Mechanism of constrictive vascular remodeling by homocysteine: role of PPAR

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Mujumdar, Vibhas S., Chandra M. Tummalapalli, Giorgio M. Aru, and Suresh C. Tyagi. Mechanism of constrictive vascular remodeling by homocysteine: role of PPAR. Am J Physiol Cell Physiol 282: C1009–C1015, 2002.—To test the hypothesis that homocysteine induces constrictive vascular remodeling by inactivating peroxisome proliferator-activated receptor (PPAR), aortic endothelial cells (ECs) and smooth muscle cells (SMCs) were isolated. Collagen gels were prepared, and ECs or SMCs (10^6) or SMCs + ECs (10^4) were incorporated into the gels. To characterize PPAR, agonists of PPAR-α [ciprofibrate (CF)] and PPAR-γ [15-deoxy-12,14-prostaglandin J_2 (PGJ_2)] were used. To determine the role of disintegrin metalloproteinase (DMP), cardiac inhibitor of metalloproteinase (CIMP) was used in collagen gels. Gel diameter at 0 h was 14.1 ± 0.2 mm and was unchanged up to 24 h as measured by a digital micrometer. SMCs reduce gel diameter to 10.5 ± 0.4 mm at 24 h. Addition of homocysteine to SMCs reduces further the gel diameter to 8.0 ± 0.2 mm, suggesting that SMCs induce contraction and that the contraction is further enhanced by homocysteine. Addition of ECs and SMCs reduces gel diameter to 12.0 ± 0.3 mm, suggesting that ECs play a role in collagen contraction. Only PGJ_2, not CF, inhibits SMC contraction. However, both PGJ_2 and CF inhibit contraction of ECs and SMCs + ECs. Addition of anti-DMP blocks SMC-as well as homocysteine-mediated contraction. However, CIMP inhibits only homocysteine-mediated contraction. The results suggest that homocysteine may enhance vascular remodeling by inactivating PPAR-α and -γ in ECs and PPAR-γ in SMCs.

aorta; arteriosclerosis; hypertension; peroxisome proliferator-activated receptor; fibrate; prostaglandin; endothelial cell; smooth muscle cell

ARTERIAL WALL REMODELING is one of the most important factors regulating lumen diameter after acute and/or chronic vascular injury (7, 28, 29, 44). Smooth muscle cells (SMCs) remodel the existing and new extracellular matrix (ECM). In response to ECM degradation, SMCs alter phenotype (43). The consequences of remodeling may lead to alterations in arterial wall geometry and lumen diameter (7, 28, 29, 44). Although the extracellular environment strongly influences cell behavior, it is unclear whether the changes in matrix composition affect connective tissue shrinkage. Hyperhomocysteinemia is associated with hypertension (39) and increases vascular intimal-medial thickness (26, 34). Homocysteine causes arteriosclerosis (19, 36, 40), endothelial cell desquamation (38), thromboresistance (22), SMC proliferation (41, 45), collagen synthesis (23, 45), oxidation of low-density lipoprotein (12), increased monocyte adhesion to the vessel wall (20), platelet aggregation (6), coagulation (34), blood rheology (8, 25), and activation of plasminogen and metalloproteinase (18, 47), the two neutral proteinases associated with remodeling. Previous studies from our laboratory have identified a redox-sensitive homocysteine receptor in SMC. This receptor regulates collagen expression (45). Primarily, there are two nuclear transcription factor (NF) receptors that control the redox state of the cell. NF-κB is induced by homocysteine (3, 49). Peroxisome proliferator-activated receptor (PPAR) is a proantioxidant. In addition, a negative correlation between high levels of homocysteine and PPAR expression has been demonstrated (4, 14). PPAR, upon induction, promotes the synthesis of superoxide dismutase (SOD) and catalase (16, 33). Meanwhile, PPAR decreases NADH/NADPH oxidase (15, 16). The high levels of homocysteine are associated with increased oxyradical generation (1) and oxidative injury (30, 52). The agonists of PPAR decrease the oxidative stress and metalloproteinase activity in macrophages (24, 35), decrease the mRNA of plasminogen activator and increase the mRNA of plasminogen activator inhibitor (50), and decrease the intimal-medial thickness (27). It is unclear, however, whether the induction of PPAR regresses intimal-medial thickness by decreasing homocysteine-mediated metalloproteinase activation. The hypothesis is that homocysteine induces constrictive collagen remodeling by antagonizing PPAR and increasing disintegrin metalloproteinase (DMP) activity.

MATERIALS AND METHODS

Cell cultures. A segment of human aorta was obtained at the time of cardiac transplant from patients with idiopathic

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DMEM, 10% FCS, 100 IU/ml penicillin/streptomycin at 37°C plated onto 6-cm culture dishes. The cells were cultured in The cells were washed two times with DMEM before they were cultured in The cells were passaged when nearly confluent. aortic medial SMCs or SMCs plus 10^4 endothelial cells. Serum-deprived cells were incorporated within collagen gels containing 10^5 SMCs alone or SMCs plus 40 μM homocysteine [H(e)] and allowed to float free in DMEM containing 0.2% FCS for 0, 6, and 18 h.

dilated cardiomyopathy who had apparently normal vessels. An Institutional Review Board waiver was obtained before the tissue was collected. The aorta was opened longitudinally, placed in Dubecco's minimal essential medium (DMEM), and used within 30 min. Endothelial cells (ECs) were removed by gently scraping the lumen with a cotton swab. The swab was immersed in DMEM containing 10% fetal calf serum (FCS). The SMCs were isolated from medial tissue after the adventitia had been carefully separated and the tissue had been minced with collagenase (20 μg/mg of tissue) in DMEM at 37°C for 2 h. The cells were washed two times with DMEM before they were plated onto 6-cm culture dishes. The cells were cultured in DMEM, 10% FCS, 100 IU/ml penicillin/streptomycin at 37°C and 5% CO2. The cells were passaged when nearly confluent and studied between passages 3 and 6. The ECs were characterized by their cobblestone appearance and positive staining for von Willebrand factor VIII (46). The SMCs were identified by their spindle shape and positive staining for smooth muscle α-actin (46).

Collagen gel contraction assay. Collagen gels were prepared as described (45). Briefly, 24-well plates were precoated with 1% agarose to promote gel detachment (48). Type I collagen (Southern Biochemical) was diluted with 4 × DMEM and cell suspensions so that the final mixture resulted in 1.25 mg/ml collagen. Gelation occurred within 10 min at 37°C. The confluent cells, serum-deprived for 24 h, were suspended in collagen gel suspension. DMEM containing 0.2% FCS was added to each well. The gels were lifted off the bottom of the wells and allowed to float freely. The cells treated with homocysteine were added to the collagen suspension. Gel diameters were measured by micrometers. Our experience with these cells suggests that ECs and SMCs tend to aggregate in collagen gel (42). Therefore, asymmetric contraction was observed. However, to minimize contribution due to asymmetric contraction, we measured diameter in two perpendicular directions and recorded the average of the two. Also, to enhance the reliability/reproducibility of the experiments, all the measurements of gel diameters were recorded in a blind fashion by a technician who was unaware of the experimental protocol. For the treatment with PPAR agonist, homocysteine and PPAR agonists were added to the cell suspension at the same time. Because homocysteine interacts with thiols in the proteins (17), it is possible that homocysteine modifies the collagen and induces conformational changes that affect the physical properties of collagen. We measured collagen gel diameters at 24 h in both the presence and absence of homocysteine (40 μM) and observed no difference in the diameters. All assays were repeated three times with triplicate wells per experimental condition.

Effects of EC, homocysteine, and PPAR agonist. The collagen gels containing 10^5 ECs or SMCs with or without homocysteine (40 μM) were prepared. The gel diameters were measured at 24 h. To determine the optimal dose of homocysteine, different concentrations of homocysteine were added. The effect of ECs on SMC-mediated collagen gel contraction was measured by adding 10^4 ECs to 10^5 SMCs. The time dependence of contraction was measured. The role of PPAR agonist was determined by adding ciproflurbate (CF; Sigma Chemical) as PPAR-α agonist and 15-deoxy-12,14-prostaglandin J2 (PGJ2; CalBiochem) as PPAR-γ agonist to the gel.

Analysis of DMP in SMCs. To determine whether the contraction of SMCs induced by homocysteine is mediated by DMP, the expression of DMP in SMCs treated with or without homocysteine was measured. Confluent cells were deprived of serum for 24 h and were treated with DMEM containing 0.2%...
RESULTS

Homocysteine-mediated collagen gel contraction. Human aortic ECs and SMCs maintain their cobblestone and spindle-like phenotype, respectively, for up to four and five passages. SMCs induce contraction in collagen gel (i.e., decrease in gel diameter). Collagen gel diameters at 0 and 24 h were 14.1 ± 0.2 and 14.0 ± 0.2 mm, respectively. Addition of homocysteine to SMCs further increased collagen gel contraction (Fig. 1). The dose-response curve generated for homocysteine-mediated collagen gel contraction suggests that a pathophysiological concentration of homocysteine (20–60 μM) induces significant contraction in collagen gel (Fig. 2A).

Addition of ECs ameliorated SMC-mediated collagen gel contraction. The addition of ECs to SMCs decreased collagen gel contraction (i.e., increase in gel diameter) compared with SMCs alone. Homocysteine induced collagen gel contraction in SMCs in the presence of ECs (Fig. 2B). However, the contraction was smaller in SMCs plus ECs than in SMCs alone (Fig. 2B). These results suggest that factors released from ECs may inhibit the collagen gel contraction by SMCs.

Role of PPAR. Addition of CF, an agonist of PPAR-α, to the homocysteine plus SMC in collagen gel did not affect the contraction. However, the addition of PGJ2, an agonist of PPAR-γ, decreased the homocysteine- and SMC collagen gel contraction (Fig. 3A). The results suggest that PPAR-γ regulates the SMC collagen gel contraction and that PPAR-α has no effect on contraction by SMCs (Fig. 3A). The homocysteine also...
induced collagen contraction by ECs (Fig. 3B), but to a lesser degree than SMCs. The addition of CF or PGJ₂ to EC plus homocysteine in collagen gel ameliorated the homocysteine-mediated collagen gel contraction. These results suggest that PPAR-α and -γ are involved in EC collagen contraction. Both CF and PGJ₂ inhibited the collagen gel contraction induced by SMC plus ECs (Fig. 4), considering the fact that both PPAR-α and -γ are present on ECs and may modulate the SMC contraction.

Expression of DMP in SMCs. To determine whether homocysteine induces DMP in SMCs, the levels of DMP in cell homogenate were measured by Western blot analysis. The results suggest that homocysteine increases DMP twofold in SMCs. The cotreatment of homocysteine with 12 μM PGJ₂ inhibits the homocysteine-mediated DMP induction in SMCs (Fig. 5). These results suggest that homocysteine induces DMP in SMCs by activation of PPAR-γ.

Role of DMP and CIMP. To determine whether DMP plays a role in SMC-mediated collagen gel contraction, the SMCs were treated with anti-DMP antibody, or CIMP, in collagen gel. Treatment with anti-DMP inhibited both contraction by SMCs alone as well as contraction by homocysteine plus SMCs (Fig. 6A). Treatment with CIMP had no effect on SMC contraction, although it inhibited the homocysteine-mediated SMC collagen gel contraction (Fig. 6B). These results suggest that integrin may play an important role in contraction by SMCs in the presence or absence of homocysteine. However, metalloproteinase plays a significant role only in homocysteine-mediated SMC collagen gel contraction.

DISCUSSION

Toward the understanding of the mechanism by which homocysteine mediates vascular contractile remodeling, we have demonstrated that homocysteine increases collagen gel contraction by ECs as well as by SMCs. However, contraction by ECs is much less significant than that by SMCs. The addition of ECs to SMCs ameliorates the SMC-mediated contraction, but it is still higher than contraction by ECs alone. These
results suggest that ECs partially inhibit SMC collagen gel contraction and that they may be related to the endothelial nitric oxide (NO) generation. However, it is also possible that certain components of ECM remodeling, such as DMP, are necessary for collagen contraction. Contraction is facilitated by PPAR-α and γ in ECs and by PPAR-γ in SMCs. Homocysteine induces DMP in SMCs by activation of PPAR-γ. Inhibition of integrin and metalloproteinase blocks homocysteine-mediated collagen gel contraction by SMCs.

Homocysteine impairs EC-dependent vasodilation (5) and induces vasoconstriction (32). To our knowledge, this is the first time that homocysteine has been shown to instigate vascular remodeling by contracting collagen gels with SMCs (Figs. 1 and 2). Previous results from our laboratory demonstrated an increase in SMC number after 24-h homocysteine treatment (45). It is likely that, during collagen contraction, SMC number is also increased. Although SMCs and ECs (Fig. 3) are known to induce collagen gel contraction separately (48), it was unclear whether ECs played any role in SMC-mediated collagen gel contraction. Our results demonstrate that ECs inhibit SMC-mediated collagen gel contraction (Fig. 2B). In pathogenesis of chronic homocysteinemia, a concentration of homocysteine in the 9–13 μM range induces hypertension and leads to chronic arteriosclerosis (39). However, levels of homocysteine of >20 μM decrease the incidence of survival by 35% due to coronary artery disease (31). Our results suggest that homocysteine mediates collagen gel constriction and that they may be related to the endothelial response in SMC-mediated collagen gel contraction (Figs. 3 and 4). Although agonists of PPAR-α and γ induce apoptosis in transformed ECs (13) or ECs undergoing either tumorigenesis/angiogenesis (2) or hepatocarcinogenesis (10), it is unclear whether these agonists regulate normal EC and SMC phenotype. Collagen breaks are required for opening of new integrin binding sites for cell survival. However, a complete disconnect of cell and the ECM leads to apoptosis. It is quite possible that these agonists induce apoptosis in a cell of ECM disconnect. However, in normal cells, these agonists increase cell survival.

Integrins are the primary cellular receptors for collagen, and an antagonist of β1-integrin completely abolished contraction of collagen gel by SMCs (21). Our results suggest that homocysteine induces a DMP in SMCs (Fig. 5). Also, we have demonstrated that DMP contributes significantly to collagen gel contraction. The inhibition of DMP by anti-DMP antibody blocks the SMC-mediated collagen gel contraction (Fig. 6A). Remodeling implies synthesis and degradation of ECM and SMC hypertrophy (43). Metalloproteinases are increased in the vessel wall after injury (51). Homocysteine causes redox injury in the vessel wall and induce metalloproteinase activity (47). A broad-spectrum inhibitor of metalloproteinase, batimastat, has been shown to reduce the extent of wall shrinkage after angioplasty in pigs (7). A metalloproteinase inhibitor has been shown to decrease collagen gel contraction by fibroblasts (37). Our results suggest that the inhibitor of metalloproteinase, CIMP, does not block SMC contraction. However, CIMP blocks homocysteine-mediated collagen gel contraction (Fig. 6B), suggesting that homocysteine-mediated collagen gel constriction is modulated by metalloproteinase activity. Previously, we investigated the effect of high homocysteine concentration on biomechanical characteristics of the vasculature (i.e., passive distensibility and media-to-lumen ratio) (30). The effects of homocysteine described in vitro in the present study may result in vascular remodeling and narrowing of the arterial/arteriolar lumen in vivo.

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