TRANSLATIONAL PHYSIOLOGY

Growth factor-mediated reversal of senescent dysfunction of ischemia-induced cardioprotection

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Submitted 9 May 2005; accepted in final form 22 September 2005


Based on the role of tumor necrosis factor-α (TNF-α) in ischemic preconditioning (IPC) and the age-associated loss of both TNF-α-induced platelet-derived growth factor-AB (PDGF-AB)-mediated cardioprotection and IPC-mediated cardioprotection, we hypothesized that targeting of PDGF-AB-based pathways would restore cardioprotection by IPC in the aging heart. To study this, IPC was induced in 4- and 24-mo-old F344 rats. Sections of young hearts isolated 1 day post-IPC revealed increased TNF-α compared with controls. In old rats, TNF-α was higher at baseline than IPC young rats and was not significantly altered after IPC. Treatment of old rats with PDGF-AB with vascular endothelial growth factor and angiopoietin-2 (a combination termed PVA), but not PDGF-AB alone, at the time of IPC decreased TNF-α. In addition, when compared with young hearts, IPC induced greater apoptosis in the old hearts, which was decreased with PVA treatment but was markedly increased with PDGF-AB. To test the significance of these findings, additional rats underwent permanent coronary ligation 1 day post-IPC. IPC was cardioprotective in young rats [14 days postmyocardial infarction (MI), fractional shortening 29 ± 6% vs. control MI 17 ± 4%, P < 0.05; Masson’s trichrome stain MI size: 13 ± 2% vs. control MI 17 ± 4% left ventricular area (LVA); P < 0.05]. In old rats, however, IPC reduced the post-MI 14-day survival (33% vs. controls 67%; P < 0.05). Treatment of IPC-aging rats with PVA, but not PDGF-AB-alone, reversed IPC-induced mortality (PVA-IPC-MI survival, 88%; PDGF-AB-IPC-MI, 14%) and reduced myocardial injury (fractional shortening: PVA-IPC, 31 ± 1% vs. control MI, 21 ± 6%, P < 0.05; MI size: PVA-IPC, 12 ± 2% vs. control MI, 18 ± 3% LVA, P < 0.05) and thus demonstrated that PDGF-AB-based pathways can reverse the senescent impairment in IPC-mediated cardioprotection.

aging; myocardial infarction; preconditioning

AGE-RELATED ALTERATIONS in tumor necrosis factor-α (TNF-α) pathways may contribute to the increased risk of cardiac disease and more severe cardiovascular pathophysiology in the geriatric population. Specifically, the recent results from the ABC Health Study revealed that in persons over 65 yr of age, TNF-α is more predictive of future cardiovascular events compared with C-reactive protein (CRP) (5). Moreover, clinical studies of inflammatory markers have linked elevations in TNF-α with an increased incidence of congestive heart failure after myocardial infarction (25). These findings in conjunction with the age-related increases in TNF-α in the systemic circulation (3) as well as the coronary arteries (7) suggest that strategies targeted at the actions of TNF-α in the older heart may have a significant impact on cardiovascular disease in the aging population.

Recently, we demonstrated that TNF-α-induced cardioprotection pathways are impaired in the aging rodent heart (4). Specifically, these studies revealed that a downregulation in a subpopulation of TNF receptor (TNFR) cardiac microvascular endothelial cells is associated with the loss of TNF-α-mediated cardioprotection in an experimental myocardial infarction model. In the young rat heart, intramyocardial injection of TNF-α reduced myocardial injury after acute coronary occlusion. TNF-α injection in older hearts, however, results in significant apoptosis and mortality after subsequent acute coronary occlusion. Importantly, codelivery of platelet-derived growth factor (PDGF)-AB can reverse these impairments and restores cardioprotection to the aging heart. Mechanistically, the actions of PDGF-AB are mediated in combination with its induction of both vascular endothelial growth factor (VEGF) and angiopoietin (Ang)-2 (but not VEGF and Ang-2 without PDGF-AB) to acutely suppress apoptosis and reduce myocardial infarction (27). From these findings, we hypothesized that strategies targeting PDGF-AB-based pathways (PDGF-AB alone or in combination with VEGF and Ang-2) would improve functional outcomes under conditions associated with dysregulation of TNF-α-mediated cardioprotection in the aging heart.

Previous experimental studies in young animal models have demonstrated the essential role of TNF-α induction in the cardioprotection provided by transient episodes of myocardial hypoxia, referred to as ischemic preconditioning (IPC) (9, 16, 20, 28). Moreover, clinical trials have demonstrated that cardiac IPC can reduce cardiac injury induced by subsequent coronary occlusion and that this benefit may be impaired in the aging heart (for review see Refs. 13 and 29). Thus, based on experimental studies revealing an age-associated loss in IPC-mediated cardioprotection (1, 22, 24), we investigated the potential role of PDGF-AB and/or PDGF-AB/VEGF/Ang-2 (PVA) to restore IPC cardioprotection in the aging rat heart.

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METHODS

Procedures utilizing animal models were approved by and performed according to the guidelines of the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University and conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

Ischemia preconditioning. A late model of IPC was induced in both 4- and 24-mo-old F344 rats (National Institute on Aging Rodent Colony, Indianapolis, IN) similarly to previously described techniques (14). After anesthesia with ketamine (90 mg/kg ip) and xylazine (4 mg/kg ip), a tracheotomy was performed, and the animal was intubated and ventilated (Harvard Rodent Ventilator model 683) with room air. A left intercostal thoracotomy was performed at the fourth and fifth intercostal space. Upon identification of the left anterior descending artery (LAD), a 7-0 suture was secured 2-0.2 mm below the level of the tip of the normally positioned left auricle, and four rounds of 10-min ischemia and reperfusion were induced. After the conclusion of the fourth reperfusion, the suture was removed, the chest wall was closed, and the animal was removed from the ventilator and placed under a heat lamp to recover. The above procedure was also performed in young and old rats without LAD occlusion as controls. Additional groups of old rats received intramyocardial injections of PDGF-AB alone or a combination termed PVA of PDGF-AB, VEGF, and Ang-2 [each factor at a dose of 100 ng as previously employed (27) in a total volume of 50 μl: two injections, 25 μl on either side of the LAD, using a 30-gauge needle syringe] that were given after the first ischemic episode. The sample size was ≥3 rats per test condition. Rats were euthanized 24 h after the injection, and the hearts were excised, fixed, and sectioned for immunohistochemistry staining rabbit or goat antibodies directed to TNF-α (sc-1351), Bcl-2 (sc-492), and Bax (sc-6236) (Santa Cruz Biotechnology) were used as primary antibodies and visualized using the ABC staining method (Santa Cruz, ABC Staining Kit) and counterstained with methyl green. Heart sections were also analyzed for apoptotic cell death detected by terminal deoxynucleotidyl transferase-mediated dUTP (TUNEL) staining (Roche In situ Cell Death Kit) and counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Positively stained cells were assessed in sections at the midpapillary level of each heart, and all stained luminal structures in a total of six

![Fig. 1. Ischemic preconditioning (IPC) induction of tumor necrosis factor-α (TNF-α) in rat hearts. Representative chromogenic histology of immunostains of (A) TNF-α, (B) Bcl-2, and Bax (methyl green nuclear counterstain), and (C) fluorescent histology of terminal deoxynucleotidyl transferase-mediated dUTP (TUNEL) reactions [4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) nuclear counterstain] in midpapillary sections in rat hearts treated with IPC (four rounds of 10 min ischemia and reperfusion mid left anterior descending) or sham operation 24 h before euthanization in 4- and 24-mo-old hearts (n ≥ 3, each). Additional sets of 24-mo-old rats received intramyocardial injection platelet-derived growth factor-AB (PDGF-AB) alone (100 ng) or a combination termed PVA of PDGF-AB (100 ng), VEGF (100 ng), and angiopoietin-2 (Ang-2, 100 ng) (n ≥ 3, each) after the first ischemic episode. D: quantification of the immunostaining and TUNEL density in the IPC and sham heart sections. Bar = 20 μm (A), 30 μm (B), 40 μm (C). *P < 0.05.

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high-power fields (×40 magnification) per section were identified in a blinded analysis as previously described (11).

Myocardial infarction after ischemic preconditioning. Additional sets of 4- and 24-mo-old rats underwent repeat operations after sham or IPC procedures to permanently ligate the LAD (n = 7, each group). Briefly, 24 h after an IPC or sham operation, the rat was reanesthetized and the LAD was permanently ligated with 8-0 nylon sutures. The chest wall was closed, and the rats were returned to their cages. The above procedure was also performed with groups of old rats receiving an intramyocardial injection PDGF-AB alone (n = 7) or the PVA combination (n = 8), as described above.

Myocardial assessment after myocardial infarction. To directly measure the effect of IPC on cardiac function following coronary ligation, echocardiograms were performed to measure left ventricular fractional shortening (FS). At 14 days postligation, the rats were anesthetized as described above. Before the hearts were harvested for histological analysis, M-mode echocardiograms were recorded of the left ventricle at the midpapillary muscle level employing an Acuson-SEQUOIA C256 with a 10-MHz intracardiac probe for transesophageal left ventricle at the midpapillary muscle level employing an Acuson-SEQUOIA C256 with a 10-MHz intracardiac probe for transesophageal echocardiography and expressed as a percentage of the total left ventricular myocardial area, as previously described (4, 11).

Statistics. Data are presented as means ± SD. Differences in echocardiographic and histological measurements were tested for significance by ANOVA and the Student’s t-test. The survival rate difference was tested by χ²-test. A value of P < 0.05 was considered significant.

RESULTS

TNF-α and apoptotic protein and TUNEL patterning after IPC. Immunohistology revealed increased cardiac patterning of TNF-α after IPC in the young rat heart (Fig. 1). These findings were in agreement with previous reports (9, 20, 28), confirming the validity of the model to investigate approaches to counter the age-associated actions of TNF-α in the senescent loss of cardioprotection. In the aging hearts, however, TNF-α patterning was significantly more extensive in the sham-operated animal compared with both control and IPC-treated young rats, which was not significantly increased in the IPC-treated aging rats. Delivery of PVA, but not PDGF-AB, resulted in a decreased patterning of TNF-α in the IPC-treated old hearts.

IPC also induced significant changes in apoptotic regulatory proteins and TUNEL staining, albeit with an age-dependent shift in the pro- and anti-apoptotic protein patterns in the IPC-treated hearts. The young hearts demonstrated a significant increase in Bcl-2 with minimal change in the patterning of Bax. IPC in the aging heart, however, resulted in a decrease of anti-apoptotic Bcl-2 while markedly increasing the pro-apoptotic protein Bax. Notably, in the IPC-treated old hearts receiving PVA, but not PDGF-AB, the Bcl-2 and Bax patterns were similar to those of the young hearts, with significant increases in Bcl-2 and decreases in Bax. Moreover, TUNEL stains performed 24 h post-IPC revealed a significantly greater distribution pattern in the hearts of the 24-mo-old IPC hearts compared with age-matched sham controls or 4-mo-old IPC hearts. In the IPC-treated old hearts, injection with PDGF-AB resulted in a significant increase in TUNEL staining, whereas those treated with the PVA combination had patterns that were similar to the old control hearts.

Age-associated myocardial infarction survival after IPC. Functionally, the induction of myocardial infarction revealed a significant age-associated loss in IPC-mediated cardioprotection. Permanent coronary occlusion 1 day after IPC induction resulted in extensive mortality in the 24-mo-old rats compared with the same age rats pretreated with sham operations before myocardial infarction (Fig. 2). IPC did not alter survival in the 4-mo-old rats. Moreover, IPC in the young rats resulted in improved cardiac function (Fig. 3) and decreased the extent of myocardial injury (Fig. 4), which was measured 14 days after induction of myocardial infarctions. Indeed, IPC improved the architecture of the myocardial infarctions, converting complete transmural injury patterns to predominantly nontransmural, subendocardial myocardial infarctions.

PVA restores IPC survival and protection in aging rats. Delivery of PDGF-AB in combination with VEGF and Ang-2 (PVA), but not PDGF-AB alone, during IPC induction restored survival of the old rats after myocardial infarction (Fig. 2). The PVA combination improved cardioprotection to the level observed in the young IPC rats as measured by echocardiography (Fig. 3) and histology (Fig. 4). Moreover, similar to the actions of IPC in the young hearts, the delivery of PVA with IPC reduced the transmural myocardial injury patterns to predominantly subendocardial myocardial infarctions. The significant mortality in the control IPC and the IPC-PDGF-AB groups limited the analysis of the functional measurements in these groups.

DISCUSSION

Our studies highlight the impact of cardioprotection strategies targeted at changes in the older heart to promote the survival and reduce myocardial injury in the aging heart after acute coronary occlusion. Specifically, our studies reveal the age-associated dysregulation of cardioprotective TNF-α induction in the rodent IPC model and its link to impaired survival after myocardial infarction. Moreover, targeting with the com-

![Graph](image-url)
Combination of PDGF-AB, VEGF, and Ang-2 reversed the pathophysiology associated with IPC to fully restore survival and cardioprotection in the aging heart.

Our studies confirm the association of TNF-α pathways in the induction of cardioprotection in the young heart after acute coronary occlusion following episodes of transient ischemia (7, 15, 21), which is required for experimental IPC-mediated cardioprotection (16). In the aging hearts, however, the induction of IPC promotes a shift to pro-apoptotic pathways and leads to the death of the majority of older rats after subsequent coronary occlusion, similar to what was observed after intramyocardial delivery of TNF-α before myocardial infarction in previous cytokine pretreatment studies (4). The increased mortality after IPC was associated with an increase in the cardiac patterns of TNF-α in the aging hearts that was present after sham operations, suggesting that the repetitive induction of hypoxia in the setting of age-associated increases in TNF-α may contribute to the increased mortality in the aging rats. Indeed, previously we demonstrated that either delivery of supraphysiological concentrations of TNF-α or permanent coronary occlusion ischemia did not affect the survival of aging rats (4). The combination of both interventions, however, resulted in a significant age-related mortality, suggesting that hypoxia may further enhance the pathological shift in senescent TNF-α-induced pathways, resulting in extensive apoptosis and decreased survival after myocardial infarction.

The differences between the benefits observed with PDGF-AB after TNF-α pretreatment and the requirement of the PDGF-AB-VEGF-Ang-2 combination may be temporally related to the initial episode of hypoxic induction in the aging hearts. To this end, we have previously demonstrated that the delayed kinetics of PDGF-AB-mediated cardioprotection can be eliminated by the codelivery of PDGF-AB and the growth factors downstream from PDGF-AB in cardiac angiogenic pathways at the time of acute

Fig. 3. A: representative M-mode transesophageal echocardiograms of 14 days post-MI in aging rats treated sham IPC and PVA-IPC 24 h before permanent occlusion of the mid-LAD arteries. B: fractional shortening (%) measurements 4- and 24-mo-old rats treated with or without IPC and growth factors before MI (n > 6, each). *P < 0.05. ND, not determined because of excessive mortality.

Fig. 4. Age-associated IPC cardioprotection. A: representative Masson’s trichrome staining of 4- and 24-mo-old rat hearts 14 days post-MI (acute LAD ligation) 24 h after sham operation (4- and 24-mo-old controls) or IPC (4-mo-old) or IPC with PVA treatment (24-mo-old rats). Arrows, transmural myocardial injury; open arrowheads, nontransmural subendocardial myocardial injury. B: measurements of cardiac injury (% left ventricular area) in 4- and 24-mo-old rats treated with or without IPC and growth factors before MI (n = 6, each). *P < 0.05.
coronary occlusion to act synergistically to reduce cardiac cell death and decrease myocardial injury (27). Indeed, the significant increase in TUNEL staining after PDGF-AB injection in the old IPC rats is consistent with the diminished survival of this treatment group after permanent LAD occlusion compared with the rats receiving IPC alone. Thus the results with the PVA combination, which increased the Bcl-2-to-Bax ratio and reduces the density of TUNEL+ cells after IPC, highlight the importance of targeting angiogenic pathways to counter the pro-apoptotic shift in the aging heart.

Mechanistically, the synergism between myocardial hypoxia and alterations in TNF-α signaling may be related to reactive oxygen species (ROS)-induced apoptotic pathways. Indeed, though we previously demonstrated an association between the loss of subendocardial TNF-R1 endothelial cells and the impairment in TNF-α induction of PDGF-B in the old rodent heart (4), the actions of TNF-α-mediated cardioprotection likely involves multiple and potentially counterbalancing pathways to reduce myocardial injury after acute coronary occlusion. The loss of these cells and the age-related changes in TNF-α function may be due to enhanced oxidation pathways and associated lipid peroxidation induced by TNF-α in the senescent cardiac microvasculature (12). Moreover, enhancement in ROS pathways may mediate TNF-α signaling, either directly or through changes in the receptor pathways transactivated by ROS, to promote apoptotic pathways (2, 10, 23), and further contribute to the loss of the cells mediating the cardioprotective actions of TNF-α. Indeed, lipid peroxidation products themselves may directly promote vascular aging and apoptosis, in part through a reduction in endothelial Bcl-2 (17). Such changes in TNF-α pathways, as well as the age-related increase in cardiac inflammation (18, 19) and increases in the TNF-α-converting enzyme in the cardiac vasculature (8), may chronically elevate TNF-α levels to further induce the altered receptor and signaling pathways to impair cardioprotection in the older heart, which may be balanced by the addition of the PVA combination with IPC. Indeed, the delivery of PVA in the IPC-treated old hearts resulted in significantly less TNF-α staining compared with the old IPC and IPC-PDGF-AB treatment groups. Notably, however, the extensive apoptosis associated with PDGF-AB injection in the old IPC hearts was not coupled with an increase in TNF-α. These findings, in conjunction with the results of recent clinical trials revealing a lack of cardiac benefit from antibody-based approaches aimed at countering TNF-α (6), suggest that the actions of PVA may be more likely due to its affects on the ratio of pro- and anti-apoptotic pathways than on TNF-α levels or Bcl-2-to-Bax ratios. Indeed, our data suggest that pathways underlying the deleterious results associated with PDGF-AB injection in the IPC-treated hearts are specifically blocked and/or compensated for by the addition of VEGF and Ang-2.

The results of our studies suggest that therapies targeting the cardioprotective pathways of the PVA combination may provide a unique approach to restore IPC-mediated benefits in the older heart. It is anticipated that identification of the mechanisms mediating the prosurvival and anti-apoptotic actions of this growth factor combination may provide unique insights into the functional alterations in the age-associated impairment in IPC cardioprotection. Further studies to determine the optimal ratios and kinetics of the growth factor delivery as well as the development of pharmacological or other therapeutic approaches based on such advances may provide an important foundation for the development of novel approaches in the treatment of cardiovascular disease in older persons.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grants AG-19738, AG-20918, and HL-67839 and by an American Federation for Aging Research-Paul Beeson Physician Faculty Scholar in Aging Research Award.

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