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Hyperoxia and transforming growth factor β1 signaling in the post-ischemic mouse heart

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ABSTRACT

Aims: Following ischemic injury, myocardial healing and remodeling occur with characteristic myofibroblast trans-differentiation and scar formation. The current study tests the hypothesis that hyperoxia and nitric oxide (NO) regulate TGF-β1 signaling in the post-ischemic myocardium.
Main methods: C57BL/6 wild-type (WT), endothelial and inducible nitric oxide synthase knockout ($eNOS^{-/-}$ and $iNOS^{-/-}$) mice were subjected to 30-min left anterior descending coronary artery occlusion followed by reperfusion. Myocardial tissue oxygenation was monitored with electron paramagnetic resonance oximetry. Protein expressions of TGF-β1, receptor-activated small mothers against decapentaplegic homolog (Smad), p21 and α-smooth muscle actin (α-SMA) were measured with enzyme-linked immunosorbent assay (ELISA), Western immunoblotting, and immunohistochemical staining.
Key findings: There was a hyperoxic state in the post-ischemic myocardial tissue. Protein expressions of total and active TGF-β1, p-Smad2/3 over t-Smad2/3 ratio, p21, and α-SMA were significantly increased in WT mice compared to Sham control. Knockout of eNOS or iNOS further increased protein expression of these signals. The expression of α-SMA was more abundant in the infarct of $eNOS^{-/-}$ and $iNOS^{-/-}$ mice than WT mice. A protein band indicating nitration of TGF-β type-II receptor (TGFβRII) was observed from WT heart. Carbogen (95% O_2 plus 5% CO_2) treatment increased the ratio of p-Smad2/t-Smad2, which was inhibited by 10006329 EUK (EUK134) and sodium nitroprusside (SNP). In conclusion, hyperoxia up-regulated and $NO/ONOO^-$ inhibited cardiac TGF-β1 signaling and myofibroblast trans-differentiation.
Significance: These findings may provide new insights in myocardial infarct healing and repair.

Introduction

Acute myocardial ischemia and ultimately myocardial infarction claims more than 1.5 million American lives every year (Tavazzi, 1999; Weir et al., 2006). Reperfusion not only reduces the ischemic injury, but also induces "reperfusion injury" (Rapapaport, 1989; Prasad et al., 2009). The mechanisms underlying ischemia and reperfusion injury (I/R) are associated with reactive oxygen/nitrogen species (ROS/RNS), which causes oxidative and nitrative damages to the inflicted myocardium (Bolli, 1996; Ferdinandy and Schulz, 2003; Kin et al., 2008; Liu et al., 2009; Zweier et al., 1994, 1988).

Immediately following I/R injury, the necrosis of myocytes sets into motion a cascade of inflammatory signals leading to degradation of cellular debris and infarct healing and remodeling with fibrotic scar formation (Becker et al., 1999; Boyle and Weisman, 1993; Factor et al., 1987; Frangogiannis, 2006; Honan et al., 1990; Kim and Braunwald, 1993; Tyagi, 1997; Zhao et al., 1987). Adequate reparative fibrosis in the infarct is beneficial to preventing either adverse fibrotic remodeling or cardiac rupture (Hutchins and Bulkley, 1978; Jugdutt, 2003b; Weber et al., 1992; Weisman and Healy, 1987).

In the ischemic and reperfused myocardium, infiltrated fibroblasts produce most of the matrix macromolecules including collagen (Eghbali et al., 1988; Miller and Gay, 1987; Fan et al., 2012), and contribute to reactive fibrosis. However, α-smooth muscle actin (α-SMA)-expressing myofibroblast trans-differentiation mediates scar contraction (Dobaczewski et al., 2010; Eghbali et al., 1991; Marijianowski et al., 1997; Rhaleb et al., 2001; Sun and Weber, 2000). It has been

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demonstrated that transforming growth factor-β1 (TGF-β1) is a critical mediator of hyperoxia-induced remodeling in epithelial cells and fibroblasts (Corroyer et al., 1996; Jugdutt, 2003a). Under hyperoxia, cell-cycle inhibiting protein cyclin dependent kinase inhibitor p21 was up-regulated, resulting in the cessation of fibroblast proliferation and initiation of its trans-differentiation (Roy et al., 2007). However, the mechanisms responsible for TGF-β1 activation and myofibroblast trans-differentiation in the infarcted heart are poorly understood (Birdsall et al., 1997; Desmouliere et al., 1993; Frangogiannis, 2006; Frantz et al., 2008).

Ligand binding of TGF-β1 to its type-II and type-I receptors (TGFβRII and TGFβRI) leads to the phosphorylation and nuclear translocation of receptor-activated small mothers against decapentaplegic homolog (Smads), which modulate the transcription of a number of genes, including α smooth muscle actin (α-SMA) (Martin et al., 2007; Saura et al., 2005). Nitric oxide (NO) has been reported to induce the degradation of Smad2/3 resulting in the suppression of TGF-β1-induced signaling in endothelial cells (Lizarbe et al., 2008; Saura et al., 2005). NO has also been shown to increase the release of the active form of TGF-β1 in cultured myocytes (Mehta et al., 2002). However, the role of NO on ventricular infarct zone fibrosis and remodeling still remains undecided (Jugdutt, 2003b).

Recently, our laboratory has demonstrated that NO, superoxide, and their derivative peroxynitrite ($ONOO^-$) suppressed oxygen consumption leading to myocardial tissue hyperoxia (Xu et al., 2008; Zhao et al., 2005; Zhu et al., 2007). NO/$ONOO^-$-induced protein tyrosine nitration may be responsible for the reduced mitochondrial respiration (Liu et al., 2009). However, little is known about the role of tissue hyperoxia on myocardial healing and repair.

In the current study, an electron paramagnetic resonance (EPR) oximetry technique was employed to measure the in vivo myocardial tissue oxygenation (He et al., 2002). Enzyme-linked immunosorbent assay (ELISA), Western immunoblotting, and immunohistochemical staining were used to determine the expressions of TGF-β1, Smad2/3, p21 and α-SMA in the post-ischemic mouse heart. To determine the effect of post-ischemic hyperoxic stress on TGF-β1 signaling and the mechanistic role of ROS or NO/$ONOO^-$, mice were also treated with Carbogen, EUK134, and SNP. With these measurements, the regulation of cardiac TGF-β1 signaling by hyperoxia and NO/$ONOO^-$ was examined. These results may provide new insights in myofibroblast trans-differentiation in the healing myocardial infarct.

Materials and methods

Animals and chemicals

Male C57BL/6 wild-type (WT), endothelial and inducible NO synthase knockout ($eNOS^{-/-}$ and $iNOS^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed under a 12:12-h light–dark cycle and were provided with water and food ad libitum. All procedures were performed with the approval of the Institutional Animal Care and Use Committee at the Ohio State University, Columbus, Ohio, and conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. 10006329 EUK134 (EUK134, Cayman Chemical, Ann Arbor, MI) and sodium nitroprusside (SNP, Sigma-Aldrich, St. Louis, MO) solutions were prepared in phosphate buffered saline (PBS).

In vivo myocardial I/R model

In vivo myocardial I/R model was prepared similarly to the methods described previously (Xu et al., 2008; Zhao et al., 2005; Zhu et al., 2007). Briefly, mice were anesthetized with ketamine (55 mg/kg) and xylazine (15 mg/kg) through intraperitoneal (i.p.) injection. Atropine (0.05 mg subcutaneous (s.c.)) was administered to reduce airway secretions. Mice were orally intubated with polyethylene (PE)-90 tubing and connected to a mouse mini-ventilator (model 845; Harvard Apparatus) with a tidal volume of 250 μl and a respiratory rate of 120 breath/min. Isofluorane was used in all the experiments to maintain a stable anesthetic status. Core body temperature was maintained at 37 ± 0.5 °C with a thermo heating lamp and monitored with a rectal thermometer. After median thoracotomy, the left anterior descending coronary artery (LAD) was visualized and ligated for 30 min by tightening a 7–0 silk suture over a length of PE-10 tubing beneath the LAD at points 1–2 mm inferior to the left auricle. The suture was similarly placed in the sham group but without LAD occlusion. At the end of 30-min ischemia, the ligature was removed, and reperfusion was visually confirmed.

In vivo EPR oximetry

For the in vivo measurement of myocardial tissue oxygenation (Po_2), EPR oximetry was used as described previously (Xu et al., 2008; Zhao et al., 2005). Specifically, about 10 μg of lithium phthalocyanine (LiPc), an oxygen-sensitive probe, was loaded in a 27-gauge needle and implanted in the area at risk (AAR) after the heart was exposed. The location of the probe was confirmed by histology in the mid-myocardium. Then the mouse was transferred to an L-band EPR spectrometer (Magnettech GmbH, Germany). After 30 min equilibration of the probe with the surrounding tissue, EPR spectra were collected before and during the 30-min ischemia, and during the 60-min reperfusion. Then the chest was closed and the mouse was allowed to recover. Additional EPR oximetry was performed at days 1, 3, 5, 7, and 14 after reperfusion with the mouse re-anesthetized. The following are the EPR parameters: frequency, 1.1 GHz; microwave power, 16 mW; and modulation amplitude, 0.045 G. The sensitivity of the probe to oxygen is 5.8 mG/mm Hg.

ELISA and Western immunoblotting analyses

To measure protein expression levels, frozen tissue from the AAR was thawed, finely minced and homogenized. After centrifugation, the supernatant was collected. Protein concentration was determined with the bicinchoninic acid (BCA) kit (PIERCE). Total and active TGF-β1 was measured using a commercially available ELISA kit (Quantikine mouse/rat/canine TGF-β1, R&D Systems). For Western immunoblotting assay, tissue homogenate was boiled in the NuPAGE®LDS sample buffer (Invitrogen) at 70 °C for 10 min. Proteins of the homogenate were subjected to electrophoresis on NuPAGE® Novex 4–12% Bis-Tris gels (Invitrogen) and transferred onto nitrocellulose membranes (Amersham Biosciences). After blocking with 5% dry milk in Tween-20- and Tris-buffered saline (TTBS) for 1 h at room temperature, the membranes were incubated with rabbit anti-p-Smad2/3 (Ser 423/425) polyclonal antibody, rabbit anti-Smad2/3 polyclonal antibody, rabbit anti-p21 polyclonal antibody, goat anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibody (1:1000, Santa Cruz) and mouse anti-α-SMA monoclonal antibody (1:1000, Sigma). After incubation, the membranes were washed with TTBS and exposed to the antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Proteins were detected by use of chemiluminescence Western immunoblotting detection reagents (Amersham Biosciences). Densitometric analyses of the immunoblots were performed using an Alpha Imager 3300 system (Alpha Innotech, San Leandro, CA).

IP for 3-NT formation

Heart tissue from the AAR was collected and homogenized in RIPA buffer supplemented with protease inhibitor cocktail (1:40). The suspension was collected with centrifugation at 13,000 *g* for 10 min. Concentration of the sample was adjusted to 8 mg/ml, and 0.5 ml of the solution was incubated with anti-TGFβRII and anti-TGFβRI polyclonal antibodies (1:100; rabbit immunoglobulin G (IgG)). Then, 60 μl of protein A agarose beads was added. After centrifugation at