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

Oki Masami, Jesmin Subrina, Islam Md. Majedul, Mowa Chishimba Nathan, Khatun Tanzila, Shimojo Nobutake, Sakuramoto Hideaki, Kamiyama Junko, Kawano Satoru, Miyauchi Takashi, Mizutani Taro

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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)-α, 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-α 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.

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.
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 vasconstrictor 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. Endotoxia was induced by administering bacterial LPS (Escherichia coli 055: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 endotoxia induction.

These animals were killed by 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)-α (R&D Systems, MN, USA) in heart tissues and in serum.

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 ± 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 PaO2 was found to be significantly (p < 0.041) decreased in LPS administered rats. However, arterial PaO2 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-α, VEGF and ET-1 in serum and plasma

Serum TNF-α 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 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-α and ET-1 levels

In all the three experimental groups, cardiac levels of TNF-α 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.

Table 1

<table>
<thead>
<tr>
<th>Effect</th>
<th>Control</th>
<th>LPS</th>
<th>LPS + SB-209670</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.45 ± 0.02</td>
<td>7.49 ± 0.02</td>
<td>7.44 ± 0.02</td>
</tr>
<tr>
<td>PaCO2 (mm Hg)</td>
<td>34.1 ± 3.4</td>
<td>26.4 ± 1.3</td>
<td>28.3 ± 4.5</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>100.9 ± 3.5</td>
<td>87.2 ± 3.7*</td>
<td>105.4 ± 4.1*</td>
</tr>
<tr>
<td>HCO3 (mmol/l)</td>
<td>23.1 ± 1.46</td>
<td>20.0 ± 0.89</td>
<td>19.3 ± 2.77</td>
</tr>
<tr>
<td>Base excess (mmol/l)</td>
<td>0.2 ± 0.1</td>
<td>3.7 ± 3.9</td>
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</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>6.0 ± 0.2</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>128 ± 5.75</td>
<td>89 ± 4.45**</td>
<td>91 ± 4.60**</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>97 ± 6.28</td>
<td>75 ± 3.13**</td>
<td>73 ± 2.03**</td>
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<tr>
<td>HR (bpm)</td>
<td>432 ± 17.5</td>
<td>402 ± 8.56*</td>
<td>470 ± 7.75</td>
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Abbreviations: systolic BP, systolic blood pressure; diastolic BP, diastolic blood pressure; HR, heart rate; LPS, lipopolysaccharide.

Data are mean ± SE.

* p < 0.05 vs. control.

** p < 0.01 vs. control.

### Table 1

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Data are mean ± SE.

* p < 0.05 vs. control.

** p < 0.01 vs. control.
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 microvascular vasoconstriction (McKinnon et al., 2006). Altered 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 compromised microvascular vasconstriction (McKinnon et al., 2006). Although we show here that levels of cardiac NO, the final 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 vasconstriction (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;
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

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References


