Published in final edited form as:

Cell Signal. 2019 December ; 64: 109394. doi:10.1016/j.cellsig.2019.109394.

PAI-1 contributes to homocysteine-induced cellular senescence

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Abstract

Cellular Senescence is associated with organismal aging and related pathologies. Previously, we reported that plasminogen activator inhibitor-1 (PAI-1) is an essential mediator of senescence and a potential therapeutic target for preventing aging-related pathologies. In this study, we investigate the efficacies of PAI-1 inhibitors in both *in vitro* and *in vivo* models of homocysteine (Hcy)-induced cardiovascular aging. Elevated Hcy, a known risk factor of cardiovascular diseases, induces endothelial senescence as evidenced by increased senescence-associated β-Gal positivity (SA-β-Gal), flattened cellular morphology, and cylindrical appearance of cellular nuclei. Importantly, inhibition of PAI-1 by small molecule inhibitors reduces the number of SA-β-Gal positive cells, normalizes cellular morphology and nuclear shape. Furthermore, while Hcy induces the levels of senescence regulators PAI-1, p16, p53 and integrin β3, and suppresses catalase expression, treatment with PAI-1 inhibitors blocks the Hcy-induced stimulation of senescence cadres, and reverses the Hcy-induced suppression of catalase, indicating that PAI-1 specific small molecule inhibitors are efficient to prevent Hcy-induced cellular senescence. Our *in vivo* study shows that the levels of integrin β3, a recently identified potential regulator of cellular senescence, and its interaction with PAI-1 are significantly elevated in Hcy-treated heart tissues. In contrast, Hcy suppresses antioxidant gene regulator Nrf2 expression in hearts. However, co-treatment with PAI-1 inhibitor completely blocks the stimulation of Hcy-induced induction of integrin β3 and reverses Nrf2 expression. Collectively these *in vitro* and *in vivo* studies indicate that pharmacological inhibition of PAI-1 improves endothelial and cardiac health by suppressing the prosenescence effects of hyperhomocysteinemia through suppression of Hcy-induced master regulators of cellular senescence PAI-1 and integrin β3. Therefore, PAI-1 inhibitors are promising drugs for amelioration of hyperhomocysteinemia-induced vascular aging and aging-related disease.

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Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109394.

Declaration of Competing Interest

None.

Keywords

Cellular senescence; Homocysteine; Vascular endothelial cells; TM5441; TM5A15; PAI-1; Heart; Integrin β3; Catalase; Nrf2

1. Introduction

Senescence, one of the major cellular processes, is associated with both chronological and accelerated aging process and age-associated pathologies. Senescent cells become irreversibly growth arrested, but not metabolically inactive rather gene expression profile is significantly different from quiescent and proliferating cells. This special gene pool expressed in senescent cells are the markers and regulators of senescence and collectively called senescence-messaging secretome (SMS)/senescence-associated secretory phenotype (SASP). Therefore, prevention or reversal of senescence is an ideal approach to prevent or slow down organismal aging process and aging-related diseases [1,2]. Recently, enormous attentions have been paid to identify the crucial regulators of cellular senescence in a context-dependent manner. We have demonstrated that plasminogen activator inhibitor-1 (PAI-1) is a marker and master regulator of senescence and thus is a potential therapeutic target for prevention or reversal of aging-associated morbidity and mortality [3–6]. Based on existing data in literature, stress-induced ROS upregulates PAI-1 levels that inhibits fibrinolytic system and controls the activities of other downstream senescence regulators including IGFBP3, p16, p21 and p53 [2]. Thus pharmacological inhibition of PAI-1 may be an ideal approach to control stress-induced cellular senescence and accelerated aging process.

Interestingly, epigenetic modification of PAI-1 by methylation controls its circulating levels. Lu and colleagues recently elegantly demonstrated that DNA methylation levels of PAI-1 gene are strongly associated with human lifespan, comorbidity and type 2 diabetes [7]. A recent study demonstrated that integrin β3 also controls cellular senescence. This study further showed that proliferative cells have very low levels of integrin αvβ3 and the levels of integrin β3 are significantly elevated in senescent cells due to downregulation of CBX7, a polycomb protein that represses integrin β3. Importantly, integrin β3 is highly expressed in tissues from aged organisms [8]. Mechanistically, elevated integrin αvβ3 expression increases the release of TGFβ that augments cellular senescence in an autocrine and paracrine manner *via* p21 and p53 pathway [8].

Another newly identified senescence regulator in fibroblasts is nuclear factor erythroid 2-related factor 2 (Nrf2). Although Nrf2 is regarded as an antioxidant and downregulates the levels of reactive oxygen species (ROS), a recent study showed that sustained overexpression of Nrf2 induces senescence in fibroblasts [9]. Interestingly, this study using several biochemical and genetic approaches further demonstrated that fibroblasts with activated Nrf2 deposit a senescence-augmenting matrix, with PAI-1, a marker and master regulator of the senescence program [9]. The pivotal role of PAI-1 in multiple types of stress-induced-senescence associated with accelerated and chronological aging processes is well-documented [2]. An increased blood level of homocysteine (Hcy), a cysteine

homologue and an intermediate product of methionine metabolism, leads to hyperhomocysteinemia, a disease associated with vascular senescence and accelerated aging processes [10,11]. It is known that elevated level of homocysteine is an inducer of PAI-1 [12] and suppressor of tPA binding to its cellular binding site [13] that causes endothelial dysfunction. Elevated Hcy causes oxidative stress in cells by auto-oxidation of its sulphydryl group and increases the production of ROS. Here, we hypothesized that PAI-1 plays an important role in Hcy-induced cellular senescence; and pharmacological inhibition of PAI-1 is sufficient to block Hcy-induced cellular senescence. To test the hypothesis, we used both *in vitro* and *in vivo* approaches and assessed the efficacies of PAI-1 inhibitors TM5441 and TM5A15 in prevention of Hcy-induced cellular dysfunction and senescence.

2. Materials and methods

2.1. Endothelial cell culture: treatment with Hcy and small molecule inhibitors of PAI-1

Primary cultures of Human Coronary Artery Endothelial Cells (HCAEC) (Cell Applications; Cat # 300–05a) and EA.hy926 (ATCC cat #CRL-2922) were grown in MesoEndo Cell Growth Media and Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin and streptomycin respectively and maintained at 37 °C in a 5% CO_2 incubator. We tested the efficacy of PAI-1 inhibitor in suppression of Hcy-induced endothelial senescence using EA.hy926 endothelial cells and primary endothelial cell line HCAEC. The EA.hy926 cell line was established by fusing human umbilical vein endothelial cells with thioguanine-resistant clone of A549 (ATCC). This human hybrid somatic cell line possesses endothelial traits and are widely used for endothelial cell biology study ([5] and references therein). Both HCAEC and EA.hy926 cells are commonly used for *in vitro* studies on cardiovascular biology [5]. Confluent cultures of endothelial cells (3rd to 8th passages) were typsinized and subcultured in 12-well clusters. After 24 h, cells were pretreated either with TM5441 (10 μM) or TM5A15 (10 μM), structurally similar another inhibitor [received from our collaborator and coauthor Dr. Toshio Miyata, Japan) or equal amount of DMSO for 24 h followed by treatment with Hcy (750 μM) (DL-Homocysteine, Sigma Cat # H4628 and TCI, Cat# H0159) [12] and $CuSO_4$ (4 μM) [14] for 4–5 days. $CuSO_4$ is used in this study because it controls several biological effects of homocysteine [14–16]. The doses of PAI-1 inhibitors and Hcy were selected based on our and other investigator's published papers [5,12].

2.2. SA-β-Gal assay and nuclear morphology study in endothelial cells

At the end of treatment period, endothelial cells HCAEC and EA.hy926 were stained for SA-β-Gal, a widely used biomarker for cellular senescence, using Senescence Assay Kit for HCAEC (BioVision, Milpitas, CA) and for EA.hy926 (Abcam). Control and treated cells were washed with PBS and fixed with fixative solution for 15 min. Fixed cells were washed twice with PBS and stained with staining solution containing staining supplement and X-Gal overnight at 37 °C incubator. The SA-β-Gal positive blue cells were visualized under a microscope and photographed. To determine the nuclear shape and size in control and treated endothelial cells (HCAEC and EA.hy926), cells were pretreated with nonionic detergent Triton X-100 (0.2%) followed by staining with DAPI.

2.3. MTT Assay of endothelial cells treated with PAI-1 inhibitor and homocysteine

Primary cultures of HCAEC were subcultured in 12-well clusters. Cells were grown in MesoEndo Cell Growth Media and maintained at 37 °C in a 5% CO_2 incubator. After 24 h, cells were pretreated with different concentration of TM5441 (5, 10, 20, 30, 50 μM) or equal amount of DMSO in triplicate for 24 h followed by treatment with Hcy (750 μM) and $CuSO_4$ (4 μM) for 5 days. At the end of treatment, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed using MTT assay kit (Abcam). In brief, cell culture media was replaced with 300 μL of serum free media and 300 μL of MTT reagent and incubated at 37 °C for 3 h. Then 900 μL of MTT solvent was added to each well and incubated for 15 min on a shaker and immediately read in a plate reader at 590 nm. Cell viability in treatment groups were compared to control group.

2.4. Preparation of endothelial cell lysates and Western blot analysis

HCAEC and EA.hy926 cells were cultured in 12-well clusters. After 24 h, cells were pretreated with TM5441 or TM5A15 (10 μM) followed by treatment of Hcy (750 μM) in the presence of $CuSO_4$ (4 μM) in triplicate for 4–5 days. Cells treated with DMSO and $CuSO_4$ were used as controls. At the end of treatment period, cells harvested from three wells were pooled. Whole cell lysates were prepared using RIPA lysis buffer supplemented with protease inhibitor cocktail (cOmplete) and phosphatase inhibitor cocktail (PhosSTOP) (Sigma). Equal amount of proteins were subjected to gel electrophoresis, transfer to PVDF membrane and subjected to western blot using antibodies against p16, p21, p53, PAI-1 (Santa Cruz), PAI-1 (Molecular Innovations), catalase (Abcam), integrin β3, pERK1/2 (Cell signaling) and Actin (Abcam) and HRP-tagged corresponding secondary antibodies. The membranes were developed with ECL reagents (Luminata Forte, Millipore, Billerica MA) and images of protein bands were captured at BIORAD molecular imager ChemiDoc XRS system (BIORAD, CA).

2.5. Animal study: treatment of mice with Hcy and PAI-1 inhibitor TM5A15

8–10 weeks old male wildtype C57Bl/6 mice were used for this study. All mouse protocols were approved by the Northwestern University Animal Care and Use Committee. Mice were divided into 4 groups. Group #1: mice were fed with regular chow and drinking water (n = 5); Group #2: mice were fed with regular chow and received Hcy (2 g/L in drinking water) (n = 5); Group #3: mice were fed with chow containing TM5A15 (10 mg/kg/day) and regular drinking water (n = 5); Group #4: mice were fed with chow containing TM5A15 (10 mg/kg/day) and received Hcy (2 g/L in drinking water) (n = 5). The Hcy dose was selected based on previous published paper [17]. The dose of Hcy represents high dose in mouse study (1.8 g/L or 2 g/L). However, based on plasma levels of Hcy in mice treated with Hcy for short period reflects mild hyperhomocysteinemia ([17] and our data, Fig. S2). The dose of PAI-1 inhibitor was selected based on previously published work using structurally similar PAI-1 inhibitors [4,18]. At the end of 8-week treatment period, hearts were perfused and collected. Ventricles were snap frozen in liquid nitrogen and stored at −80 °C for further biochemical assays.

2.6. Heart and kidney tissue lysates preparations and western blot analysis

Frozen heart or kidney tissues were submerged in tissue lysis buffer supplemented with protease inhibitor cocktail (cOmplete) and phosphatase inhibitor cocktail (PhosSTOP) (Sigma). Tissue lysates were prepared by homogenization using Pestle Mixer (Argos Tech.) and centrifugation at 10,000 rpm for 15 min at 4 °C. The supernatant was used as source of protein. Equal amount of heart or kidney tissue lysates from each mouse were pooled ($n = 5$) and loaded on 4–12% gradient gel. Equal amount of heart lysate proteins from each mouse ($n = 4–5$/group) were also subjected to western blot analysis. The proteins were transferred to PVDF membranes and processed for immunoblot analysis using antibodies against integrin αV, integrin β3 (Abcam), CBX7 (Abcam), Nrf2 (Abcam), pERK1/2, p-p38 (Cell Signaling) and α-tubulin (GenScript). For coupled immunoprecipitation and immunoblot assay, 1 mg of total heart tissue lysates pooled from 5 mice (200 μg/mouse) were immunoprecipitated with PAI-1 antibody (Molecular Innovations) and Protein A-Agarose beads overnight at 4 °C. The beads-bound proteins were spinned down and washed with lysis buffer for three times and beads were resuspended in LDS loading buffer (Invitrogen), denatured at 95 °C and loaded on 4–12% gradient gel and subjected to western blot using antibodies against integrin αV, integrin β3, protein phosphatase 1 (PP1), and PAI-1.

2.7. Measurement of plasma levels of Hcy in control and Hcy-fed mice

Mouse plasma was obtained from retro-orbital blood samples that were collected in EDTA containing tubes and spun at 2000 ×*g* for 20 min. The metabolites were extracted from plasma samples ($n = 5$) using 80% methanol. Plasma samples mixed with 80% ethanol were incubated at −80 °C for 4 h and then centrifuged at 20,000 ×*g* for 15 min at 4 °C. The metabolite-containing supernatant was subjected to Liquid chromatography–mass spectrometry (LC–MS) at Northwestern Metabolomics Developing Core Facility. The relative abundance of Hcy is presented as ion intensity (peak area) of Hcy normalized by the Total Ion Current (TIC).

2.8. Statistical analysis

Data are presented as Mean ± SD or Mean ± SEM. The significance of differences between controls and experimental groups were determined by statistical analysis using student's *t*-test and a value of $P < .05$ by student's *t*-test was considered statistically significant. Statistical analyses were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

3. Results and discussion

Senescent cells directly contribute to organismal chronological as well as accelerated aging and age-related syndromes [19–21]. Thus prevention of cellular senescence or elimination of senescent cells is an ideal approach to extend healthy lifespan. Previously, we and others demonstrated that PAI-1 is a key regulator of cellular senescence and aging [2,22]. In this research communication, we have tested the efficacies of small molecule inhibitors of PAI-1, TM5441 and TM5A15, on prevention of homocysteine (Hcy)-induced vascular endothelial senescence. Elevated levels of Hcy in circulation of hyperhomocysteinemia patients develop cardiovascular diseases. In order to test the efficacy of small molecule inhibitors of PAI-1,

endothelial cells were pretreated with PAI-1 inhibitors TM5441 or TM5A15, or vehicle DMSO as control. After 24 h, cells were treated with Hcy for 4–5 days and then tested for the effect of PAI-1 inhibitors on Hcy-induced endothelial senescence. Light microscopy images reveal the changes in cellular morphology (flattened) in Hcy-treated endothelial cells, a trait of senescent cells (Fig. 1A,C). Importantly, PAI-1 inhibitor TM5441 significantly normalizes the Hcy-induced cellular morphological changes. SA-β-Gal assay results reveal that Hcy treatment leads to significantly increased number of SA-β-Gal positive cells compared to DMSO or TM5441 alone treated cultures of HCAEC and EA.hy926 cells (Fig. 1A,D). Significantly, pretreatment of endothelial cells with PAI-1 inhibitor TM5441 reduces the number of SA-β-Gal positive endothelial cells indicating Hcy augments endothelial senescence and PAI-1 inhibitor is highly effective in prevention of Hcy-induced endothelial senescence (Fig. 1A–E). We have further tested the induction of endothelial senescence by Hcy and the efficacy of PAI-1 inhibitor in prevention of senescence by evaluating senescence-activated nuclear morphological changes. Treatment of endothelial cells (HCAEC and EA.hy926 cells) with Hcy leads to nuclear shape change from an oval shape to a more cylindrical shape and pretreatment with TM5441 partially reduces Hcy-induced nuclear shape change indicating TM5441 is efficient in prevention of Hcy-induced endothelial senescence (Fig. 2A,B). The results of MTT analysis reveal that cell survival of HCAEC are not significantly influenced by exposure to different doses of TM5441(5 μM–50 μM) for 5 days (Fig. S1).

As senescence regulators are a group of cell cycle regulators and growth factors, next we investigated the expression levels of these regulators in Hcy-induced senescent endothelial cells and whether PAI-1 inhibitor imparts any influence on these regulators. Results reveal that high level of Hcy stimulates the levels of important known cadre of senescence markers and well-characterized regulators PAI-1, p16, p53 and p21 in human endothelial cells. Notably, pretreatment with TM5441 reduces the levels of senescence-controlling cadres PAI-1, p16, p53, p21 and integrin β3 (Fig. 3A, B, D, E and 3A', B', D', E'). The levels of pERK1/2 MAPK are modestly elevated in Hcy-treated HCAEC and importantly, that stimulation was blocked by the presence of TM5441 (Fig. 3D). We further tested the efficacy of another potent PAI-1 inhibitor TM5A15 in prevention of endothelial senescence. Results reveal that, like TM5441, TM5A15 efficiently blocks the expression of senescence marker and regulator p53 in the absence and presence of Hcy (Fig. 3C and C′) indicating both PAI-1 inhibitors TM5441 and TM5A15 are highly efficient in blocking Hcy-induced endothelial senescence through suppression of important senescence regulators (Fig. 3A–E and 3A′–E'). Together, these results further indicating the potentiality of PAI-1 as an ideal druggable target for suppression of cellular senescence under hyperhomocysteinemia.

Several studies reported that senescence induction and progression is controlled by intracellular and extracellular signals and vastly depends on the interaction of the cells with ECM-receptors like integrins, which are heterodimeric cell-surface transmembrane receptors that provide cellular adhesion [23]. To further extend our *in vitro* observation, on homocysteine-induced vascular senescence and PAI-1 inhibitors, we investigated the efficacy of a potent PAI-1 inhibitor TM5A15 on Hcy induced cardiac senescence *in vivo*. Mice were treated with Hcy in drinking water in the absence or presence of TM5A15 in chow for 9 weeks. Mice fed with regular water and standard chow were used as controls. In

order to confirm that the biological effects in Hcy fed mice are due to elevated levels of Hcy, the plasma levels of Hcy were measured in mice from all four groups by LC-MS technique. Results reveal that the blood levels of Hcy increased in Hcy fed mice compared to control mice (Fig. S2).

Next, we focused on the biochemical analysis of senescence regulators in heart lysates. In order to examine the interaction of PAI-1 with integrins αVβ3, equal amounts of pooled proteins from each group ($n = 5$) were immunoprecipitated with PAI-1 antibody and immunoprecipitated proteins were subjected to immunoblot with antibodies against integrin αV, integrin β3, PP1 and PAI-1. Results reveal that PAI-1 interacts with integrin αVβ3 and PP1. Interestingly, Hcy treatment enhances the physical interactions. However, co-treatment with TM5A15 reduces this increased interaction of PAI-1-integrin β3 and PAI-1-PP1 but not PAI-1-integrin αV by Hcy indicating interaction of two potent senescence regulators PAI-1 and integrin β3 may play an important role in Hcy-induced cardiac senescence (Fig. 4A,A'). Increased interaction of PP1 with PAI-1 in Hcy-treated cardiac tissues is consistent with a recent observation made by Yao et al. [24] who identified PP1 as a new member of PAI-1 interacting proteins and involved in controlling cellular proliferation and apoptosis. This is also consistent with the observation that in doxorubicin-induced senescent endothelial cells, the PAI-1 interacts with PP1 and PAI-1 inhibitor disrupts this interaction (unpublished data). At present the significance of this PAI-1-PP1 interaction in Hcy-induced cellular senescence is not clear.

Next we asked the most obvious question whether Hcy-induced increased interaction of PAI-1 and integrin β3 is due to posttranslational modification of these factors or simply due to increased expression of integrin β3 in Hcy treated hearts. Western blot analysis of equal amount of whole tissue lysates pooled from five mice in each group reveal that Hcy treatment causes increased levels of integrin β3. Most importantly Hcy fails to stimulate the levels of integrin β3, another senescence regulator, in mice co-treated with TM5A15 indicating inhibition of PAI-1 has dual beneficial effect in the context of prevention of cellular senescence, major contributor in aging process and related pathologies (Fig. 4B, B',D,D'). This *in vivo* data is consistent with our *in vitro* observation that Hcy induces the levels of integrin β3 in endothelial cells and TM5441 blocks that stimulation (Fig. 3E,E'). In contrast, the levels of integrin αV remained unaltered in Hcy and TM5A15 treated heart tissues compared to controls (Fig. 4B). These results also indicate that the increased PAI-1-integrin β3 interaction is simply due to presence of elevated levels of integrin β3 in Hcy treated murine cardiac tissues. In order to determine whether the levels of integrin β3 is also increased in Hcy treated kidneys, we collected kidneys from all four groups and kidney lysates were processed for western blot analysis. Interestingly, the quantification of integrin β3 relative to loading control α-Tubulin reveal that the levels of integrin β3 are also elevated in Hcy treated kidneys compared to control kidneys. Importantly, TM5A15 attenuated that stimulation (Fig. S3) indicating pharmacological inhibition of PAI-1 effectively normalize the expression levels of integrin β3 in both heart and kidney. In this context, it is worth mentioning that a previous report demonstrated, that the levels of integrin β3 is 16-fold higher in buffy-coat cells derived from rat model of folate deficiency-induced hyperhomocysteinemia [25]. It is important to note that the levels of integrin αV remain unaltered in the presence or absence of TM5A15. The rationale behind the investigation on

involvement of integrin β3 in Hcy induced senescence came from a recent study as mentioned earlier that documented the essential role of integrin β3 in cellular senescence [8]. This study demonstrated that the levels of integrin β3 are significantly elevated in old human primary fibroblasts and induced senescent fibroblasts. Furthermore, in the absence of integrin β3, senescence inducer fails to induce fibroblast senescence. This study showed that in response to senescence inducer the levels of CBX7, a chromatin organization modifier or transcriptional repressor that mediates monoubiquitation of H2A and promotes H3K9 trimethylation, are decreased and thus in the absence of transcriptional repressor, the levels of integrin β3 increases, and elevated integrin β3 increases the release of TGF-β that activates senescence in an autocrine and paracrine manner [8]. It is known that polycomb CBX7 extends cellular lifespan through downregulation of p16/p53/p21 pathway [26]. Although the levels of integrin β3 are elevated in cardiac tissues, we are unable to demonstrate the significant alteration of CBX7 in murine heart tissues in the presence of elevated levels of Hcy (Fig. 4B).

As mentioned earlier that although Nrf2 is considered as an antioxidant and downregulates the levels of ROS, a recent study by Hiebert et al. showed that sustained overexpression of Nrf2 induce senescence in fibroblasts through induction of PAI-1 [9]. This prompted us to examine the effect of Hcy and PAI-1 inhibitor on Nrf2 expression in murine heart tissue lysates. Results reveal that the levels of antioxidant gene regulator Nrf2 is modestly decreased in Hcy-treated mice hearts compared to control (Fig. 4C,C') and most importantly, co-treatment with TM5A15 and Hcy prevents the Hcy-induced suppression of Nrf2. In this context it is important to note that Hcy inhibits the protein levels of antioxidant catalase in endothelial cells and co-treatment with Hcy and PAI-1 inhibitor rescue the expression of catalase further indicating beneficial effect of PAI-1 inhibitor on Hcy-induced endothelial dysfunction (Fig. 3A). These results are consistent with our previous reports on the effect of PAI-1 inhibitor on expression of antioxidant catalase in doxorubicin-induced senescent endothelial cells [5]. Our observation is also consistent with a recent study that showed that Nrf2 deficiency is associated with cellular senescence in cerebral vessels and may contribute to age-related cerebrovascular pathology [27]. Significantly, pharmacological inhibition of PAI-1 recovers the levels of Nrf2 even in the presence of senescence inducer Hcy. As pERK1/2 MAPK [28] and p-p38 [29] are known to be induced in response to Hcy and may contribute to cellular senescence, we next investigated the levels of pERK1/2 MAPK and p-p38 MAPK in ventricular tissue lysates. Although the levels of pERK1/2 are elevated by Hcy and that stimulation is abrogated by TM5441 in endothelial cells *in vitro* (Fig. 3D), the *in vivo* results reveal that there are no significant changes in the levels of phospho-ERK1/2 MAPK levels in Hcy and or PAI-1 inhibitor treated murine hearts (Fig. 4B). This may be due to the fact that heart tissues lysates are derived from different cardiac cell types compared to lysates from *in vitro* cultured endothelial cells.

4. Conclusion

In summary, we have presented substantial evidence that metabolites like Hcy induces endothelial senescence, a major trait of endothelial dysfunction *in vitro* and *in vivo*. Most important and significant finding of the present study is that PAI-1 inhibitor (TM5441/TM5A15) efficiently inhibits senescence marker SA-β-Gal and senescence regulators

including PAI-1, p16, p21, p53, integrin β3 and thus Hcy-induced endothelial senescence. This report highlighted further the expression of other contributing regulators of senescence pathways like integrin β3, CBX7, PP1, Nrf2, pERK1/2 MAPK and p-p38 MAPK of which integrin β3, PP1 and Nrf2 may contribute in Hcy-induced regulatory pathways involved in cardiac senescence. In conclusion, PAI-1 contributes to Hcy-induced cellular senescence through activation of senescence regulators including integrin β3 and thus PAI-1 is an ideal druggable target for therapeutic approach to control metabolite-induced cellular and organismal aging and associated pathologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH-NHLBI grants (5R01HL051387) and American Heart Association (AHA) (16GRNT31130010 and 18IPA34170365). We would like to thank Dr. Panagiotis D Flevaris for providing integrin αV and integrin β3 antibodies.

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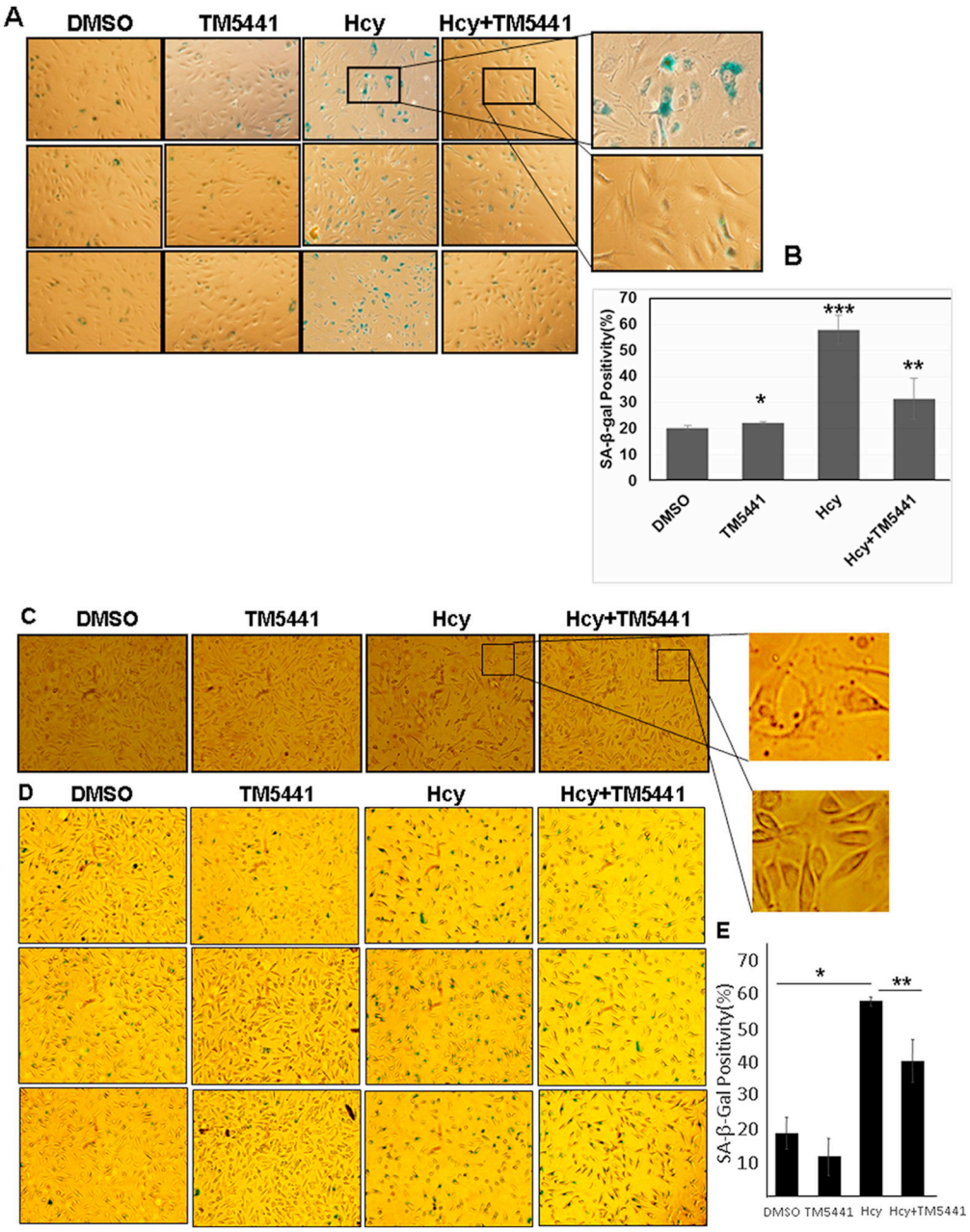

Fig. 1. PAI-1 inhibitor blunts Homocysteine-induced senescence in vascular endothelial cells as evidenced by SA-β-Gal activity.

Cultures of HCAECs (A,B) and EA.hy926 endothelial cells (C-E) were pretreated in triplicate with TM5441 followed by Homocysteine (Hcy) treatment for 4–5 days. At the end of incubation, the cells were subjected to SA-β-Gal assay and photographed. Images showing morphology and SA-β-Gal positive cells in DMSO, TM5441, Hcy and Hcy + TM5441 treated cultures of HCAECs (**A**) and EA.hy926 cells (**C,D**). Quantification data are shown in B. * denotes $p = 0.0376$ (DMSO *vs* TM5441); ** denotes $p = 0.0087$ (Hcy *vs* Hcy + TM5441); *** denotes $p = 0.0003$ (DMSO *vs* Hcy). Quantification data are shown in E. * denotes p=0.0029 (DMSO vs Hcy); ** denotes p=0.035 (Hcy vs Hcy+TM5441).

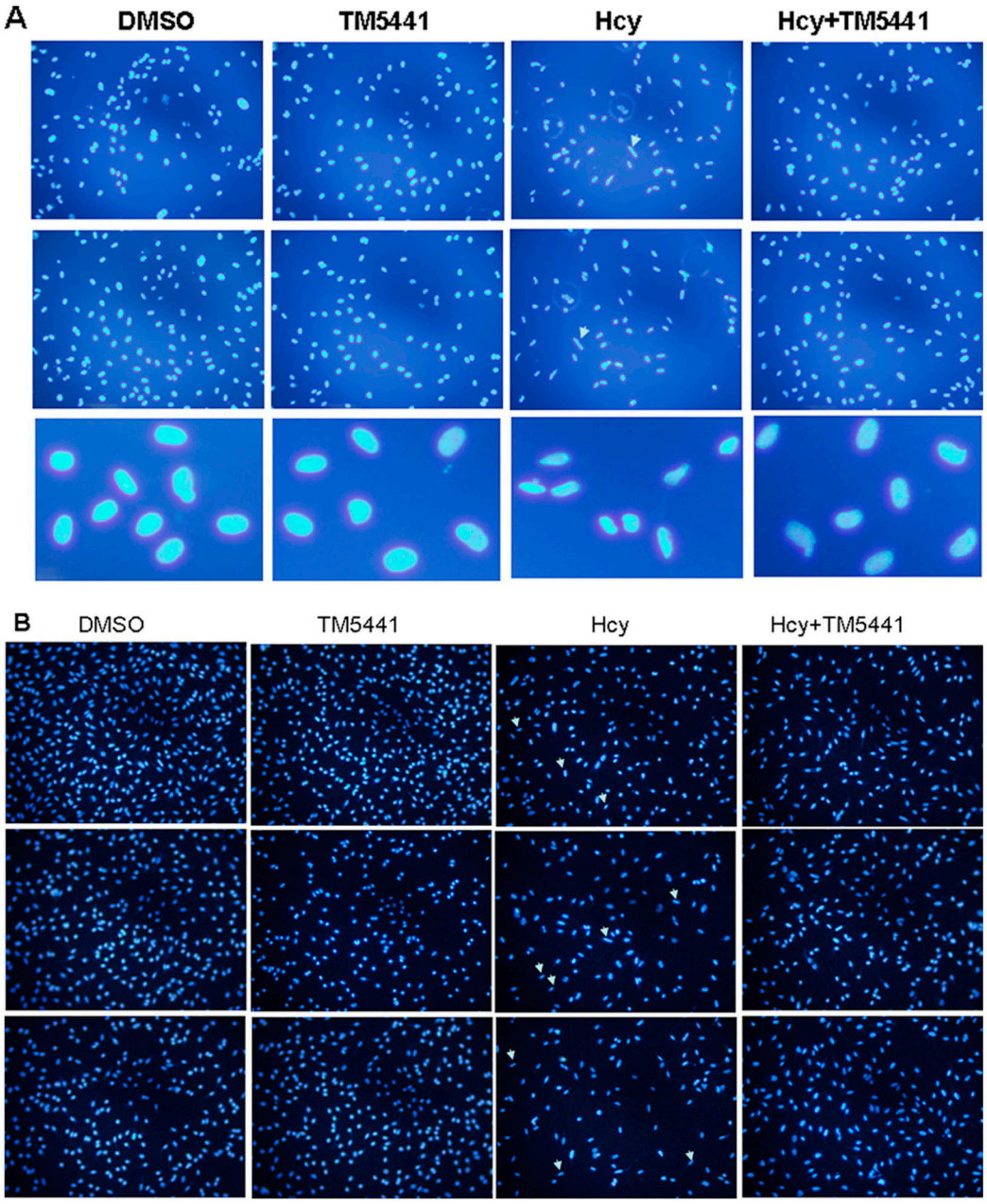

Fig. 2. PAI-1 inhibitor blunts Homocysteine-induced senescence in vascular endothelial cells as evidenced by nuclear morphology.

Cultures of HCAECs (A) and EA.hy926 endothelial cells (B) were pretreated with TM5441 followed by Homocysteine (Hcy) treatment for 4–5 days. At the end of incubation, the cells were subjected to nuclear morphology analysis by Triton X-100 pretreatment followed by DAPI staining and photographed. Nuclear morphology in DMSO, TM5441, Homocysteine and Homocysteine+TM5441 treated cultures are shown. Examples of cylindrical shaped nuclei are shown by arrows.

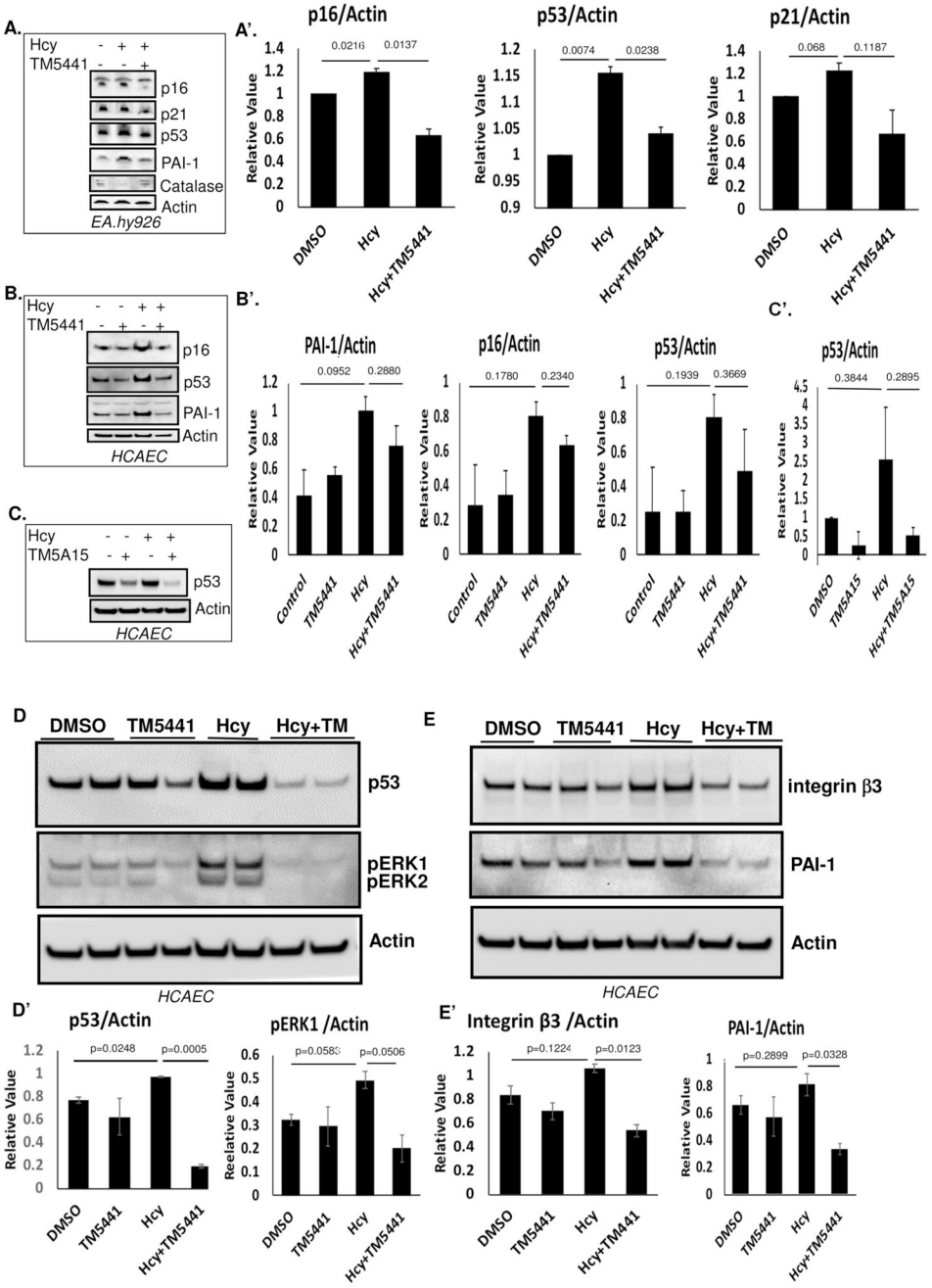

Fig. 3. PAI-1 inhibitors reduce Homocysteine-induced senescence regulators in vascular endothelial cells.

Cultures of EA.hy926 endothelial cells (A) and HCAEC (B, C) were pretreated with TM5441 (10 μM) (A, B) or TM5A15 (10 μM) (C) in triplicate followed by Homocysteine (Hcy) treatment for 4–5 days. Whole cell extracts were prepared and equal amount of pooled proteins from three wells were subjected to Western blot analysis for senescence markers and regulators using specific antibodies as indicated (A–C). Bar represents mean ± sem. Quantitative data are shown on the right (A'-C'). The levels of at least 2–3 senescence markers were determined in repeat experiments. D, E. Whole cell extracts (HCAEC) were

prepared from two separate experiments and equal amount of pooled proteins from three wells were subjected to Western blot analysis for senescence markers and regulators p53 and pERK1/2 (D), integrin β3 and PAI-1 (E) using specific antibodies. Quantitative data in the lower panel showing the levels of each regulator relative to loading control Actin (D', E').

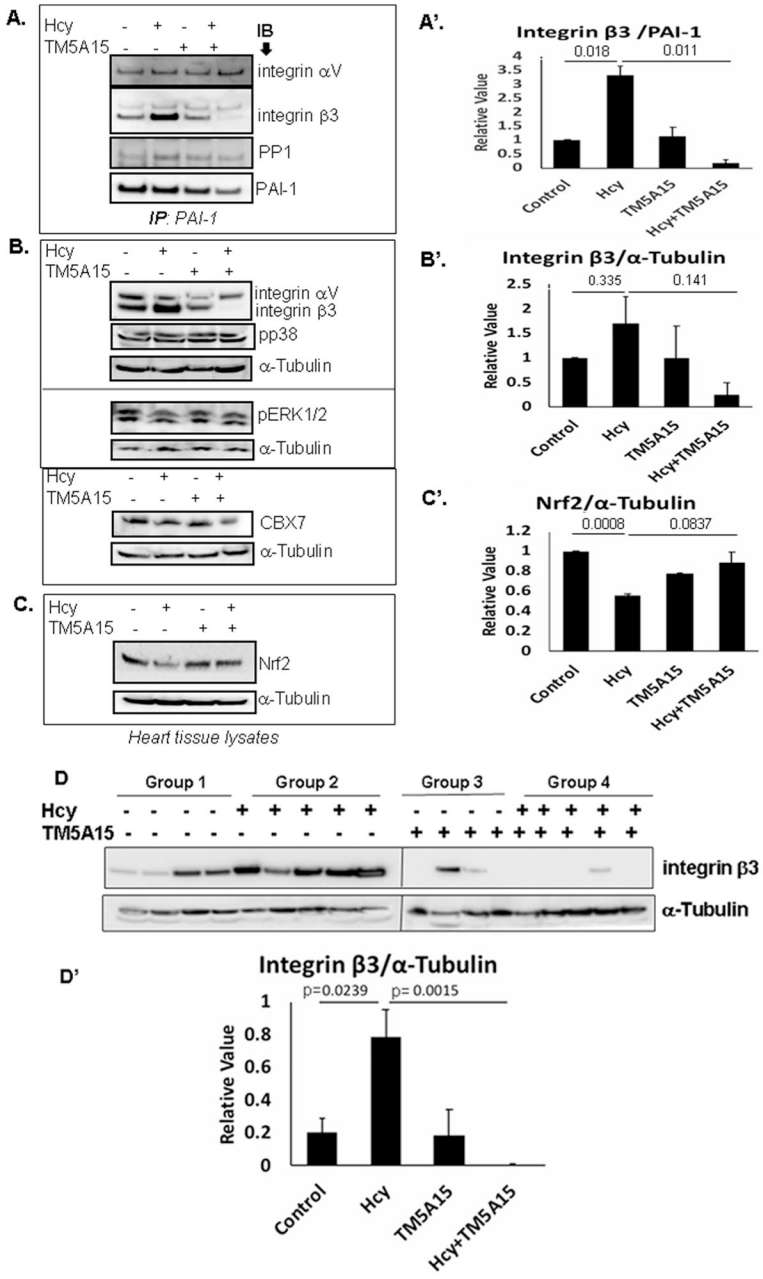

Fig. 4. Homocysteine induces the levels of senescence regulator integrin β3 and its interactions with PAI-1 in murine hearts and PAI-1 inhibitorTM5A15 completely blunts that stimulation of integrin β3.

Mice received Hcy (2 g/L) in drinking water for 9 weeks in the absence and presence of PAI-1 inhibitor TM5A15-containing chow. Heart tissues were lysed and equal amount of tissue lysate protein pooled from each mouse per group (n = 5/group) were subjected to coupled Immunoprecipitation: - Immunoblot analysis (IP:IB) (A) and immunoblot analysis (B and C) using antibodies as indicated (A–C). - Quantitative data of integrin β3 andNrf2 are shown on the right (A'-C'). Data represents mean ± sem. The levels of at least 2–3 senescence markers were determined in repeat experiments. D. Equal amount of tissue

extracts from each mouse were subjected to immunoblot analysis using integrin β3 and mα-Tubulin antibodies. Group 1 control (n=4); Group 2 Hcy (n=5); Group 3 TM5A15 (n=4) and Group 4Hcy+TM5A15 (n = 5). The quantitative data are presented in D'. Bar represents mean ± sem.