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Activation of JAK-STAT and Nitric Oxide Signaling as a Mechanism for Donor Heart Dysfunction

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Abstract

Background—Donor heart dysfunction (DHD) precluding procurement for transplantation occurs in up to 25% of brain dead (BD) donors. The molecular mechanisms of DHD remain unclear. We investigated the potential role of myocardial interleukin (IL)-6 signaling through the JAK2-STAT3 pathway which can lead to the generation of nitric oxide (NO) and decreased cardiac myocyte contractility.

Methods—Hearts were procured using standard technique with UW solution from 14 donors with a left ventricular (LV) ejection fraction of <35% (DHD). Ten hearts with normal function (NF) after BD served as controls. LV IL-6 was quantitated by ELISA and JAK2-STAT3 signaling was assessed by expression of phosphorylated STAT3. Inducible NO synthase (iNOS) and caspase-3 were measured by activity assays.

Results—Myocardial IL-6 expression was 8-fold greater in the DHD group vs. NF controls. Phosphorylated STAT3 expression was 5-fold higher in DHD vs. NF indicating increased JAK2-STAT3 signaling. LV activity of iNOS was 2.5-fold greater in DHD vs. NF. LV expression of the proapoptotic gene Bnip3 and caspase-3 activity were 3-fold greater in the DHD group vs. NF.

Conclusions—Myocardial IL-6 expression is significantly higher in the setting of DHD compared to hearts procured with normal function. This may lead to increased JAK2-STAT3 signaling and upregulation of iNOS which has been shown to decrease cardiac myocyte contractility. Increased NO production may also lead to increased apoptosis through upregulation of Bnip3 gene expression. Increased iNOS signaling may be an important mechanism of DHD and represents a novel therapeutic target to improve cardiac function after BD.

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Introduction

Cardiac transplantation remains the best long-term therapy for patients with end-stage heart failure. Despite the benefits of transplant for this patient population, this option continues to be extremely limited by the number of organ donors. In addition, up to 25% of potential cardiac donors are not utilized due to severe ventricular dysfunction in the absence of structural or ischemic heart disease, known as donor heart dysfunction (DHD). Several mechanisms for DHD have been investigated including ischemic injury [1], direct catecholamine-induced injury [2], impaired β -adrenergic receptor signaling [3], and the release of inflammatory mediators [4]. Recent work has demonstrated that myocardial mRNA levels of tumor necrosis factor (TNF)- α and interleukin-6 (IL-6) are elevated in DHD compared to brain dead donors with normal cardiac function [5]. TNF- α is known to depress ventricular function through myocardial TNFR1 and TNFR2 receptors [6]. The role of IL-6 in DHD remains unclear as myocardial protein levels of this cytokine have not been reported following brain death and the mechanism by which IL-6 may cause cardiac dysfunction in this setting has not been described. Apoptosis has also been shown to be present in the heart following BD as cleaved, or activated, caspases-9 and -3 are upregulated and may also contribute to DHD [7]. Recent work has shown that IL-6 decreases cardiac contractility via a nitric-oxide (NO)-dependent pathway [8,9]. In adult rat cardiac myocytes, the mechanism for this IL-6-mediated activation of iNOS and decrease in contractility was mediated by signaling through the JAK2-STAT3 pathway [10]. The primary objective of this study is to further investigate the role of inflammatory signaling in human donor heart dysfunction with a specific focus on the Janus-activated kinase (JAK)-signal transducer and activation of transcription (STAT) pathway which may be an important mechanism of impaired cardiac function following BD through the generation of nitric oxide (NO).

Methods

Study population

Patients with a clinical diagnosis of brain death and who had consented to organ donation underwent routine cardiac evaluation including echocardiography and cardiac catheterization in selected cases. Those with no evidence of structural heart disease and no prior cardiac medical history who had a left ventricular ejection fraction of less than 35% on serial echocardiography were included in this study. Standard techniques of myocardial preservation with University of Wisconsin (UW) solution and donor cardiectomy were utilized in this DHD group of patients. Right and left ventricular biopsies were snap frozen in liquid nitrogen following procurement. Patients with normal cardiac function where the heart was not procured for transplantation for non-cardiac factors served as controls. This study was approved by our local organ procurement organization and by the Institutional Review Board at the University of Cincinnati College of Medicine.

Myocardial Interleukin-6 Quantitation

Left ventricular tissue was homogenized in 10mM 3-[N-Morpholino]propanesulfonic acid (MOPS) buffer containing protease inhibitor cocktail (Complete Mini, EDTA-free protease inhibitors, Roche Diagnostics, Indianapolis, IN) with 200mg tissue/mL of buffer. Interleukin-6 was measured in the homogenates by enzyme-linked immunosorbent assay (ELISA) using a human IL-6 kit (BioSource International, Inc., Camarillo, CA).

JAK2/STAT3 signaling

Signaling through the JAK2-STAT3 pathway was assessed by protein immunoblotting for the activated, or phosphorylated, form of STAT3 (pSTAT3) as well as total STAT3. Whole tissue extracts were prepared from left ventricular biopsies by homogenization in ice-cold lysis buffer

(25 mmol/L Tris-HCl [pH7.5], 5 mmol/L EDTA, 5 mmol/L EGTA, 10 µg/mL leupeptin, 20 µg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). The immunodetection of myocardial levels of STAT3 and pSTAT3 was performed using a polyclonal antibody for each molecule (Cell Signaling Technology). Equal amounts of protein (50 µg) were electrophoresed through 10% Tris/glycine gels and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dried milk in 0.1% Tween 20 in PBS (PBS-T) for 1 hour at room temperature. The protein was visualized using a horseradish peroxidase-linked secondary antibody and ECL detection (Amersham). Expression of GAPDH was utilized as an internal control for equal protein loading in all immunoblotting experiments.

Expression of iNOS and BNIP3

Frozen tissue (100–120 mg) was homogenized in 2 ml of lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Na deoxycholate, 10 mmol/L NaF, 0.1% SDS, 1 mmol/L PMSF and Complete Protease Inhibitor Cocktail (Roche Biochemicals)). The lysates were placed on ice for 30 minutes followed by centrifugation at 42,000g at 4°C to remove cellular debris. The supernatants were collected and stored at –20°C or used immediately. Protein (100 µg) was separated on 12% polyacrylamide-SDS gels and transferred onto nitrocellulose membranes (Invitrogen, Carlsbad, CA). After blocking with TBS-Tween/5% skim milk, the membranes were incubated overnight at 4°C with primary antibodies against iNOS (1:1000 dilution) or BNIP-3 (1:1000 dilution, Abcam, Cambridge, MA) followed by secondary antibodies conjugated with horseradish peroxidase (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. Signals were detected with ECL (Amersham).

Nitric Oxide (NO) Production

NO production was assessed by measuring total nitrate and nitrite (NO_x) in myocardial tissue extracts. Whole tissue extracts were prepared from left ventricular biopsies by homogenization in ice-cold lysis buffer (25 mmol/L Tris-HCl [pH7.5], 5 mmol/L EDTA, 5 mmol/L EGTA, 10 µg/mL leupeptin, 20 µg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride). Protein concentration was measured for each sample and equal protein content was utilized for each sample. The concentration of NO_x in the tissue extracts was determined using a colorimetric assay kit with a detection limit of 2.5 µM (Cayman Co., Ann Arbor, MI). Nitrate/nitrite concentrations were averaged from triplicate measurements and reported as nmol NO_x per mg tissue protein.

Caspase Activity

Caspase-3 activity in myocardial tissue was measured using a Caspase-Glo assay kit (Promega) and modified protocol. Briefly, the proluminescent substrate is cleaved by caspase-3. After Caspase cleavage, a substrate for luciferase (aminoluciferin) is released; this results in the luciferase reaction and the production of luminescent signal. Cytosolic extracts from ventricular tissue were prepared by homogenization in hypotonic extraction buffer (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, 1 mM Pefablock, and 1 µg/ml each pepstatin, leupeptin, and aprotinin) and subsequently centrifuged (15 minutes, 13,000 rpm, 4°C). The protein concentration of supernatant was adjusted to 1 mg/ml with extraction buffer and stored at –80°C. An equal volume of reagents and 10 mg/ml cytosolic protein were added to a white-walled 96-well plate and incubated at room temperature for 1 hour. The luminescence of each sample was measured in a plate-reading luminometer.

Statistical analysis

Data are expressed as mean ± SEM. Analyses were conducted using Statview 4.01 software (Abacus Concepts Inc., Berkeley, CA). Experimental groups were compared using Student's t

test or 1-way ANOVA, as appropriate. *P* values of less than 0.05 were considered statistically significant.

Results

Patient demographics are summarized in Table 1. Ten patients had normal right and left ventricular function by transthoracic echocardiography which was performed after consent was obtained from family for organ donation. These hearts were not utilized for transplant for non-cardiac reasons and served as controls with normal function (NF). The mean duration to procurement following the diagnosis of brain death (BD) was 18.3 ± 3.5 hours. In the study group, fourteen patients had severely depressed global ventricular function following BD with a left ventricular ejection fraction of $26.5 \pm 3.4\%$ by echocardiography. The time from diagnosis to procurement was not different between groups. At the time of multi-organ procurement, the donor cardiectomies were performed using identical surgical technique as for hearts which would have been procured for transplantation. There was no significant difference between groups in age, sex, and etiology of BD (Table 1). None of the donors in the DHD group suffered a cardiac arrest prior to organ procurement.

Previous studies have shown that myocardial IL-6 mRNA levels are significantly higher (2.4-fold) in donor hearts with severe ventricular dysfunction compared to those with normal function (NF) [5]. Protein levels of IL-6 in the heart following BD have not been previously reported. There was a significantly higher (8-fold) myocardial level of IL-6 protein in the DHD group compared to the NF group [76.5 ± 4.1 versus 9.5 ± 1.4 pg/mg protein] as measured by ELISA (Figure 1). This marked difference in myocardial IL-6 levels is not primarily due to BD as both groups had a clinical diagnosis of BD and the timing from BD to procurement was not different between groups. These data demonstrate that elevated levels of IL-6 in the heart may play a role in donor heart dysfunction.

IL-6 has been shown to activate the JAK2-STAT3 pathway through the IL-6 receptor and gp130, a signal transducing receptor, in adult ventricular myocytes. This leads to a decrease in ventricular myocyte contractility which is mediated by JAK2-STAT3 signal transduction [10]. We studied activation of the JAK2-STAT3 signaling pathway by measuring left ventricular protein expression of activated, or phosphorylated, STAT3 (pSTAT3). Phosphorylated STAT3 expression in the left ventricle was elevated 3-fold in the nuclear fraction in the DHD group compared to the NF group (Figure 2). Total myocardial STAT3 expression was not different between groups (data not shown) indicating that the increase in nuclear pSTAT3 in the DHD group is due to phosphorylation and translocation of STAT3 from the cytoplasmic fraction to the nuclear fraction.

Recent studies have shown that IL-6 decreases cardiac contractility via a nitric oxide (NO)-dependent pathway [9]. We studied left ventricular inducible NO synthase (iNOS) expression and activity in the NF and DHD groups as IL-6 has been shown to induce iNOS expression via JAK2-STAT3. Myocardial iNOS expression was significantly upregulated by nearly 2-fold in the DHD group as compared to the NF group (Figure 3). More importantly, NO production, as measured directly, was 2.5-fold greater in the DHD group compared to NF controls [259.2 ± 24.3 versus 101.4 ± 11.1 nmol NO/mg protein, $P < 0.01$] (Figure 4). These data indicate that donor heart dysfunction is associated with increased iNOS expression and activity which can lead to a decrease in cardiac myocyte contractility. This may be due to the increased JAK-STAT signaling that was present in the DHD group.

Apoptosis may play an important role in DHD after brain death. We investigated the expression of myocardial BNIP3, a proapoptotic gene, and caspase-3 activity, which is involved in the terminal part of the apoptotic pathway. Left ventricular expression of BNIP3 was 3-fold greater

in the DHD group compared to NF (Figure 5). Caspase-3 activity was also significantly greater in the DHD group compared to NF controls as measured by luminescence [743.2 ± 92.3 versus 235.0 ± 31.4 relative light units, $P < 0.005$]. These data confirm that there is a greater degree of apoptosis in the setting of DHD relative to brain death with preserved cardiac function and that apoptosis may be an important contributing mechanism of impaired cardiac function after BD.

Discussion

Donor heart dysfunction (DHD) precluding procurement for transplant in the absence of structural heart disease occurs in up to 25% of potential donors, however, the mechanisms of DHD remain poorly understood [11,12]. Activation of myocardial inflammatory pathways has been shown to be present following brain death (BD) and particularly in the setting of DHD. Myocardial tumor necrosis factor (TNF)- α mRNA and protein expression is greater in donor hearts with poor function (DHD) compared to those utilized for transplant [5]. Myocardial IL-6 mRNA was also upregulated in donor hearts with severe ventricular dysfunction [5], however, the mechanism by which IL-6-mediated signaling may depress cardiac function in this setting is unclear.

Proteins of the IL-6 family bind to receptors in the plasma membrane. Subsequent signal transduction involves activation of the janus kinase (JAK) and signal transducer and activator of transcription (STAT) proteins. STAT proteins are translocated into the nucleus, where they bind to the promoter region of target genes and regulate transcription [13]. All seven STAT isoforms have been identified in cardiac myocytes, fibroblasts, endothelial cells, and smooth muscle cells [14,15,16]. Studies analyzing the role of STAT proteins in cardiac function have focused on STAT3 as the JAK-STAT pathway is involved in ischemia/reperfusion injury and hypertrophy. In acute processes, non-transcriptional effects of the JAK-STAT proteins such as posttranslational modifications of downstream proteins are likely to be more significant than regulating gene transcription [17].

In vitro studies have shown that IL-6 acutely decreases cardiac contractility via a nitric oxide (NO)-dependent pathway [8,9]. IL-6 has also been shown to increase expression of inducible NO synthase (iNOS) as the mechanism for enhanced NO production [10]. Recent studies in adult cardiac myocytes have shown that activation of IL-6 receptors leads to downstream activation of the JAK-STAT pathway, specifically the JAK2-STAT3 isoforms, which mediates the NO-associated decrease in contractility [10]. Significant increases in serum levels of IL-6 and its mRNA and protein expression in the myocardium have been reported in patients with congestive heart failure [18,19], myocarditis [20], myocardial infarction [21,22], and injury associated with ischemia/reperfusion [23] and cardiopulmonary bypass [24]. Thus, it has been hypothesized that IL-6 plays an important role in the pathophysiology of these cardiac disorders.

Our primary aim in this study was to determine the potential role of IL-6 and JAK-STAT signaling in DHD and to delineate a potential mechanism by which this cytokine may contribute to cardiac dysfunction after BD. There was a nearly 8-fold increase in left ventricular IL-6 protein expression in the DHD group compared to NF control hearts. We found a significant increase in STAT3 activation in the DHD group compared to normally functioning control hearts after brain death. Similar to the previous in vitro studies in adult cardiac myocytes, this led to a significant increase in myocardial iNOS expression and activity in the DHD group relative to the NF hearts. These data indicate that DHD may be mediated, at least in part, by enhanced JAK2-STAT3 signaling and increased iNOS expression, resulting in increased NO production and decreased cardiac contractility. It remains unclear why this DHD subgroup of patients have such high levels of inflammatory signaling via IL-6 or other cytokines in the heart following BD as it does not appear to be a result of BD alone.

Previous studies of human DHD by Yacoub and colleagues have demonstrated that apoptosis may contribute to DHD as myocardial expression of activated caspase-9 and caspase-3 was significantly greater in donor hearts not utilized for transplant due to severe ventricular dysfunction [7]. NO has recently been shown to increase expression of the proapoptotic gene BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein 3) in macrophages, leading to death of these cells in vitro [25]. Although this has not been studied specifically in cardiac myocytes in vitro, we found a significant upregulation in myocardial expression of BNIP3 in the DHD group which may be mediated by increased NO synthesis. Thus, increased intracellular NO may also be an important mechanism of apoptosis which could contribute to decreased cardiac function in patients with DHD.

We have demonstrated for the first time that activation of JAK2-STAT3 signaling, potentially by IL-6, leading to induction of iNOS and NO synthesis may be an important mechanism of human DHD. In addition, NO may also be contributing to apoptotic signaling through increased expression of BNIP3 in these patients with severe ventricular dysfunction following BD. Targeted inhibition of STAT3 activation or NO production following BD may represent a novel strategy to improve or maintain donor cardiac function and may also decrease the incidence of graft failure following transplant which is a major cause of post-transplant mortality. Further study is necessary to determine why myocardial levels of IL-6, STAT3 activation, iNOS induction, and apoptosis are significantly greater in a subset of patients following brain death. Interestingly, a common single nucleotide polymorphism (G/C) in the IL-6 gene promoter at position -174 has been reported to influence IL-6 expression, with the G allele being associated with higher expression levels in response to stimulation [26]. The IL-6-174 GG genotype is associated with greater IL-6 plasma levels after coronary artery bypass grafting and a significantly greater incidence of peri-operative death, myocardial infarction, and stroke after surgical coronary revascularization relative to the IL-6-174 GC genotype [27]. Novel approaches to increase the number of cardiac donors after BD are critical to providing heart transplantation to an increasing population of patients with advanced heart failure.

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Figure 1. Left ventricular IL-6 expression after brain death in normal function (NF) and donor heart dysfunction (DHD) groups. * $P<0.02$ versus NF; N=10 in NF and 14 in DHD.

Figure 2. Myocardial phosphorylated STAT3 expression after brain death in normal function (NF) and donor heart dysfunction (DHD) groups. * $P < 0.01$ versus NF; N=10 in NF and 14 in DHD.

Figure 3. Myocardial iNOS expression after brain death in normal function (NF) and donor heart dysfunction (DHD) groups. * $P < 0.03$ versus NF; $N = 10$ in NF and 14 in DHD.

Figure 4. Left ventricular NO production in NF and DHD groups. * $P < 0.001$ versus NF; $N = 10$ in NF and 14 in DHD.

Figure 5. Myocardial expression of the proapoptotic gene Bnip3 after brain death. NF;normal function (N=10), DHD;donor heart dysfunction (N=14). * $P<0.04$ versus NF.

Figure 6. Left ventricular caspase-3 activity after brain death

NF;normal function (N=10), DHD;donor heart dysfunction (N=14). * $P<0.01$ versus NF. RLU;relative light units.

TABLE 1

Clinical profile for patients in this study

	NF (N=10)	DHD (N=14)	P value
Age (years)	44.7±13.2	29.8±5.1	NS
Sex (M/F)	6/4	8/5	NS
Mechanism	5 GSW 5 SAH	7 GSW 6 SAH	NS
LV EF	64.3±5.3%	26.5±3.4%	<0.01
Drugs	T ₄ , PE	T ₄ , PE	NS

GSW: Gun shot wound to the head, SAH: sub-arachnoid hemorrhage

T₄: thyroxine, PE: phenylephrineNS: not significant, $P>0.05$