

# Dual blockade of endothelin action exacerbates up-regulated VEGF angiogenic signaling in the heart of lipopolysaccharide-induced endotoxemic rat model

著者別名	荒井 サブリナ, 下條 信威, 河野 了, 宮内 卓, 水谷 太郎
journal or publication title	Life sciences
volume	118
number	2
page range	364-369
year	2014-11
権利	(C) 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license ( <a href="http://creativecommons.org/licenses/by-nc-nd/.0/">http://creativecommons.org/licenses/by-nc-nd/.0/</a> ).
URL	<a href="http://hdl.handle.net/2241/00123595">http://hdl.handle.net/2241/00123595</a>

doi: 10.1016/j.lfs.2014.02.008



## Dual blockade of endothelin action exacerbates up-regulated VEGF angiogenic signaling in the heart of lipopolysaccharide-induced endotoxemic rat model



Masami Oki<sup>a</sup>, Subrina Jesmin<sup>a</sup>, Md. Majedul Islam<sup>a</sup>, Chishimba Nathan Mowa<sup>b</sup>, Tanzila Khatun<sup>a</sup>, Nobutake Shimojo<sup>a</sup>, Hideaki Sakuramoto<sup>a</sup>, Junko Kamiyama<sup>a</sup>, Satoru Kawano<sup>a</sup>, Takashi Miyautchi<sup>c</sup>, Taro Mizutani<sup>a,1</sup>

<sup>a</sup> Department of Emergency and Critical Care Medicine, Faculty of Medicine, University of Tsukuba, Tsukuba, Ibaraki, Japan

<sup>b</sup> Department of Biology, Appalachian State University, Boone, NC, USA

<sup>c</sup> Centre for Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, Tsukuba, Ibaraki, Japan

### ARTICLE INFO

#### Article history:

Received 1 November 2013

Accepted 6 February 2014

Available online 16 February 2014

#### Keywords:

Endotoxemic heart

Lipopolysaccharide

Vascular endothelial growth factor

Endothelin receptor antagonist (SB-209670)

### ABSTRACT

**Aims:** Sepsis is a cluster of heterogeneous syndromes associated with progressive endotoxemic developments, ultimately leading to damage of multiple organs, including the heart. However, the pathogenesis of sepsis-induced myocardial dysfunction is still not fully understood. The present study is the first to examine alterations in expression of key angiogenic signaling system mediated by vascular endothelial growth factor (VEGF) in septic heart and the effects of endothelin dual blocker (ETDB) on it.

**Main methods:** Normal Wistar rats were either administered with: a) vehicle only (control group), b) lipopolysaccharide only (LPS: 15 mg/kg) and then sacrificed at different time points (1 h, 3 h, 6 h and 10 h), and c) the last group was co-administered with LPS and ETDB (SB-209670, 1 mg/kg body weight) for 6 h and then sacrificed.

**Key findings:** Administration of LPS resulted in increases in levels of: a) serum tumor necrosis factor (TNF)- $\alpha$ , b) serum VEGF and c) serum endothelin (ET)-1 levels accompanied by up-regulation of cardiac VEGF and its downstream angiogenic signaling molecules. While cardiac TNF- $\alpha$  level was unchanged among experimental groups, cardiac ET-1 level was significantly higher in LPS-administered group.

**Significance:** We conclude that elevation in VEGF angiogenic signaling may be triggered by diminished oxygenation in the myocardium following LPS administration as a consequence of sepsis-induced microvascular dysfunction. Because of this cardiac dysfunction, oxygen supply may be inadequate at microregional level to support the normal heart metabolism and function. ETDB at 6 h further increased the elevated levels of VEGF angiogenic signaling in endotoxemic heart.

© 2014 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>).

### Introduction

Sepsis is a complex syndrome characterized by an imbalance between pro- and anti-inflammatory responses and the development of progressive damage in multiple organs that ultimately leads to organ failure (Hotchkiss and Karl, 2003; Vandijck et al., 2006). The systemic inflammatory response maybe initiated by entry of bacterial lipopolysaccharide (LPS) or other microbial components into the lymphatic and circulatory systems. Once the sepsis cascade is triggered, a systemic inflammatory response will ensue and, if unregulated, will

lead to multiple organ failure, which is associated with a high mortality rate in humans. Clinically, this condition is characterized by liver, pulmonary, cardiovascular, renal and gastrointestinal dysfunctions (Bone et al., 1997; Wheeler and Bernard, 1999).

Cardiac diastolic and myocardial dysfunctions commonly occur in patients with severe sepsis. Early diagnosis and aggressive supportive therapy are critical in preventing mortality, which is high in patients with septic shock (Annane et al., 2005). Key cardiovascular changes during septic shock include cardiovascular collapse and peripheral vascular dysfunction, which can result in heterogeneous microcirculatory flow and can frequently induce myocardial depression. Cardiovascular collapse can increase the risk of death in sepsis by as much as two fold, and myocardial depression occurs in almost 40% of septic patients. To date, the pathogenesis of sepsis-induced myocardial dysfunction is still not fully understood.

<sup>1</sup> Address for requests for reprints: Dept. of Emergency and Critical Care Medicine, Faculty of Medicine University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan. Tel.: +81 29 853 3210, 3081; fax: +81 29 853 3092.

Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen that is important in neovascularization under both physiological and pathophysiological conditions, plays a crucial role in blood vessel formation during development and also in the regulation of hypoxia-induced tissue angiogenesis (Banai et al., 1994a; Ferrara and Davis-Smyth, 1997). VEGF possibly exerts these biological processes through three mechanisms of action, namely by: (1) increasing blood flow to the tissue via vasodilation, (2) reducing the distance between the cells in the tissue and the nearest blood vessel by stimulating angiogenesis and (3) increasing the permeability of the blood vessels to plasma, small solutes and macromolecules. VEGF is a unique molecule in that is up-regulated in all known endogenous physiological and pathological forms of angiogenesis, can stimulate angiogenesis directly (Ferrara and Bunting, 1996), is a potent vasodilator (Ku et al., 1993) and is able to increase vascular permeability (Bates and Curry, 1996). In the normal heart, the growth of new blood vessels is a rare occurrence, however, chronic ischemia may stimulate VEGF synthesis resulting in angiogenesis and coronary collateral formation (Banai et al., 1994b; Sabri et al., 1991).

Endothelin (ET)-1, the most potent vasoconstrictor peptide known to date (Mitaka et al., 1993; Yanagisawa et al., 1988) has been shown to be significantly higher in plasma of septic patients (Battistini et al., 1996) and a clear correlation exists among ET plasma levels, morbidity and mortality in septic patients, a fact that suggests involvement of ET in human septic shock (Pittet et al., 1991; Weitzberg et al., 1991). Further, ET has been suggested to contribute to dysfunction of several vital organ systems in septic shock.

In the present study, we intended to investigate whether LPS administration in rat causes any changes in myocardial VEGF system expression and whether blockade of ET could have any effect on altered VEGF system in the heart in sepsis.

## Materials and methods

### Animal preparation

Male Wistar rats (200–250 g, 8 weeks old) were used in all experiments described in the present study. Endotoxemia was induced by administering bacterial LPS (*Escherichia coli* O55: B5) (15 mg/kg in sterile saline) intra-peritoneally (IP), a dose that was sufficient to induce heart injury, as well as trigger an inflammatory cytokine response. The control group (n = 26) received an equal volume of vehicle (sterile saline; 2 ml/body weight), without LPS. And the rats received rehydration therapy during the endotoxemia induction.

These animals were killed by Nembutal (sodium pentobarbital, IP, 80 mg/kg/body weight) at specific time points after treatment (LPS or vehicle only), namely, 1, 3, 6, and 10 h post-treatments (n = 22 for each time point). The blood samples were collected by cardiac puncture for blood gas analysis, and heart tissues were harvested with care, snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . All animal care procedures were in compliance with the institutional guidelines, and the experiments performed were approved by the Ethics Committee of the Animal Resource Centre of the University of Tsukuba. In the second part of the study, we investigated whether ET plays a specific role in the pathogenesis of endotoxin using a LPS-induced endotoxemic rat model administered with the dual ET antagonist (SB-209670) (GlaxoSmithKline plc, Great West Road, Brentford, Middlesex, TW8 9GS, United Kingdom). SB-209670 [(+)-(1S, 2R, 3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid] is a non-peptide ET receptor antagonist which was rationally designed using conformational models of a natural ligand, ET-1. SB-209670 is one of the most potent ET receptor antagonist identified to date and has been used to elucidate a role for ET as a mediator of cardiovascular pathophysiology (Ohlstein et al., 1994). Prior to treatments, rats were anesthetized with urethane [35% ethyl carbamate (Wako Pure Chemical Industries, Osaka, Japan) + 4% alpha-chloralose (Wako) saline wt./vol., 0.4 to 0.8 g/kg, IP], and the left jugular vein was

cannulated for drug (LPS, dual ET antagonist) administration. All drugs were administered intravenously as a slow bolus injection. Based on our previous and pilot studies, the LPS-only treated group (15 mg/kg, intravenous) was killed 6 h after treatment.

Lastly, the dual ET blocker group was first treated with the blocker (SB-209670) followed by LPS 15 min after the dual ET blocker was administered intravenously (15 mg/kg in saline). After LPS administration, dual ET blocker was continuously infused (1 mg/kg/h) through the jugular vein with a pump for 6 h (n = 18) for each group. Vehicle only-treated rats were used as a control.

### Measurements of hemodynamic parameters

The rats were anesthetized with Nembutal (40 mg/kg/body weight, IP) and a microtip pressure transducer catheter (SPC-320, Millar Instruments, Houston, TX, USA) was inserted into the left carotid artery, as described in our previous study (Jesmin et al., 2007; Sakai et al., 1996). Then arterial blood pressure and heart rate were monitored with a pressure transducer (model SCK-590, Gould, Ohio, USA) and recorded with the use of a polygraph system (amplifier, AP-601 G, Nihon Kohden, Tokyo, Japan; Tachometer, AT-601 G, Nihon Kohden; and thermal-pen recorder, WT-687 G, Nihon Kohden).

### Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA), a sensitive technique for determining tissue protein concentration, was used to determine levels of VEGF, endothelial nitric oxide synthase (eNOS) and tumor necrosis factor (TNF)- $\alpha$  (R&D Systems, MN, USA) in heart tissues and in serum.

### Enzyme-linked immunosorbent assay for plasma and cardiac ET-1 levels

Concentration of ET-1 in plasma and heart tissue extracts was determined using a Quantikine ET-1 Enzyme Immuno Assay Kit (R&D Systems, MN, USA), according to the manufacturer's protocol. A 4.5 h solid phase ELISA was used, and contained synthetic ET-1 and antibodies raised against synthetic ET-1. This immunoassay has been shown to accurately quantitate synthetic and naturally occurring ET-1. A monoclonal antibody specific for ET-1 was pre-coated onto a microplate. Standards and samples were pipetted into the wells and if present, ET-1 antigen was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific to ET-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and a color developed in proportion to the amount of ET-1 bound in the initial step. The color development was then stopped and its intensity measured. The ET-1 concentration of each sample was calculated with a standard curve constructed by plotting the absorbance of each standard solution.

### Nitric oxide colorimetric assay

Nitric oxide (NO) was indirectly detected in cardiac tissue extracts as nitrite using a NO Colorimetric Assay kit (Roche Diagnostics, Mannheim, Germany). In this method, the nitrate present in the sample was reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase. The nitrite formed reacted with sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride to give a red-violet diazo dye. The diazo dye was measured at 550 nm, on the basis of its absorbance within the visible range.

### Statistical analysis

The results were expressed as mean  $\pm$  SE, and the means were compared by a one-way factorial analysis of variance, followed by Tukey's or Bonferroni for multiple comparisons. For nonparametric

statistical analysis, the Kruskal–Wallis test was performed. Differences were considered significant at  $p < 0.05$ . The statistical analysis and calculations were performed using the SPSS 21.0 software package (SPSS, Inc., Chicago, IL, USA).

## Results

### Blood gas and hemodynamic parameters (Table 1)

Arterial PaO<sub>2</sub> was found to be significantly ( $p < 0.041$ ) decreased in LPS administered rats. However, arterial PaO<sub>2</sub> increased remarkably ( $p < 0.027$ ) following SB-209670 injection in endotoxemic rats. Blood lactate concentrations were increased dramatically after LPS ( $p < 0.035$ ) was given and increased ( $p < 0.027$ ) further after SB-90670 treatment in septic rats. Base excess was markedly ( $p < 0.042$ ) lowered in LPS administered group compared to control group. Both the systolic and diastolic blood pressures significantly decreased ( $p < 0.002$ ) in LPS administered rats at 6 h compared to the control rats. Heart rate was significantly higher ( $p < 0.025$ ) in LPS administered rats compared to the control rats and treatment with SB-209670 had no significant effect on this elevated heart rate in endotoxemic rats.

### Expression of TNF- $\alpha$ , VEGF and ET-1 in serum and plasma

Serum TNF- $\alpha$  level in rat administered with LPS was significantly ( $p < 0.001$ ) elevated compared to the control group and decreased sharply ( $p < 0.013$ ) following administration of dual ET blocker (Fig. 1A). Consistent with our previous studies, serum VEGF levels in the present study increased ( $p < 0.008$ ) after LPS administration in the endotoxemic rat model and was unchanged following the treatment of the ET blocker (Fig. 1B). The plasma level of ET-1 (Fig. 1C) was significantly higher ( $p < 0.003$ ) in LPS administered group compared to the control group and was further up-regulated with the blockade of ET receptors.

### Expression of cardiac VEGF, eNOS and NO levels

VEGF and eNOS/NO-related pathway promotes angiogenesis via activation of an angiogenic signaling cascade. VEGF, which is considered the most potent angiogenic growth factor for the heart tissue, appeared to be significantly elevated ( $p < 0.004$ ) in the heart of the LPS-administered endotoxemic rat, and was (VEGF) further elevated ( $p < 0.025$ ) after animals were treated with ET blocker (Fig. 2A). Consistent with VEGF expression, levels of cardiac eNOS (Fig. 2B) were found to be higher ( $p < 0.004$ ) in LPS-administrated rats compared to control group and increased further after treatment with ET blocker, as demonstrated by ELISA analysis. Although concentration

**Table 1**  
Effects of LPS on arterial blood gas and hemodynamic parameters.

	Control	LPS	LPS + SB-209670
pH	7.45 $\pm$ 0.02	7.49 $\pm$ 0.02	7.44 $\pm$ 0.02
PaCO <sub>2</sub> (mm Hg)	34.1 $\pm$ 3.4	26.4 $\pm$ 1.3	28.3 $\pm$ 4.5
PaO <sub>2</sub> (mm Hg)	100.9 $\pm$ 3.5	87.2 $\pm$ 3.7*	105.4 $\pm$ 4.1#
HCO <sub>3</sub> <sup>-</sup> (mmol/l)	23.1 $\pm$ 1.46	20.0 $\pm$ 0.89	19.3 $\pm$ 2.77
Base excess (mmol/l)	-0.2 $\pm$ 1.1	-3.7 $\pm$ 1.0*	-5.1 $\pm$ 2.8
Lactate (mmol/l)	0.6 $\pm$ 0.2	1.7 $\pm$ 0.2*	1.9 $\pm$ 0.5*
Systolic BP (mm Hg)	128 $\pm$ 5.75	89 $\pm$ 4.43**	91 $\pm$ 4.60**
Diastolic BP (mm Hg)	97 $\pm$ 6.28	75 $\pm$ 3.13*	73 $\pm$ 2.03**
HR (bpm)	432 $\pm$ 17.5	492 $\pm$ 8.56**	470 $\pm$ 7.75

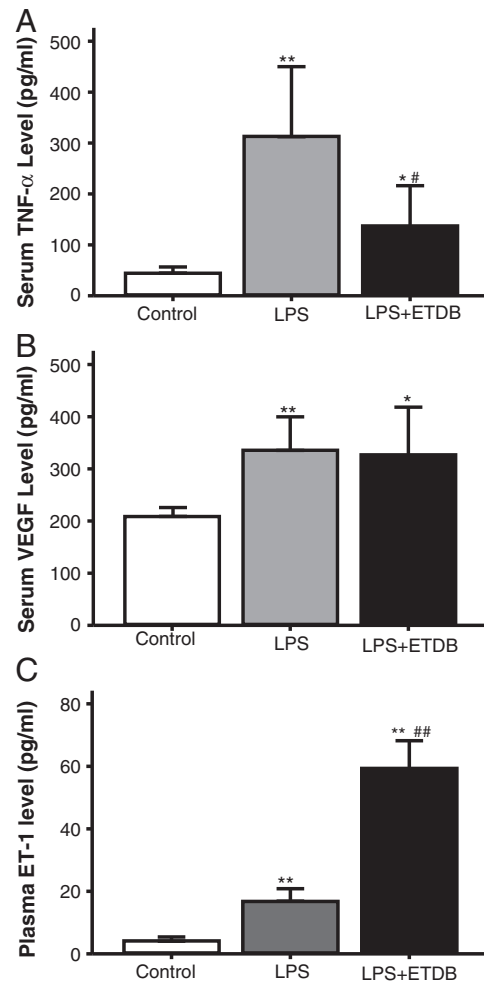
Abbreviations: systolic BP, systolic blood pressure; diastolic BP, diastolic blood pressure; HR, heart rate; LPS, lipopolysaccharide.

Data are mean  $\pm$  SE.

\*  $p < 0.05$  vs. control.

\*\*  $p < 0.01$  vs. control.

#  $p < 0.05$  vs. LPS.



**Fig. 1.** Expression levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; A), vascular endothelial growth factor (VEGF; B) and endothelin-1 (ET-1; C) in serum or plasma from control rats, lipopolysaccharide-administered (LPS) rats and LPS-administered rats treated with dual endothelin blocker (LPS + ETDB). White bars, control subjects; gray bars, LPS-administered rats; black bars, LPS + ETDB treated rats. Values are mean  $\pm$  SE. \* $p < 0.05$  vs. control; \*\* $p < 0.01$  vs. control; # $p < 0.05$  vs. LPS; ## $p < 0.01$  vs. LPS.

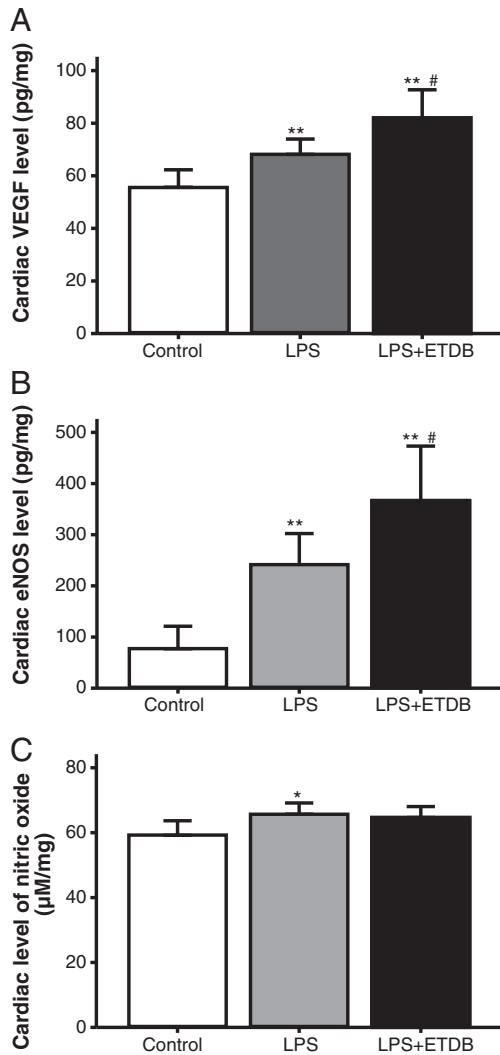
of NO, which is the final downstream molecule of VEGF angiogenic signaling, was significantly increased ( $p < 0.046$ ) in the heart of the endotoxemic rat, ET blocker failed to further induce increase in the concentration of cardiac NO in LPS-treated group, implying the presence of a non-functional and disrupted VEGF angiogenic signaling in ET blocker-treated heart (Fig. 2C).

### Expression of cardiac TNF- $\alpha$ and ET-1 levels

In all the three experimental groups, cardiac levels of TNF- $\alpha$  level were statistically found to be similar (Fig. 3A). However, levels of cardiac ET-1 were found to be significantly up-regulated ( $p < 0.008$ ) in LPS-administered rats and were further up-regulated ( $p < 0.005$ ) following treatment with ET blocker (Fig. 3B).

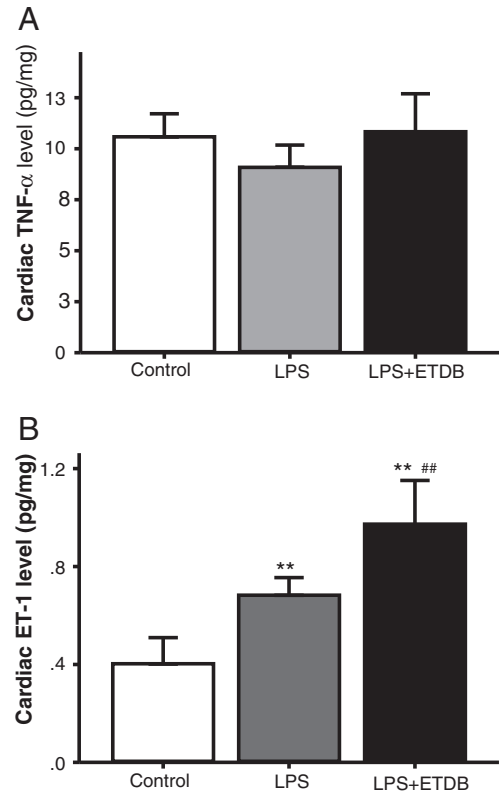
## Discussion

The key findings of the present study are that: 1) the components of VEGF angiogenic signaling are significantly up-regulated in the heart at 6 h after LPS administration; and 2) dual blockade of ET action for 6 h exacerbates components of the up-regulated VEGF angiogenesis signaling system in cardiac tissues of the endotoxemic rats.



**Fig. 2.** Expression levels of components of vascular endothelial growth factor (VEGF) angiogenic signaling pathway in cardiac tissues as demonstrated by ELISA: protein expression levels of VEGF (A), endothelial nitric oxide synthase (eNOS) (B) and nitric oxide (NO) (C) in heart tissues obtained from control rats, lipopolysaccharide-administered (LPS) rats and LPS-administered rats treated with dual endothelin blocker (LPS + ETDB). White bars, control subjects; gray bars, LPS-administered rats; black bars, LPS + ETDB treated rats. Values are mean  $\pm$  SE. \* $p < 0.05$  vs. control; \*\* $p < 0.01$  vs. control; # $p < 0.05$  vs. LPS.

Inadequate oxygen supply is probably the most important pathophysiological mechanism that leads to myocardial dysfunction (Jurgensen et al., 2004). It (low oxygen concentration) may also play an important role in sepsis pathophysiology and subsequent decrease in heart function. Specifically, progressive hypoxia reduces left ventricle contractility (Walley et al., 1988). Although direct evidence of tissue hypoxia within the septic heart has been lacking (Avontuur et al., 1995; Herbertson et al., 1995; Hotchkiss et al., 1991) evidence showing diminished blood flow via autoregulation (Avontuur et al., 1997), as well as loss of myocardial capillary density (Barroso-Aranda et al., 1991), suggests disruption of capillary blood flow (Ellis et al., 2002) in the septic heart and overall compromise of microvascular oxygen transport (Bateman et al., 2003). Because of sepsis-induced microvascular dysfunction, oxygen supply may be inadequate to support normal heart metabolism and function. Physical stress, ischemia or infection induces a remodeling process of the cardiovascular system through activation of cardiac myocytes, fibroblasts, endothelial cells, and smooth muscle cells. Locally expressed growth factors including platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and VEGF are known to play key roles in these processes. It is very likely that the heart of the endotoxemic rat



**Fig. 3.** Expression levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and endothelin-1 (ET-1) in the cardiac tissues, as revealed by ELISA: protein expression levels of TNF- $\alpha$  (A) and ET-1 (B) in heart tissues obtained from control rats, lipopolysaccharide-administered (LPS) rats and LPS-administered rats treated with dual endothelin blocker (LPS + ETDB). White bars, control subjects; gray bars, LPS-administered rats; black bars, LPS + ETDB treated rats. Values are mean  $\pm$  SE. \*\* $p < 0.01$  vs. control; ## $p < 0.01$  vs. LPS.

in the present study might have been hypoxic, which in turn could have enhanced the expression of VEGF in the cardiac tissues following LPS administration. Such a speculation is consistent with data from a recent study that demonstrated that HIF-1 $\alpha$  expression is up-regulated in a heart of an endotoxemic rat, 5 h post-LPS administration (Bateman et al., 2007). Another important downstream molecule of VEGF angiogenic signaling that was found to be up-regulated in the present study, following LPS administration, was eNOS. At the local level, the microcirculation regulates and distributes red blood cells and oxygen throughout the tissue to maintain tissue oxygen concentration (Bateman et al., 2003). However, despite normal or enhanced cardiac output during sepsis, distribution and regulation of local tissue oxygen delivery are compromised by decreased functional capillary density (Bateman et al., 2001; Boczkowski et al., 1992; Goldman et al., 2004; Lam et al., 1994) and diminished microvascular vasoconstriction (McKinnon et al., 2006). Although we show here that levels of cardiac NO, the final downstream molecule of VEGF angiogenic signaling, increase after LPS administration, for now we are unclear on the source of the up-regulated NO, i.e., whether it is from eNOS or iNOS, or the state of its biological activity. Thus, we suggest that in the heart of an endotoxemic rat, both VEGF and its downstream molecule eNOS, are up-regulated by hypoxia. However, because of the absence of a functionally active NO in the heart, the up-regulated VEGF angiogenic signaling could not correct the compromised coronary microcirculation in endotoxemia.

Another important finding of the present study is the data showing that dual blockade of ET further up-regulated the already high levels of VEGF angiogenic signaling molecules. The key role played by ET in the pathogenesis of sepsis has been adequately described previously. Endotoxins increase plasma ET-1 levels, along with increased mRNA expression of preproET-1 in the lungs and heart (Hemsen, 1991;



Kaddoura et al., 1996). Experiments involving infusion of ET-1 show signs of cardiovascular complications generally associated with septic shock (Weitzberg et al., 1991, 1993), suggesting that ET mechanisms may be the central factor behind the dysfunction of vital organs during sepsis (Oldner et al., 1999; Pittet et al., 1991). Consistent with these earlier findings, here we show that plasma and cardiac ET-1 levels are significantly higher in the LPS-induced endotoxemic rat model. Further up-regulation of cardiac ET-1 level in the heart of LPS-administered rat with the treatment of dual ET blocker suggests that effective ET blockade has been achieved in the present study. Overexpression of cardiac ET-1 triggers an increase in inflammatory cytokines, such as TNF- $\alpha$ , IL-1, and IL-6, as well as interstitial inflammatory infiltration and an inflammatory cardiomyopathy that subsequently leads to heart failure and death (Shindo et al., 1998). Here, serum TNF- $\alpha$  levels in LPS-administered rats were significantly elevated compared to control group and were remarkably decreased by dual blockade of ET. However, in the present study, we could not observe a parallel relationship between the expression of cardiac ET-1 and TNF- $\alpha$ . TNF- $\alpha$  is also capable of inducing gene expression of the pro-angiogenic molecules, such as VEGF and its receptors (VEGFRs) (Giraud et al., 1998; Ristimaki et al., 1998). However, the pattern of TNF- $\alpha$  and VEGF expression was reversed in the heart of an endotoxemic rat. Although the reverse relationship between cardiac VEGF and TNF- $\alpha$  cannot be adequately explained at present, we have reported similar data previously (Jesmin et al., 2012) using a LPS-induced lung injury model. In LPS-induced lung injury model, we demonstrated a time-dependent decrease in VEGF expression in pulmonary tissue compared to that of control rats. In contrast, pulmonary levels of TNF- $\alpha$  showed a significant up-regulation up to the 3 h time point and then returned to almost control levels at 6 h and 10 h after LPS administration (Jesmin et al., 2012). Indeed, reverse relationships that have been described before between TNF- $\alpha$  and VEGF receptors (Patterson et al. 1996). Patterson et al. (1996) have demonstrated that TNF- $\alpha$  is capable of significant anti-angiogenic activity by modulating VEGF receptor expression in cultured human vascular endothelial cells. TNF- $\alpha$  is widely accepted as the central proinflammatory cytokine mediating cellular responses to both endogenous and exogenous stimuli. Although responsible for significant VEGF release from a myriad of cell types, it's (TNF- $\alpha$ ) primary effects in high concentrations (as occurs in infection and malignancy) is anti-angiogenic (Patterson et al., 1996). Thus, high local or circulating levels of TNF- $\alpha$  may, in part, attenuate tissue repair by causing functional down-regulation of VEGF receptors on endothelial cells in these circumstances. But the reverse relationship between cardiac VEGF and TNF- $\alpha$  observed in the present study, as well as our previous study (Jesmin et al., 2012), needs further studies for more clarification.

The interaction between VEGF and ET-1 in vascular endothelial and smooth muscle cells is well documented. ET-1 has been documented to enhance VEGF mRNA expression via activation of ETA receptors in rat vascular smooth muscle cells (Matsuura et al., 1998). ET-1 and VEGF apparently play a complementary and coordinated role during neovascularization and malignant ascites formation in ovarian carcinoma (Salani et al., 2000a). The endothelial autocrine regulatory role of ET-1, as a putative angiogenic factor in the process of neovascularization, has been recently reported (Salani et al., 2000b). In contrast with those reports, ET receptor blockade with bosentan shows a marked pro-angiogenic effect in an ischemic leg after femoral occlusion, and this effect appears to be directly dependent on the VEGF/eNOS pathways (Iglarz et al., 2001). Bosentan was considered to modulate ischemia-induced angiogenesis by increasing the speed of revascularization and maintaining sustained activation of the process (Iglarz et al., 2001). However, several lines of evidence suggest cell mitogen and proliferating effects of ET-1 in *in vitro* studies (Hirata et al., 1989; Wren et al., 1993; Yang et al., 1999). We clearly demonstrate that ET receptor blockade with SB-209670 exacerbates cardiac VEGF angiogenic signaling in endotoxemia. In sepsis, plural stimuli may be involved in the angiogenic response, depending on the tissue type or organ

investigated. Future studies should aim to shed more light on the mechanisms underlying the further up-regulation of VEGF signaling by ET receptor antagonism in sepsis.

Besides, the effects of SB-209670 on the up-regulated VEGF signaling cascade in endotoxemic heart as observed in the present study, SB-209670 did not exert any effect on pH and lactate levels. On the other hand, Andersson et al. (2008) demonstrated improved pH and arterial lactate values using tezosentan. For now, we cannot fully account for this discrepancy. Study designs, including the types of ET blocker used, treatment duration, and types of experimental animals used, might be responsible for such discrepancy between the present study and the study by Andersson et al. (2008).

One of the important limitations of the present study is that we did not investigate the effects of using a long term treatment period for SB-209670 and the subsequent effects on cardiac VEGF signaling in endotoxemic rats. In our unpublished observation we found that VEGF did not show any clear time-dependent expression profile in endotoxemic heart. To the contrary, a biphasic pattern has been observed (Oki et al., unpublished observation, 2013). Thus, future studies should carefully examine the effects, if any, of long term SB-209670 treatment on VEGF signaling in an endotoxemic rat heart and the in depth investigations should address the observed effects of SB-209670 in the hearts for both hemodynamic compensatory and decompensatory phases of sepsis. Lastly, the present study could not clarify the role of blood pressure in the observed effects of cardiac VEGF signaling by SB-209670 in endotoxemic rat heart, thus warranting further studies.

## Conclusion

The up-regulation of VEGF angiogenic signaling as observed in the present study may be due to diminished oxygenation of myocardium in LPS-administered rats, as a consequence of sepsis-induced microvascular dysfunction, implying that oxygen supply may have been inadequate at the local level to support normal heart metabolism and function. Dual blockade of ET for 6 h further elevated the VEGF angiogenic signaling in endotoxemic heart.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

## Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research B and C from the Ministry of Education, Culture, Sports, Science and Technology of Japan (23406037, 23406016, 23406029, 24406026, 25462812 and 25305034) and Japan Society for the Promotion of Science.

## References

- Andersson A, Fenhammar J, Frithiof R, Weitzberg E, Sollevi A, Hjelmqvist H. Mixed endothelin receptor antagonism with tezosentan improves intestinal microcirculation in endotoxemic shock. *J Surg Res* 2008;149:138–47.
- Annane D, Bellissant E, Cavaillon JM. Septic shock. *Lancet* 2005;365:63–78.
- Avontuur JA, Bruining HA, Ince C. Inhibition of nitric oxide synthesis causes myocardial ischemia in endotoxemic rats. *Circ Res* 1995;76:418–25.
- Avontuur JA, Bruining HA, Ince C. Nitric oxide causes dysfunction of coronary autoregulation in endotoxemic rats. *Cardiovasc Res* 1997;35:368–76.
- Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, et al. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation* 1994a;89:2183–9.
- Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E. Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. *Cardiovasc Res* 1994b;28:1176–9.
- Barroso-Aranda J, Schmid-Schonbein GW, Zweifach BW, Mathison JC. Polymorphonuclear neutrophil contribution to induced tolerance to bacterial lipopolysaccharide. *Circ Res* 1991;69:1196–206.
- Bateman RM, Jagger JE, Sharpe MD, Ellsworth ML, Mehta S, Ellis CG. Erythrocyte deformability is a nitric oxide-mediated factor in decreased capillary density during sepsis. *Am J Physiol Heart Circ Physiol* 2001;280:H2848–56.

- Bateman RM, Sharpe MD, Ellis CG. Bench-to-bedside review: microvascular dysfunction in sepsis—hemodynamics, oxygen transport, and nitric oxide. *Crit Care* 2003;7:359–73.
- Bateman RM, Tokunaga C, Kareco T, Dorscheid DR, Walley KR. Myocardial hypoxia-inducible HIF-1 $\alpha$ , VEGF, and GLUT1 gene expression is associated with microvascular and ICAM-1 heterogeneity during endotoxemia. *Am J Physiol Heart Circ Physiol* 2007;293:H448–56.
- Bates DO, Curry FE. Vascular endothelial growth factor increases hydraulic conductivity of isolated perfused microvessels. *Am J Physiol* 1996;271:H2520–8.
- Battistini B, Forget MA, Laight D. Potential roles for endothelins in systemic inflammatory response syndrome with a particular relationship to cytokines. *Shock* 1996;5:167–83.
- Boczkowski J, Vicaut E, Aubier M. In vivo effects of *Escherichia coli* endotoxemia on diaphragmatic microcirculation in rats. *J Appl Physiol* (1985) 1992;72:2219–24.
- Bone RC, Grodzin CJ, Balk RA. Sepsis: a new hypothesis for pathogenesis of the disease process. *Chest* 1997;112:235–43.
- Ellis CG, Bateman RM, Sharpe MD, Sibbald WJ, Gill R. Effect of a maldistribution of microvascular blood flow on capillary O<sub>2</sub> extraction in sepsis. *Am J Physiol Heart Circ Physiol* 2002;282:H156–64.
- Ferrara N, Bunting S. Vascular endothelial growth factor, a specific regulator of angiogenesis. *Curr Opin Nephrol Hypertens* 1996;5:35–44.
- Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev* 1997;18:4–25.
- Giraud E, Primo L, Audero E, Gerber HP, Koolwijk P, Soker S, et al. Tumor necrosis factor- $\alpha$  regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. *J Biol Chem* 1998;273:22128–35.
- Goldman D, Bateman RM, Ellis CG. Effect of sepsis on skeletal muscle oxygen consumption and tissue oxygenation: interpreting capillary oxygen transport data using a mathematical model. *Am J Physiol Heart Circ Physiol* 2004;287:H2535–44.
- Hemsen A. Biochemical and functional characterization of endothelin peptides with special reference to vascular effects. *Acta Physiol Scand Suppl* 1991;602:1–61.
- Herbertson MJ, Werner HA, Russell JA, Iversen K, Walley KR. Myocardial oxygen extraction ratio is decreased during endotoxemia in pigs. *J Appl Physiol* (1985) 1995;79:479–86.
- Hirata Y, Takagi Y, Fukuda Y, Marumo F. Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis* 1989;78:225–8.
- Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med* 2003;348:138–50.
- Hotchkiss RS, Rust RS, Dence CS, Wasserman TH, Song SK, Hwang DR, et al. Evaluation of the role of cellular hypoxia in sepsis by the hypoxic marker [<sup>18</sup>F]fluoromisonidazole. *Am J Physiol* 1991;261:R965–72.
- Iglarz M, Silvestre JS, Duriez M, Henrion D, Levy BI. Chronic blockade of endothelin receptors improves ischemia-induced angiogenesis in rat hindlimbs through activation of vascular endothelial growth factor–NO pathway. *Arterioscler Thromb Vasc Biol* 2001;21:1598–603.
- Jesmin S, Zaedi S, Shimojo N, Iemitsu M, Masuzawa K, Yamaguchi N, et al. Endothelin antagonism normalizes VEGF signaling and cardiac function in STZ-induced diabetic rat hearts. *Am J Physiol Endocrinol Metab* 2007;292:E1030–40.
- Jesmin S, Zaedi S, Islam AM, Sultana SN, Iwashima Y, Wada T, et al. Time-dependent alterations of VEGF and its signaling molecules in acute lung injury in a rat model of sepsis. *Inflammation* 2012;35:484–500.
- Jurgensen JS, Rosenberger C, Wiesener MS, Warnecke C, Horstrup JH, Grafe M, et al. Persistent induction of HIF-1 $\alpha$  and -2 $\alpha$  in cardiomyocytes and stromal cells of ischemic myocardium. *FASEB J* 2004;18:1415–7.
- Kaddoura S, Curzen NP, Evans TW, Firth JD, Poole-Wilson PA. Tissue expression of endothelin-1 mRNA in endotoxaemia. *Biochem Biophys Res Commun* 1996;218:641–7.
- Ku DD, Zaleski JK, Liu S, Brock TA. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am J Physiol* 1993;265:H586–92.
- Lam C, Tymk K, Martin C, Sibbald W. Microvascular perfusion is impaired in a rat model of normotensive sepsis. *J Clin Invest* 1994;94:2077–83.
- Matsuura A, Yamochi W, Hirata K, Kawashima S, Yokoyama M. Stimulatory interaction between vascular endothelial growth factor and endothelin-1 on each gene expression. *Hypertension* 1998;32:89–95.
- McKinnon RL, Lidington D, Bolon M, Ouellette Y, Kidder GM, Tymk K. Reduced arteriolar conducted vasoconstriction in septic mouse cremaster muscle is mediated by nNOS-derived NO. *Cardiovasc Res* 2006;69:236–44.
- Mitaka C, Hirata Y, Nagura T, Tsunoda Y, Amaha K. Circulating endothelin-1 concentrations in acute respiratory failure. *Chest* 1993;104:476–80.
- Ohlstein EH, Nambi P, Douglas SA, Edwards RM, Gellai M, Lago A, et al. SB 209670, a rationally designed potent nonpeptide endothelin receptor antagonist. *Proc Natl Acad Sci U S A* 1994;91:8052–6.
- Oldner A, Wanecek M, Weitzberg E, Rundgren M, Alving K, Ullman J, et al. Angiotensin II receptor antagonism increases gut oxygen delivery but fails to improve intestinal mucosal acidosis in porcine endotoxin shock. *Shock* 1999;11:127–35.
- Patterson C, Perrella MA, Endege WO, Yoshizumi M, Lee ME, Haber E. Downregulation of vascular endothelial growth factor receptors by tumor necrosis factor- $\alpha$  in cultured human vascular endothelial cells. *J Clin Invest* 1996;98:490–6.
- Pittet JF, Morel DR, Hemsén A, Gunning K, Lacroix JS, Suter PM, et al. Elevated plasma endothelin-1 concentrations are associated with the severity of illness in patients with sepsis. *Ann Surg* 1991;213:261–4.
- Ristimaki A, Narko K, Enholm B, Joukov V, Alitalo K. Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem* 1998;273:8413–8.
- Sabri MN, DiSciascio G, Cowley MJ, Alpert D, Vetrovec GW. Coronary collateral recruitment: functional significance and relation to rate of vessel closure. *Am Heart J* 1991;121:876–80.
- Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K, Sugishita Y. Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature* 1996;384:353–5.
- Salani D, Di Castro V, Nicotra MR, Rosano L, Tecce R, Venuti A, et al. Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol* 2000a;157:1537–47.
- Salani D, Taraboletti G, Rosano L, Di Castro V, Borsotti P, Giavazzi R, et al. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am J Pathol* 2000b;157:1703–11.
- Shindo T, Kurihara H, Kurihara Y, Morita H, Yazaki Y. Upregulation of endothelin-1 and adrenomedullin gene expression in the mouse endotoxin shock model. *J Cardiovasc Pharmacol* 1998;31(Suppl. 1):S541–4.
- Vandijk D, Decruyenaere JM, Blot SI. The value of sepsis definitions in daily ICU-practice. *Acta Clin Belg* 2006;61:220–6.
- Walley KR, Becker CJ, Hogan RA, Teplinsky K, Wood LD. Progressive hypoxemia limits left ventricular oxygen consumption and contractility. *Circ Res* 1988;63:849–59.
- Weitzberg E, Lundberg JM, Rudehill A. Elevated plasma levels of endothelin in patients with sepsis syndrome. *Circ Shock* 1991;33:222–7.
- Weitzberg E, Ahlborg G, Lundberg JM. Differences in vascular effects and removal of endothelin-1 in human lung, brain, and skeletal muscle. *Clin Physiol* 1993;13:653–62.
- Wheeler AP, Bernard GR. Treating patients with severe sepsis. *N Engl J Med* 1999;340:207–14.
- Wren AD, Hiley CR, Fan TP. Endothelin-3 mediated proliferation in wounded human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 1993;196:369–75.
- Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411–5.
- Yang Z, Krasnici N, Luscher TF. Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. *Circulation* 1999;100:5–8.