2-terminal ends, resulting in loss of NH2-terminal antibody recognition. However, the antibody against domain IV recognizes both the autolyzed and unautolyzed form of calpains. We observed increased proteolytic activity of calpain-1 with Hcy treatment as evidenced by loss in NH2-terminal antibody binding. Treatment with calpeptin significantly decreased the Hcy-induced calpain-1 activation (Fig. 2). Total calpain-1 content was found to be increased with Hcy treatment (Fig. 3). No significant difference was observed in total calpain-1 content of control and calpeptin-treated cells. The proteolytic activity of calpain-2 does not increase (Fig. 2) with Hcy treatment as demonstrated by lack of changes in NH2 calpain-2 expression. There were no significant changes in calpain-2 domain IV expression levels (Fig. 2). This demonstrates that Hcy treatment specifically induces the activation of calpain-1 but not calpain-2. To determine whether the calpain activity is calcium dependent, MVEC were cultured in the presence or absence of intracellular calcium chelator (BAPTA-AM). It was observed that BAPTA-AM significantly blocked Hcy-induced calpain activity, which suggested that Hcy-induced calpain-1 activity is dependent on increased intracellular calcium (Fig. 4). The basal levels of calpain activity in the control MVEC were not affected significantly when treated with BAPTA-AM and calpain-1 inhibitor alone (data not shown).

Hcy-induced calpain activity is not mirrored by decrease in calpastatin levels. The calpain system consists of calpain isoforms and their endogenous inhibitor calpastatin. To determine the role of calpain system in Hcy-induced calpain activation, the mRNA and protein levels of calpain-1, -2, and calpastatin were checked. It was observed that Hcy treatment does not affect the calpain-1 and -2 mRNA (Fig. 3) and protein expression levels (data not shown). Interestingly, we observed no significant decrease in the calpastatin mRNA and protein levels with Hcy treatment in cultured MVEC (Fig. 3). These findings suggest that the calpain activity was not mirrored by decreased levels of the endogenous calpain inhibitor calpastatin.

Hcy-induced calpain activity lies upstream of ERK-1/2 signal pathway. Recent studies from our laboratory and others have implicated the involvement of ERK-1/2 (MAP kinase) and calpains in MMP regulation. To determine whether these are the two distinct pathways that regulate Hcy-induced MMP-9, various pharmacological inhibitors for calpain (calpeptin and calpain-1 inhibitor) and ERK-1/2 (PD-98059) were used. It was observed that the ERK-1/2 blocker (PD-98059) does not block the calpain activity (Fig. 5A). However, the
Calpain blockers significantly decreased the Hcy-induced ERK activation (Fig. 5B). There was no significant change in the basal levels of calpain activity and ERK phosphorylation when the control MVEC were treated with either ERK inhibitor (PD-98059) or calpain inhibitors alone (data not shown). Taken together, these data demonstrate that the Hcy-induced calpain activation lies upstream of ERK-1/2 signal pathway.

Hcy induces calpain-1 translocation from cytosol to mitochondria. Mitochondrial abnormalities have been identified in HHCy. Recent reports suggest calpain-like activity in isolated rat liver mitochondria. However, it is largely unknown whether the Hcy-mediated mitochondrial damage parallels the calpain activation. The serum-starved cells were treated for 1 h with Hcy. The mitochondria were isolated by differential centrifugation. Calpain-1 levels and activity were determined in the isolated mitochondrial fraction by immunoblotting with calpain-1 domain IV and NH2-terminal primary antibodies, respectively. In our experimental conditions, we observed that, before Hcy treatment, mitochondrial fraction showed significantly less recognition with calpain-1 domain IV antibody, indicating significantly low total calpain-1 levels compared with the cytosolic compartment. After Hcy treatment, mitochondrial compartment expresses calpain-1 domain IV and there was increased proteolytic activity of calpain-1, as evidenced by loss in NH2-terminal antibody recognition (Fig. 6A).

These observations suggest that Hcy induces the translocation of active calpain-1 from cytosol to mitochondria in the cultured MVEC. To support the mitochondrial translocation of active calpain-1 by Hcy treatment, the confocal laser microscopy was performed using synthetic calpain substrate, t-BOC, and the mitochondrial marker MitoTracker Red. It was observed that, with the Hcy treatment, there was an increase in calpain activity that was found to be colocalized with mitochondria (Fig. 6B).

Hcy-induced mitochondrial translocation of calpain-1 induces intramitochondrial oxidative stress. It is known that Hcy induces oxidative stress and activates MMP-9. The attention...
has been focused on mitochondria as a primary source of ROS in pathophysiological events. Our hypothesis is that Hcy-mediated mitochondrial translocation of calpain-1 induces intramitochondrial oxidative stress. Therefore, we determined the mitochondrial ROS production by spectramax M2 (Molecular Devices) using membrane-permeant DCFH-DA, which deesterifies to DCFH and oxidizes in the presence of ROS to fluorescent DCF. Since DMSO is a well-known free radical

Fig. 6. Hcy induces calpain-1 translocation from cytosol to mitochondria. A: representative Western blots from 3 different experiments are shown. MVEC cultured in T-75 cm² flask were treated with or without Hcy (120 μM) for 1 h. Mitochondria were isolated by differential centrifugation, as mentioned in MATERIALS AND METHODS. Calpain-1 translocation from cytosol to mitochondria was determined by immunoblot analysis using antibody that recognizes the NH₂ terminus of calpain-1 in the cytosolic and mitochondrial fraction. Increased calpain activation is depicted by loss of antibody recognition. Primary antibody against calpain-1 domain IV recognizes both active and inactive forms of calpains and was used to quantify total calpain content. Mitochondrial and cytosolic purity was assessed by probing the fractions with prohibitin, a mitochondrial marker, and GAPDH, a cytosolic marker, respectively. B: fluorescence staining of MVEC cells with the calpain substrate t-BOC (green fluorescence, represented by arrow) and the mitochondrial marker MitoTracker Red (red fluorescence, represented by arrowhead). Colocalization of calpain activity and mitochondria is seen as yellow. Confocal microscopy of MVEC with (Bi) or without (Bi) Hcy (120 μM) treatment for 1 h is shown. A single cell is enlarged from group of cells and shown for better resolution. Data are representative of least 6–7 experiments in which >100 cells were examined.
scavenger, the stock solution for calpain-1 inhibitor was made in methanol. We observed a significant increase in DCF fluorescence with Hcy-treated mitochondria. Moreover, Hcy-induced DCF fluorescence was ablated by the addition of calpain-1 inhibitor to the mitochondrial suspension (Fig. 7A). The data were analyzed and the variation in DCF fluorescence between the experiments was calculated in terms of fold change in DCF fluorescence intensity, after applying offset correction on the y-axis. It was observed that there was a significant increase (2- to 3-fold) in DCF fluorescence intensity with Hcy-treated mitochondrial suspension. The blocking of DCF fluorescence by calpain-1 inhibitor affirms that the response was attributable to ROS generation. We also determined the ROS production in the cultured MVEC using DCHF-DA stain by confocal microscope. FCCP, which is a potent uncoupler of mitochondrial oxidative phosphorylation, was used to determine whether the Hcy-induced oxidative burst is of mitochondrial origin. In our experimental condition, it was observed that pretreatment of the cultured MVEC with calpain-1 inhibitor and mitochondrial uncoupler (FCCP) blocked the Hcy-induced ROS generation, as evidenced by the decrease in the DCF fluorescence and punctuate staining pattern (Fig. 7B). The mitochondrial oxidative burst is caused by imbalance between the enzymes that catalyze redox reaction such as mitochondrial membrane respiratory enzymes (NOX) and its antioxidant pool, which consists of TRX. We detected NOX-4 and TRX-2 levels in the presence or absence of calpain-1 inhibitor in mitochondrial membrane by Western blot. The purity of the mitochondrial membrane was >98% as accessed by Western blot with TIM44 (inner membrane mitochondrial marker). It was observed that calpain-1 inhibition reduces significantly NOX-4 expression and increases TRX-2 expression levels (Fig. 7C). Taken together, our results suggest that the Hcy-induced ROS were of mitochondrial origin and involve calpain-1 activation in the cultured MVEC.

Hcy-induced intramitochondrial oxidative stress regulates MMP-9. We observed that Hcy treatment induces intramitochondrial oxidative stress (Fig. 7, A–C) in MVEC. To determine the role of intramitochondrial oxidative stress in Hcy-induced MMP-9 induction via ERK pathway, we treated

Fig. 7. Hcy-induced mitochondrial translocation of calpain-1 induces intramitochondrial oxidative stress. A: cells were cultured in T-75 cm2 flask, treated with or without Hcy (120 μM) for 1 h. Intramitochondrial reactive oxygen species (ROS) were detected by incubating the mitochondria with 10 μM DCFH-DA for 45 min at room temperature, and the time-based fluorescence of DCF was recorded by spectrophotometer (Fluorolog-2) in the presence or absence of calpain-1 inhibitor (1 μM) at the indicated time. CaCl2 was added to test functional integrity. Representative time-based scan for DCF fluorescence from 6 different experiments is shown. B: cells were treated with or without Hcy (120 μM) for 1 h in the presence or absence of calpain-1 inhibitor (1 μM), FCCP, a protonophoric mitochondrial uncoupler (10 μM) for 30 min. ROS production was also evaluated by staining cells with DCFH-DA. Images were acquired by laser confocal microscope (FluoView 1000) at an excitation of 488 nm and emission of 525 nm. C: cells were cultured in T-75 cm2 flask, treated with or without Hcy (120 μM) for 1 h in the presence or absence of calpain-1 inhibitor (1 μM) for 30 min. Isolated mitochondrial membranes were probed for NADPH oxidase-4 (NOX-4) and thioredoxin 2 (TRX-2) using Western blot analysis. Membrane purity was checked by immunoblotting with TIM44 (translocase of inner membrane 44), a mitochondrial inner membrane marker. Ratio of TRX-2 to NOX was plotted as %change over control. Values represent an average ± SE from 4 different experiments. *P < 0.05 compared with Hcy treatment; #P < 0.05 compared with control.
MVEC with the mitochondrial uncoupler FCCP and checked for MMP-9 and ERK-1/2 activation. We observed that FCCP treatment ablated both Hcy-induced ERK-1/2 activation (Fig. 8, A and B) and MMP-9 activation (Fig. 8, C and D). This suggests the possible involvement of intramitochondrial oxidative stress in Hcy-induced MMP-9 regulation.

**DISCUSSION**

The present study demonstrates that calpain-1 is required for Hcy-induced MMP-9 activation. Hcy activates and translocates calpain-1 from cytosol to mitochondria and causes intramitochondrial oxidative stress, leading to MMP-9 activation. Moreover, we presented evidence that calpain and ERK-1/2 (MAP kinase) are the most likely events within the Hcy/MMP-9 signal axis and that ERK-1/2 lies downstream of calpain.

The calpain system consists of the calpain isoforms calpain-1 and calpain-2 and their endogenous inhibitor calpastatin. Calpains are the family of nonlysosomal, calcium-dependent cysteine proteases and cleave a number of cellular substrates, e.g., cytoskeletal proteins and kinases (8). Previous studies from our laboratory have shown that Hcy increases the intracellular calcium levels in MVEC in a dose-dependent manner (21). The increase in cytosolic calcium is known to activate calcium-dependent cysteine proteases, calpains that have been implicated in the pathophysiology of cardiovascular events. In the present study, we studied the well-characterized and ubiquitously expressed calpain-1 and -2. We observed that Hcy specifically increases calpain-1 activity and is a calcium-dependent phenomenon in cultured MVEC. The difference in calpain isoform activation may be attributed to the selective calpain regulation under different pathophysiological events. Our study supports the proposed dissociation/autolysis mechanism of calpain activation by Suzuki and Sorimachi (29), wherein they suggested that calcium binding to calpain induces dissociation of calpain subunits (autolysis) and resulted in calpain activation. Our observations are also consistent with the findings of Stalker et al. (28), who observed the involvement of calpain-1 activation in endothelial dysfunction during Type 2 diabetes. These findings support the importance of calpain-1 in instigating signal transduction under physiological and pathological conditions (10).

The calpain activity is also regulated by the levels of the endogenous calpain inhibitor calpastatin (8). The increase in calpastatin levels reflected the loss of calpain activity during ischemia-reperfusion injury and neonatal cerebral damage (4, 27). Interestingly, in our study, we observed that calpain activity is not mirrored by the decrease in calpastatin levels. This is consistent with the observation made by Liu et al. (16), who observed that the calpain activity is not reflected by the change in calpastatin levels in renal proximal tubular (RPT) during oncosis.

There is scant literature on the role of calpain in MMP regulation. In this regard, Popp et al. (23) demonstrated that the specific calpain inhibitor CPIB reduces extracellular matrix invasion as well as MMP-2 and -9 mRNA expressions in leukemic THP-1 cells. However, the role of calpain in Hcy-induced MMP-9 activation is not known. Our results with pharmacological calpain inhibitors determined the involvement of calpain in Hcy-induced MMP-9 activation, which in accordance with the observations of Popp et al. (23). Recent reports suggested the involvement of calpain in ERK-1/2 (MAP kinase) signal pathway (9). Our previous findings demonstrated the involvement of ERK-1/2 pathway in Hcy-induced MMP-9 activation (21). It has not been deciphered to date whether these are the two distinct pathways that regulate Hcy-induced MMP-9. Our observations with different pharmacological blockers of calpain and ERK-1/2 demonstrated that calpain and ERK-1/2 (MAP kinase) are the most likely events within a single pathway and that ERK pathway lies downstream of calpain. These findings are in accordance with Veeranna et al. (36), who suggested that calpains are the upstream activators of ERK pathway during the pathophysiology of Alzheimer disease.

Recent reports suggested that the calpain-like activity in mitochondria is associated with mitochondrial dysfunction (1, 35). Wingrave et al. (37) suggested that increase in calcium activated the calpain and mediates mitochondrial damage during spinal cord injury in rats. Austin et al. (3) suggested that the mitochondrial abnormalities are associated with HHcy. They observed mitochondrial damage in Dami cells with Hcy treatment and suggested the importance of glutathione and heat shock proteins in mitochondrial protection (3). Very recently, our laboratory demonstrated that Hcy damages mitochondria by decreasing the mitochondrial membrane potential and induces endothelial apoptosis (34). It is very well established that Hcy activates MMP-9, in part, by inducing redox signaling (21). Attention has been focused on the mitochondria as a primary source of ROS in pathophysiology-related events. Whether Hcy treatment-induced mitochondrial translocation of calpain-1 parallels the mitochondrial damage by instigating the oxidative burst is not understood. Ariyoshi et al. (2) suggested that, when the human platelets were treated with thrombin, there was ~40% translocation of calpain-1 activity to the intracellular membrane fraction, and, moreover, membrane translocation of calpain-1 was completely blocked by treatment with EDTA. In the present study, our observations with mitochondrial and cytosolic compartment suggest the translocation of calpain-1 to the mitochondrial compartment with autolytic activation when treated with Hcy. This is the first study, to our knowledge, that suggests the mitochondrial translocation of calpain-1 with Hcy treatment in the cultured MVEC.
Our study corralated the mitochondrial translocation of calpain-1 with the induction of intramitochondrial oxidative burst with Hcy. The mitochondrial oxidative burst is caused by imbalance between the enzymes that catalyze redox reaction such as mitochondrial membrane respiratory enzymes (NOX) and its antioxidant counterpart, which consists of TRX (32). In this study, we employed different approaches to determine the redox status of mitochondrial in response to Hcy and confirmed that Hcy-induced ROS production was of mitochondrial origin and is calpain mediated. Furthermore, we presented evidence that intramitochondrial oxidative stress regulates Hcy-induced MMP-9 via ERK signal pathway. Taken together, our observations determine the novel mitochondrial mechanism for Hcy-induced MMP-9 activation and support our speculation (Fig. 9) that Hcy induces activation and mitochondrial translocation of calpain-1, leading to MMP-9 activation, in part, by increasing mitochondrial oxidative stress.

Clinical Significance

HHCy is a multifactorial condition and involves abnormal expression of MMP-9. This study specifically highlights the novel contribution of mitochondrial translocation of calpain-1 in Hcy-induced MMP-9 activation and may open the possibility for anti-proteolytic (anti-calpain)-based therapies for the experimental treatment of HHCy-associated pathologies such as atherosclerosis, stroke, and dementia by decreasing MMP-9 expression and function. Homocysteine and H2O2 act synergistically to enhance mitochondrial damage. J Biol Chem 273: 30808–30817, 1998.  

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