ELSEVIER

The Journal of Heart and Lung Transplantation

http://www.jhltonline.org

Antioxidant properties of pioglitazone limit nicotinamide adenine dinucleotide phosphate hydrogen oxidase and augment superoxide dismutase activity in cardiac allotransplantation

Tomomi Hasegawa, MD, PhD,[a,b] Kenji Okada, MD, PhD,[a] Yutaka Okita, MD, PhD,[a] and David J. Pinsky, MD[b]

From the [a]Division of Cardiovascular Surgery, Department of Surgery, Kobe University Graduate School of Medicine, Kobe, Japan; and the [b]Department of Internal Medicine, University of Michigan Cardiovascular Center, Ann Arbor, Michigan.

KEYWORDS:
cardiac allograft;
PPAR-gamma;
oxidative stress;
NADPH oxidase;
superoxide dismutase

BACKGROUND: Many non-immunologic factors contribute to the development of cardiac allograft vasculopathy (CAV), chief among them being ischemia-reperfusion injury associated with oxidative stress. We hypothesized that pioglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ agonist, could attenuate graft oxidant stress in cardiac transplantation.

METHODS: Experiments were performed using a murine heterotopic cardiac allotransplantation model. Pioglitazone was administered to recipients once daily, beginning 1 day before transplantation.

RESULTS: At 4 hours after transplantation, pioglitazone significantly reduced the expression of endothelial cell adhesion receptors and infiltration of polymorphonuclear leukocytes (PMNs). The anti-oxidant balance in pioglitazone-treated cardiac allografts was significantly bolstered by reduced nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase (Nox1 and $p22^{phox}$ subunits) activity and preservation of manganese superoxide dismutase (SOD) activity, resulting in the mitigation of oxidative damage at the level of lipids, proteins, and DNA. At 7 days after transplantation, PPAR-γ was significantly up-regulated by pioglitazone, but nuclear factor-κB and inducible nitric oxide synthase were significantly down-regulated. A concomitant reduction of inflammatory cytokines and chemokines and graft leukosequestration was noted. Pioglitazone consequently prolonged cardiac allograft survival and attenuated CAV development. In vitro experiments demonstrated that pioglitazone decreased transendothelial PMN migration, NADPH oxidase activity, and loss of SOD activity in PMNs and endothelial cells.

CONCLUSIONS: Pioglitazone can suppress the oxidative stress and damage and can stimulate antioxidant capacity in cardiac allografts after transplantation. Mitigation of graft oxidant stress could be an important mechanism through which pioglitazone confers benefit after cardiac transplantation.

J Heart Lung Transplant 2011;30:1186–96

Perfused tissues are often subjected to a barrage of extraneous insults that perturb homeostasis. This is particularly the case during periods in which blood flow temporarily ceases, because nutritive substrate delivery is interrupted and waste products accumulate, and even more so when blood flow resumes, in which case reactive oxygen species (ROS) are generated and inflammatory cascades are activated. Of the various endogenous defense mechanisms that are triggered to counter these extraneous perturbations, antioxidant defenses are among the most primal and most essential to protect tissue from injury. Oxidative stress with attendant tissue injury occurs as a result of an imbalance between the production and removal of ROS, which have been implicated as causally involved in the pathogenesis of

Reprint requests: Tomomi Hasegawa, MD, PhD, Department of Cardiovascular Surgery, Kobe Children's Hospital, 1-1-1, Takakuradai, Suma-ku, Kobe 654-0081, Japan. Telephone: +81-78-732-6961. Fax: +81-78-735-0910.

E-mail address: tohasegawa_kch@hp.pref.hyogo.jp

1053-2498/$ -see front matter
doi:10.1016/j.healun.2011.07.006

various cardiac diseases.[1–3] The net excess in ROS levels is important in directly oxidizing tissues and amplifies a subsequent inflammatory response that perpetuates the cycle of damage.[2,4]

In the setting of cardiac allotransplantation (CTx), exposure of the heart to the ischemia-reperfusion (I/R) environment by the harvest, preservation, and implantation procedures can act as one of the multiple potential non-immunologic adjuvants for oxidative damage. Placement of the heart into an immune milieu can accelerate further oxidative damage and consequent tissue inflammation,[5–7] driving cardiac allograft vasculopathy (CAV).

Because the inexorable CAV development has still hampered improvements in long-term patient survival despite recent remarkable advances in immunosuppressive agents,[8,9] the non-immunologic adjuvants must be indispensable targets to overcome CAV development. Actually, peri-CTx anti-oxidant treatment can elicit a striking protection against CAV development long after the period of its administration.[10–12] Although the exact mechanism underlying the protection against CAV development has not been fully defined, we have demonstrated that a suppressive interrelation among redox-sensitive transcription factor nuclear factor-κB (NF-κB) induction, inducible nitric oxide synthase (iNOS) expression, cytokine and chemokine expression, and graft leukosequestration can be a potential cascade of anti-oxidant treatment to inhibit CAV development.[11]

The peroxisome proliferator-activated receptor (PPAR) family of transcription factors, activated under discrete conditions of metabolic or ischemic stress, activate transcription of families of genes involved in organ protection. The ligand-activated PPAR-γ agonists in the thiazolidinedione class are generally used as insulin-sensitizing agents to modulate glycemic control in a clinical setting, but some experimental models have shown that one of them, pioglitazone, possesses pleiotropic cardioprotective actions such as anti-inflammatory[13–15] and anti-proliferative properties.[16,17] In experimental CTx, we[18] and other[17] have demonstrated that pioglitazone can prolong allograft survival and attenuate CAV development, but its anti-oxidant effect on the cardiac allograft has not been elucidated yet.

The present experiments were designed to establish the hypothesis that the protective effect of pioglitazone on cardiac allograft can be ascribed to its anti-oxidant properties. Particularly, we focused on CTx-induced oxidative stress in the I/R phase.

Materials and methods

The handling of the laboratory animals and their use in experiments in this study conformed to the *Guidelines for Animal Experiment* at Kobe University Graduate School of Medicine and University of Michigan. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health (NIH) Publication No. 85–23, revised 1996).

Animals

Male mice of C57BL/6J (H-2^b) and CBA/J (H-2^k) strains, 8 to 12 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME).

Antibodies

Rabbit polyclonal antibodies against PPAR-γ, Nox1, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against Cu/Zn-superoxide dismutase (SOD) and Mn-SOD from Assay Designs (Ann Arbor, MI), rat anti-mouse Ly-6G antibody from Southern Biotech (Birmingham, AL), rat anti-mouse CD4 and CD8 antibody from BD Pharmingen (San Diego, CA), and mouse monoclonal antibody for β-actin from Sigma-Aldrich (St. Louis, MO) were purchased.

Treatment protocol

Pioglitazone, provided by Takeda Chemical Industries (Tokyo, Japan), was diluted in normal saline for allograft experiments and dissolved in dimethyl sulfoxide (Sigma-Aldrich) for in vitro experiments. Pioglitazone (3 mg/kg/day; $n = 6$)[17,19] or normal saline vehicle (control; $n = 6$) was given to recipient mice by gastric gavage once daily beginning 1 day before CTx.

Heterotopic CTx

Completely allo-mismatched murine heterotopic CTx was performed as described previously.[20] C57BL/6J mice were used as donors and CBA/J mice as recipients. Recipient mice received no immunosuppressive agents. To observe the CAV development, recipient mice received transient immunosuppression using a brief pre-operative course of anti-murine CD4 (clone GK1.5) and anti-murine CD8 (clone 2.43) antibodies (Harlan Bioproducts for Science, Indianapolis, IN).

Graft survival and histomorphometric assessment

Survival and CAV area in cardiac allografts were assessed as previously described.[12] Briefly, graft survival was evaluated by daily palpitation, and absence of pulsation was interpreted as graft death. Grafts were fixed in 10% formalin, paraffin-embedded, and sectioned transversely at the maximal circumference of the ventricle. Sections were cut (5-μm thick), and the CAV area was analyzed using elastica van Gieson staining to highlight the internal elastic lamina (IEL) and external elastic lamina. The cross-sectional area of luminal occlusion was calculated using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD) as follows: luminal occlusion = [(IEL area – luminal area)/IEL area] $\times$ 100%.

Myeloperoxidase assay

Myeloperoxidase activity in cardiac allografts was assessed as previously described.[21] Data are standardized to the protein concentration of each sample as determined by a micro BCA Protein Assay Kit (Pierce, Rockford, IL).