Left ventricular mitogen activated protein kinase signaling following polymicrobial sepsis during streptozotocin-induced hyperglycemia

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Abstract

We hypothesized that sepsis during hyperglycemia would activate left ventricular (LV) mitogen activated protein kinase (MAPK) signaling mechanisms and modulate generation of endothelin-1 (ET-1) and nitric oxide (NO) that can contribute to the progression of LV dysfunction. A single injection of streptozotocin (STZ, 60 mg/kg, via tail vein) was used to produce type 2 diabetes in male SD rats. Polymicrobial sepsis and sham-sepsis were induced using single i.p. injection of cecal inoculum and sterile 5% dextrose water, respectively, on the 13th and 27th day following STZ injection. Both 2-week (2-wk) and 4-wk diabetes groups were associated with hyperglycemia and weight loss. LV end diastolic pressure (LVEDP) was significantly increased in 4-wk diabetes but not in 2-wk diabetes group. Plasma concentration of tumor necrosis factor-alpha (TNF-α) was significantly increased in 4-wk diabetes + sepsis group as compared to sham, 2-wk diabetes + sepsis and sepsis groups. Elevated plasma and LV ET-1 and NO byproducts (NOx) along with LV preproET-1 and inducible nitric oxide synthase (iNOS) protein expression were observed in 4-wk but not in 2-wk diabetes group. Sepsis further elevated LV iNOS and preproET-1 in 4-wk diabetes group. Up-regulated phosphorylation of LV p38-MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2) and heat shock protein-27 (Hsp27) was observed in 4-wk diabetes group. Sepsis caused a factorial increase in LV p38-MAPK and Hsp27 phosphorylation and iNOS up-regulation but not ERK1/2 following progression from 2-wk to 4-wk diabetes. The study provides evidence that sepsis up-regulated LV iNOS, p38-MAPK phosphorylation and elevated LVEDP during 4-wk diabetes. We concluded that sepsis contributes in the development of LVEDP dysfunction and alteration in signaling mechanisms depending upon the progression from 2-wk to 4-wk diabetes in the rat.

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Keywords: Diabetes; SIRS; Signaling; Endothelin-1; Nitric oxide synthase; Left ventricular end diastolic pressure

1. Introduction

Diabetes is a complex and multifarious group of disorders characterized by hyperglycemia that has reached epidemic proportions in the present century. Infection is a leading cause of morbidity and mortality among the diabetic population [1]. A higher frequency of occurrence of septic shock following surgical procedures has been observed in diabetic patients [2]. Infection inflicted by both gram-positive and gram-negative bacteria initiates systemic inflammatory response syndrome (SIRS) leading to sepsis, septic shock and end-organ failure [3]. Both diabetes and polymicrobial sepsis are associated with myocardial injury and characterized by elevated left ventricular end diastolic pressure (LVEDP), hypotension, depressed myocardial performance and alterations in regional vascular perfusion [4–7]. However, the influence of sepsis on diabetes-induced altered myocardial function and underlying stress-induced molecular signaling mechanisms has not been characterized yet.

Mitogen activated protein kinases (MAPKs) are implicated in the etiology of sepsis and diabetes [8,9]. MAPKs are serine-threonine protein kinases involved in cell survival, proliferation and apoptosis [10]. Three MAPK subfamilies have been well characterized: extracellular signal regulated kinase 1 and 2 (ERK1/2), c-jun N-terminal kinases (JNK) and p38-MAPK [11]. ERK1/2 is involved in the growth response of cell while p38-MAPK and JNK are associated with cellular response to stress [11], inflammation [12] and vasoactive mediators such as endothelin-1.
Also p38-MAPK activation stimulates inducible nitric oxide synthase (iNOS) expression in serum-deprived RAW 264.7 cells. These observations suggest that signaling mechanisms can regulate various vasoactive molecules and vice versa. Although a differential regulation of p38-MAPK and ERK1/2 depending upon the duration and severity of diabetes has been demonstrated, the effect of sepsis in the regulation of MAPK signaling mechanism during diabetes is not known.

We have earlier demonstrated that diabetes during coronary artery bypass grafting and chronic peritoneal sepsis produced an imbalance in the myocardial and systemic ET-1 and nitric oxide (NO) profiles. However, the precise role of ET-1 following sepsis during diabetes is unclear. Diabetes, both type 1 and type 2, is associated with decreased NO bioavailability. Conflicting reports (i.e. increased, unchanged and decreased) exist regarding the state of iNOS and endothelial NOS (eNOS) during diabetes. We have earlier reported that elevation of ET-1 and NO mechanisms, either systemically or locally, in the myocardium correlated well with the development of myocardial dysfunction during sepsis and make the heart susceptible for a myocardial injury. We anticipate that, depending upon the duration of hyperglycemia, sepsis would modulate systemic and myocardial ET-1 and NO mechanisms, and ventricular function. Therefore, in the present study we hypothesized that sepsis during hyperglycemia would activate LV MAPK signaling mechanisms and modulate the generation of ET-1 and NO that can contribute to the progression of myocardial dysfunction. To address this hypothesis, we determined: (1) if induction of sepsis depending upon the duration of hyperglycemia using a STZ-induced type 2 rat model would modulate LV p38-MAPK and ERK1/2 phosphorylation, and (2) if sepsis-induced alterations in the systemic and local levels of ET-1 and NO would correlate with LVEDP dysfunction during 2-week (wk) and 4-wk diabetes.

2. Materials and methods

Male Sprague–Dawley rats (Harlan, IN, USA) weighing 350–400 g were used in the study. The rats were acclimatized to the laboratory conditions for at least 7 days following their arrival. All animal experiments were conducted in compliance with humane animal care standards outlined in the NIH Guide for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee of North Dakota State University.

2.1. Experimental protocol

All animals were age-matched at the onset of the study. The rats were randomly divided into three groups: sham, 2-wk and 4-wk diabetic rats (n = 12 for each group) (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Treatments</th>
<th>1st day</th>
<th>13th day</th>
<th>27th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>6</td>
<td>Saline</td>
<td>D1,W</td>
<td>D1,W</td>
<td></td>
</tr>
<tr>
<td>Sepsis (24 h)</td>
<td>6</td>
<td>C.I.</td>
<td>–</td>
<td>–</td>
<td>C.I.</td>
</tr>
<tr>
<td>D2</td>
<td>6</td>
<td>STZ</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D4</td>
<td>6</td>
<td>STZ</td>
<td>–</td>
<td>–</td>
<td>C.I.</td>
</tr>
<tr>
<td>D2 + sepsis</td>
<td>6</td>
<td>STZ</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D4 + sepsis</td>
<td>6</td>
<td>STZ</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

C.I., cecal inoculum in sterile 5% dextrose water (D5W); D2, 2-wk diabetes group; D4, 4-wk diabetes group; D2 + sepsis, 2-wk diabetes + sepsis group; D4 + sepsis, 4-wk diabetes + sepsis group; STZ, Streptozotocin (60 mg/kg, iv).

2.1.1. Induction of diabetes

Diabetes was produced by a single tail vein injection of streptozotocin (STZ; 60 mg/kg, i.v.). The induction of diabetes was confirmed by blood glucose estimation (Glucometer Elite®, Bayer, IN) 24 h after STZ-injection. Diabetes was confirmed if blood glucose >200 mg/dl, and monitored at different time intervals throughout the study. The animals were not given insulin supplementation.

2.1.2. Induction of sepsis

Sepsis was induced in the animals using cecal inoculum (200 mg/kg, i.p.) as previously described. Briefly, cecal inoculum was prepared by mixing cecal contents (200 mg) obtained from donor rats (euthanitized with pentobarbital; 100 mg/kg, i.p.) with 5 ml of 5% dextrose water to yield a concentration of 40 mg/ml. The cecal inoculum was prepared fresh each day and cecal material from one donor rat was used within 2 h for three to five experimental animals.

The nondiabetic sham rats received an injection of 1 ml/kg saline. The animals in each group were then further subdivided into sham and sepsis groups. Two or four weeks post-STZ administration, recordings of systemic hemodynamics, heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP) and LV pressure were done. Arterial blood was collected in plastic tubes containing EDTA (1 mg/ml) and heparin (5 units/ml) to determine the plasma concentration of ET-1, NO by-products (NOx) and tumor necrosis factor-α (TNF-α). The animals were euthanitized by pentobarbital (100 mg/kg, iv) and LV tissue was harvested. The concentration of ET-1, NOx and protein expression of iNOS, eNOS, preproET-1, phosphorylated p38-MAPK (pp38-MAPK), total p38-MAPK, total and phosphorylated ERK1/2 and phosphorylated heat shock protein-27 (Hsp-27) were determined in LV tissue obtained from each animal.

3. Surgical protocol

Separate groups of animals (N = 6) were used for hemodynamic study. On the day of experiment, the rats were anesthetized with an intraperitoneal injection of pentobarbital.
sodium (Nembutal®, Abbott; 50 mg/kg). The animals were then laid ventrally and through a cervical midline incision right carotid artery was catheterized using a polyethylene cannula (PE-50, internal diameter 0.58 mm, external diameter 0.965 mm, Clay Adams Division, Becton Dickinson and Co., Parsippany, NJ). The catheter inserted in the right carotid artery was further advanced to the left ventricle to obtain LV pressure tracings. Once the presence of the cannula tip in the left ventricle was confirmed (as evident by the waveform tracing), the cannula was kept in position. Tail artery cannulation was performed for arterial blood pressure recording. Hemodynamic data were collected using transducers (TSD 104A) connected to LV cannula and tail artery cannula. Transducers were connected to multichannel Biopac data acquisition system attached to a computer. The data were collected at a sampling rate of 1000 Hz and analyzed by employing the software AcqKnowledge. Prior to acquisition, the transducers were calibrated at 0 and 200 mm Hg using a standard mercury sphygmomanometer. The catheters were tunneled subcutaneously and exited at the back of the neck of the animals. The surgical wounds were sutured using sterile non-absorbable silk sutures (Ethicon, Johnson & Johnson) and animals were returned to individual cages. Mean arterial pressure (MAP) was calculated as \[\text{MAP} = \left(\text{SBP} + 2 \times \text{DBP}\right)/3\]. Rate of change of LV contraction (+dP/dt) and relaxation (−dP/dt) were calculated as the first derivative of LV pressure over time. Rate pressure product (RPP), an indicator of myocardial oxygen demand, was calculated as a product of SBP and HR.

4. Biochemical estimations

4.1. Concentration of TNF-α in plasma

The plasma concentration of TNF-α was determined using a rat TNF-α EIA kit (R&D systems, Minneapolis, MN). The rat TNF-α assay has no cross-reactivity with the other cytokines like Interleukin-1 (IL-1), IL-2 and IL-4.

4.2. Concentration of ET-1 in plasma and LV tissue

The concentration of ET-1 was determined in plasma and LV tissue using a commercially available EIA kit (Assay Designs Inc., Ann Arbor, MI). Briefly, the blood samples were collected in plastic tubes containing EDTA (1 mg/ml) before euthanasia. The samples were centrifuged at 3000 × g (Centra-8R, IEC, MA) for 15 min at 4 °C and plasma were separated. Plasma was then acidified adding an equal volume of 20% acetic acid. The LV tissue samples were weighed and homogenized using Polytron homogenizer (Brinkman Instruments, Westbury, NY). The tissue homogenate was centrifuged at 49000 × g (Beckman Coulter, Palo Alto, CA) for 15 min at 4 °C and the supernatant was collected in plastic tubes. The smaller peptides were extracted from plasma and supernatants using SEP-columns and then assayed for ET-1 using an EIA kit from Assay Designs. The ET-1 assay has <0.1% cross-reactivity with related peptides like bigET-1.

4.3. Concentration of NOx in plasma and LV tissue

The concentration of NO byproducts NOx (nitrate + nitrite) was determined in LV tissue and plasma as previously described [6,23] using Griess reagent. The blood samples immediately after collection were spun down at 5000 rpm for 10 min. The plasma was then decanted and stored at −20 °C until the time of NOx determination. The LV tissue was homogenized with cold phosphate buffered saline (PBS) on ice that inhibited the activity of NOS ex vivo. The homogenate was spun down (3000 × g, 5 min, Beckman) and the supernatant was collected. The NOx concentration was then determined in the supernatant using Griess reaction method.

4.4. Immunoblot analysis

The LV tissue samples were homogenized in lysis buffer and centrifuged. Following the technique of Pollock et al. [24] the homogenized samples were electrophoresed on 10% denaturing sodium dodecyl sulfate polyacrylamide gels. The proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Gelman Sciences, Pierce, Rockford, IL). Uniform loading was assessed by β-actin protein expression. Nonspecific binding sites on the membrane were blocked with 5% nonfat dry milk in Tris–HCl containing 0.2% Tween-20 buffer (TBST) overnight at 4 °C. The membranes were then exposed to primary antibody for 1 h at room temperature. After five washings in TBST the membranes were incubated with the secondary antibody for 1 h at room temperature. Finally membranes were washed three times with TBST. The specific proteins were detected by enhanced chemiluminescence (ECL reagent, Amersham Pharmacia Biotech). The blots were analyzed using Un-Scan-It software to estimate the density of the blots in pixels.

4.5. Statistical analyses

All the data were expressed as mean ± S.E. The hemodynamic and biochemical data were analyzed using one-way ANOVA using SPSS software. When a significant F value was obtained, post hoc Students Newman Keul’s test was performed for inter- and intra-group comparisons. Statistical significance was realized at P ≤ 0.05 to approve the null hypothesis for individual parameters.

5. Results

5.1. General characteristics of animals

Diabetes induction using STZ (at both 2 wk and 4 wk) significantly increased blood glucose levels and reduced
body weight as compared to their age-matched non-diabetic sham and septic counterparts. Although the heart weights were comparable in sham, sepsis and 2-wk diabetic rat groups, the 4-wk diabetic and 4-wk diabetic + septic animals had a significantly reduced heart weight as compared to the sham group (Table 2). The mortality observed in the present study during 2-wk and 4-wk diabetes was 20% and 26%, respectively.

The septic animals uniformly displayed piloerection, lethargy, periocular discharge, and diarrhea, and post-mortem analysis revealed the presence of ascites and infarcts on peritoneal organs. Induction of sepsis in diabetic rats produced profusely ulcerated peritoneal cavity that had an extremely foul-smelling fluid with ascites.

5.2. Systemic hemodynamics

Sepsis significantly increased LVEDP at 24 h as compared to sham. Although 2-wk diabetes did not alter LVEDP, it was significantly elevated in 4-wk diabetes group as compared to sham and 2-wk diabetes group. Sepsis increased LVEDP in both 2-wk and 4-wk diabetes groups as compared to sham. LVEDP in the 4-wk diabetes + sepsis group was also significantly increased as compared to sepsis and 2-wk diabetes + sepsis groups. No differences in MAP were observed in any of the groups studied. A significant increase in HR in sepsis group was observed as compared to sham. Both 2-wk and 4-wk diabetes produced a significant decrease in HR as compared to sham. Although sepsis in 2-wk and 4-wk diabetic rats significantly increased HR as compared to respective diabetes group, the values were still significantly lower than in sham and sepsis groups. RPP was significantly decreased in 2-wk and 4-wk diabetes groups as compared to sham. Sepsis significantly decreased RPP in 2-wk and 4-wk diabetic rats as compared to sepsis group. Although there was no change in $-dP/dt$ in any of the groups, $+dP/dt$ was significantly decreased in sepsis, 2-wk diabetes and 4-wk diabetes + sepsis groups as compared to sham (Table 3).

5.3. Concentration of TNF-$\alpha$ in plasma

Sepsis produced a significant increase in plasma TNF-$\alpha$ concentration as compared to sham. Sepsis in 4-wk

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**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Heart weight (g)</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>390.1 ± 13.7</td>
<td>1.46 ± 0.07</td>
<td>121 ± 16</td>
</tr>
<tr>
<td>Sepsis (24 h)</td>
<td>358.7 ± 8.4</td>
<td>1.45 ± 0.03</td>
<td>134 ± 17</td>
</tr>
<tr>
<td>D2</td>
<td>324.5 ± 15.7*</td>
<td>1.35 ± 0.06</td>
<td>426 ± 57*</td>
</tr>
<tr>
<td>D4</td>
<td>312.1 ± 9.2*</td>
<td>1.28 ± 0.06*</td>
<td>446 ± 42*</td>
</tr>
<tr>
<td>D2 + sepsis</td>
<td>323.4 ± 15.9*</td>
<td>1.30 ± 0.1</td>
<td>381 ± 77*</td>
</tr>
<tr>
<td>D4 + sepsis</td>
<td>308.3 ± 18.3*</td>
<td>1.23 ± 0.05*</td>
<td>513 ± 28*</td>
</tr>
</tbody>
</table>

D2, 2-wk diabetes group; D4, 4-wk diabetes group; D2 + sepsis, 2-wk diabetes + sepsis group; D4 + sepsis, 4-wk diabetes + sepsis group. (N= 5 in each group).

* P ≤ 0.05 as compared to respective 2-wk diabetes group.

† P ≤ 0.05 as compared to age-matched sham group.

‡ P ≤ 0.05 as compared to age-matched sepsis group.

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**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mm Hg)</th>
<th>HR (bpm)</th>
<th>LVEDP (mm Hg)</th>
<th>RPP (× 1000)</th>
<th>$+dP/dt$ (× 1000)</th>
<th>$-dP/dt$ (× 1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>130.3 ± 3.3</td>
<td>419 ± 5</td>
<td>4.4 ± 0.4</td>
<td>67.3 ± 3.7</td>
<td>14.0 ± 0.3</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Sepsis (24 h)</td>
<td>115.3 ± 9.6</td>
<td>471 ± 11*</td>
<td>7.4 ± 0.6*</td>
<td>67.8 ± 7.5</td>
<td>9.0 ± 1.4*</td>
<td>9.2 ± 2.0</td>
</tr>
<tr>
<td>D2</td>
<td>120.4 ± 6.4</td>
<td>326 ± 7*</td>
<td>5.2 ± 0.5</td>
<td>47.9 ± 3.2*</td>
<td>9.9 ± 1.1*</td>
<td>7.2 ± 1.1</td>
</tr>
<tr>
<td>D4</td>
<td>117.4 ± 2.9</td>
<td>339 ± 10*</td>
<td>8.6 ± 0.7*</td>
<td>47.9 ± 2.7*</td>
<td>11.2 ± 0.7</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>D2 + sepsis</td>
<td>121.2 ± 8.1</td>
<td>365 ± 18*</td>
<td>7.1 ± 0.8*</td>
<td>53.3 ± 5.7*</td>
<td>11.1 ± 1.4</td>
<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>D4 + sepsis</td>
<td>116.3 ± 7.1</td>
<td>382 ± 19*</td>
<td>11.1 ± 1.9*</td>
<td>50.6 ± 3.9*</td>
<td>9.0 ± 1.1*</td>
<td>7.5 ± 1.6</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; HR, heart rate; LVEDP, left ventricular end diastolic pressure; D2, 2-wk diabetes group; D4, 4-wk diabetes group; D2 + sepsis, 2-wk diabetes + sepsis group; D4 + sepsis, 4-wk diabetes + sepsis group. (N= 5 in each group).

* P ≤ 0.05 as compared to respective 2-wk diabetes group.

† P ≤ 0.05 as compared to age-matched sham group.

‡ P ≤ 0.05 as compared to age-matched sepsis group.

§ P ≤ 0.05 as compared to age-matched 2-wk diabetes group.

# P ≤ 0.05 as compared to 4-wk diabetes group.
diabetes group significantly elevated the concentration of plasma TNF-α as compared to sham, respective 4-wk diabetes and 2-wk diabetes + sepsis groups. Plasma TNF-α concentration in 2-wk and 4-wk diabetes groups and 2-wk diabetes + sepsis group were not different than sham group (Fig. 1).

5.4. Concentration of ET-1 in plasma and LV tissue

The concentration of ET-1 in plasma and LV tissue was not altered in sepsis and 2-wk diabetes groups as compared to sham. Four-week diabetes group displayed a significant elevation in plasma and LV ET-1 levels as compared to sham. Sepsis significantly increased ET-1 concentration in plasma and LV tissue in both 2-wk and 4-wk diabetes group as compared to sham and sepsis groups (Fig. 2A and B).

5.5. Protein expression of LV preproET-1

To determine the effect of sepsis on LV ET-1 during diabetes, we determined the expression of ET-1 precursor, preproET-1 in addition to ET-1 levels. A significant elevation in LV preproET-1 protein expression was observed in 4-wk diabetes but not in 2-wk diabetes group as compared to sham and 2-wk diabetes groups. Induction of sepsis in 2-wk and 4-wk diabetes group significantly increased LV preproET-1 expression as compared to sham. However, LV preproET-1 in 4-wk diabetes + sepsis group was significantly higher as compared to sepsis and 2 wk diabetes + sepsis groups (Fig. 2C).

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Fig. 2. Effect of sepsis on (A) concentration of ET-1 in plasma (N=6) (B) concentration of ET-1 in left ventricular tissue (N=6) (C) protein expression of preproET-1 (N=3 in each group) in the left ventricular tissue obtained from sham and diabetic (2-wk and 4-wk) rats. β-Actin protein expression depicts uniform loading in gel. Data are expressed as mean ± S.E. The blot is a representative of three independent experiments. D2, 2-wk diabetes group; D4, 4-wk diabetes group; D2 + sepsis, 2-wk diabetes + sepsis group; D4 + sepsis, 4-wk diabetes + sepsis group, *P≤0.05 as compared to sham-treated group, †P≤0.05 as compared to sepsis group, *P≤0.05 as compared to respective 2-wk group, †P≤0.05 as compared to 2-wk diabetes group.
5.6. Concentration of NOx in plasma and LV tissue

A significant elevation in the concentration of NOx in plasma but not in LV tissue was observed in sepsis group as compared to sham. Two-week diabetes did not alter the concentration of NOx in plasma and LV tissue as compared to sham. However, 4-wk diabetes significantly increased the concentration of NOx in plasma and LV tissue as compared to sham and 2-wk diabetes group. Sepsis in both 2-wk and 4-wk diabetes groups further elevated plasma and LV NOx levels significantly as compared to sham, sepsis and respective diabetes groups. Also 4-wk diabetes + sepsis group significantly increased both plasma and LV NOx levels as compared to 2-wk diabetes + sepsis group (Fig. 3).

5.7. Protein expression of LV iNOS and eNOS

Sepsis significantly increased the protein expression of iNOS (Fig. 4A) and decreased eNOS (Fig. 4B) in LV tissue as compared to sham. Two-week diabetes group did not exhibit any changes in LV iNOS and eNOS as compared to sham. Four-week diabetes significantly increased iNOS and decreased eNOS expression in LV tissue as compared to sham and 2-wk diabetes groups. Sepsis in 2-wk diabetic rats significantly increased the expression of LV iNOS and decreased eNOS as compared to sham and 2-wk diabetes groups. Also in 4-wk diabetic rats sepsis significantly increased iNOS and decreased eNOS as compared to sham and sepsis groups (Fig. 4A and B).

5.8. LV total and phosphorylated p38-MAPK

To determine the effect of sepsis in 2-wk and 4-wk diabetes on p38-MAPK activation, we determined the expression of pp38-MAPK (Fig. 5A) and total p38-MAPK (Fig. 5B) in LV tissue. A significant up-regulation of total and phosphorylated LV p38-MAPK was observed in sepsis group as compared to sham. Two-week diabetes did not alter phosphorylated and total p38-MAPK as compared to sham. However, 4-wk diabetes produced a significant increase in phosphorylated and total p38-MAPK as compared to sham. Sepsis in 2-wk and 4-wk diabetic rats significantly elevated both total and phosphorylated p38-MAPK as compared to sham. Also LV p38-MAPK phosphorylation was significantly increased in 4-wk diabetic + septic rats as compared to sepsis and 2-wk diabetes + sepsis groups (Fig. 5).

5.9. LV total and phosphorylated ERK1/2

The effect of sepsis in diabetic rats on the phosphorylation of ERK1/2 was examined as two 44- and 42-kDa bands in LV tissue. Diabetes, both 2-wk and 4-wk, produced a significant increase in the protein expression of LV total (Fig. 6B) and phosphorylated ERK1/2 (Fig. 6A) as compared to sham group. Sepsis did not alter total and phosphorylated ERK1/2 in LV tissue. However, 2-wk and 4-wk diabetes + sepsis groups exhibited a significant increase in LV phosphorylated ERK1/2 as compared to sham and sepsis groups (Fig. 6).

5.10. Phosphorylation of LV Hsp27

To examine if LV p38-MAPK phosphorylation activated the downstream effectors during sepsis in diabetes, the protein expression of phosphorylated Hsp 27 was determined. Phosphorylated LV Hsp27 was significantly increased in 4-wk diabetes groups but not in the 2-wk diabetic group as compared to sham. Sepsis also increased LV phosphorylated Hsp27 expression as compared to sham group. Sepsis in 2-wk and 4-wk diabetic groups significantly increased LV phosphorylated Hsp27 expression as compared to sham. The increase in phosphorylated Hsp27 expression in 4-wk diabetes + sepsis group was
significant as compared to sepsis and 2-wk diabetes + sepsis groups (Fig. 7).

6. Discussion

The present study demonstrated that polymicrobial sepsis elevated TNF-α, ET-1 and NO generation in 4-wk diabetic rats. We further found evidence that sepsis potentiated diabetes-induced elevated LVEDP and LV p38-MAPK phosphorylation depending upon the duration of hyperglycemia in the rat. These data suggest that an up-regulation of p38-MAPK and ERK1/2 phosphorylation along with altered biosynthesis of ET-1 and NO, following progression from 2-wk to 4-wk diabetes, can contribute to secondary myocardial complications found in diabetic subjects.

STZ induced type 2 diabetes in rodents is a well-established model characterized by insulin deficiency associated with insulin resistance [25]. STZ produces pancreatic beta cell necrosis and DNA damage through superoxide production [26]. Bar-On et al. [27] reported that STZ (55 mg/kg i.v) caused increased plasma glucose levels, reduced body weight and a mortality of 17% in rats. Similarly in this study too, we have observed 20% and 26% mortality in 2-wk and 4-wk diabetes groups, respectively. The rats were hyperglycemic and lost weight 17% and 20% following 2-wk and 4-wk of diabetes induction using STZ. Although no changes in MAP and LVEDP were observed in 2-wk diabetes group, 4-wk diabetic rats exhibited an increased LVEDP. We and other research groups have reported that LVEDP alterations occur much earlier than changes in rates of LV contractility (+dP/dt and −dP/dt), and therefore can be used as an early marker for CHF [28], sepsis [29] and
related cardiovascular pathologies. The data obtained in the present study support our contention that STZ-induced hyperglycemia produced LVEDP alterations indicating an early stage of CHF during progression from 2-wk to 4-wk diabetes. Mihm et al. [30] have also reported similar hemodynamic alterations during 7 days and 35 days post-STZ. In addition, we have also observed decrease in myocardial oxygen demand (RPP) in diabetic rats which correlates well with HR. Similar observations were made by Law et al. [31] in a 4-wk diabetic canine model where they demonstrated a decreased myocardial oxygen demand.

Diabetes exacerbates infections (gram-positive and gram-negative) and renders patients susceptible to infection [32]. These infections are related to invasive procedures such as chronic peritoneal dialysis, hemodialysis and vascular access device, etc. [33]. The cecal inoculum used in the present study to induce sepsis consists of both gram-positive and gram-negative microorganisms [34]. Sepsis-induced SIRS is a complex array of clinical conditions including hypotension, blood coagulopathy and increased capillary permeability that can lead to organ failure [35]. Sepsis releases several proinflammatory cytokines like TNF-α, IL-1 and IL-6 [35]. We have also found elevated plasma TNF-α in septic rats but not in diabetic animals suggesting that progression from 2-wk to 4-wk diabetes is not associated with development of SIRS as seen during sepsis. Although sepsis did not increase the concentration of circulating TNF-α during 2-wk diabetes, it caused a ~2-fold increase in 4-wk diabetes. These data demonstrate that sepsis-induced active SIRS, characterized by elevated TNF-α, was evident at 4-wk diabetes but not at 2-wk. However, the lack of elevated TNF-α in 2-wk diabetes following sepsis could be due to activation of anti-inflammatory cytokines like IL-10 which are known to control excessive

![Figure 5](image_url)
inflammatory reactions. The data suggest that increased duration of hyperglycemia from 2 wk to 4 wk produces more severe SIRS following sepsis.

Overt myocardial dysfunction has been attributed to an active SIRS following polymicrobial sepsis and diabetes characterized by reduced LV pressure development and relaxation ($+dP/dt$ and $-dP/dt$, respectively) and reduced peak ventricular pressure development in vitro [4,6]. In a diabetic dog model Law et al. [31] have demonstrated that induction of endotoxemia produced depressed left ventricular contraction. However, the current study elucidates LV dysfunction in a more clinically relevant study as opposed to the one carried out by Law et al. In the present in vivo study sepsis produced an elevation in LVEDP that was further increased in 4-wk diabetic animals. Also, RPP was decreased following sepsis in 2-wk and 4-wk diabetes. Since RPP is a function of HR and SBP it appears that changes in RPP are being reflected due to changes in HR. In our study we have found that sepsis produced depressed left ventricular contraction in 4-wk diabetic groups suggesting a deteriorating effect of sepsis in 4-wk diabetic rats. Hence, it appears that a septic insult disposes the myocardium towards LV dysfunction during 2-wk diabetes that becomes more severe during 4-wk diabetes in the rat.

Elevated [36], unchanged [37] and attenuated [38] plasma ET-1 levels have been reported during diabetes. Although the reason for such a variation in findings appears difficult to fathom, the discrepancies in data may be attributed to differences in species of animals, duration of hyperglycemia, dose of STZ administered, etc. In a STZ-induced diabetes rat model we have observed an increase in ET-1 concentration in plasma and LV tissue during 4-wk but not in 2-wk diabetes. These results were further supported by up-regulated LV preproET-1 protein expression during

Fig. 6. Effect of sepsis on the protein expression of (A) phosphorylated extracellular signal regulated kinase (p-ERK1/2) and (B) total ERK (T-ERK1/2) in the left ventricular tissue obtained from sham and STZ-treated (2-wk and 4-wk) animals ($N=3$ in each group). Data are expressed as mean ± S.E. The blot in each case is a representative of three independent experiments. D2, 2-wk diabetes group; D4, 4-wk diabetes group; D2 + sepsis, 2-wk diabetes + sepsis group; D4 + sepsis, 4-wk diabetes + sepsis group, $^*P<0.05$ as compared to sham-treated group and $#P<0.05$ as compared to sepsis group.
4-wk diabetes. Apparently, when sepsis was induced in diabetic rats, both plasma and LV ET-1 concentrations were elevated. It appears that although sepsis can activate ET mechanisms both in 2-wk and 4-wk diabetes, the elevation of LV ET-1 and expression of preproET-1 were more pronounced in 4-wk diabetic rats. These data suggest that induction of sepsis activates LV ET-1 mechanisms during diabetes and can contribute to cardiovascular alterations such as elevated LVEDP as seen in diabetic subjects. Since ET-1 genes are expressed in heart and isolated ventricular myocytes [39], we speculate that prolonged hyperglycemia renders the heart susceptible to ET-1 gene activation via an active SIRS. Although the mechanisms of ET-1 increase during diabetes are relatively unknown, several research groups speculated that this could be due to an abnormal production by the affected endothelium [40]; or lack of suppression of ET-1 release secondary to attenuated endothelium-derived relaxing factor production [41]. However, further studies will be required to explore the mechanism of ET-1 elevation following sepsis in diabetic rats.

Besides ET-1, NO is another potent vasomediator that not only regulates vascular function but also causes myocardial depression [6,17,18]. Various studies have been carried out to explore the influence of diabetes on NOS activation. Stock-klausner-Farber et al. [19] have reported that myocardial NOS (iNOS and eNOS) activity increased and reached maximal values after diabetes duration of 4 wk and 6 wk. In the present study, progression of diabetes from 2 wk to 4 wk up-regulated iNOS while down-regulating eNOS expression that correlates well with elevated LVEDP and systemic and local increase in NOx levels. This suggests that NO stimulation in the myocardium occurs with increased duration of hyperglycemia in STZ-induced diabetic rats. Similar to our earlier studies, an increased expression of iNOS and depressed expression of eNOS was seen following sepsis [42]. Unlike 2-wk diabetic group, sepsis induction in these animals produced elevated expression of LV iNOS along with depressed eNOS and increased NOx levels. These data suggest that sepsis activated LV NO mechanisms in 2-wk diabetes. Similar results were obtained following sepsis induction in 4-wk diabetes group, which were more pronounced than in sepsis during 2-wk diabetes. These results indicate that polymicrobial sepsis in diabetic rats exacerbates iNOS activation that may account for elevated NOx concentration locally and systemically in the rat.

In the present study down-regulation of myocardial eNOS along with increased iNOS and NOx during both diabetes and sepsis is intriguing. Recent studies have demonstrated that high concentrations of NO as would be produced after iNOS induction inhibit eNOS activity and expression [43]. Similar to our findings, Zhao et al. [20] have shown this in alloxan-induced diabetes in dogs; a twofold increase in iNOS mRNA expression was accompanied with a decrease in eNOS protein expression. Similar elevation of iNOS along with depression of eNOS was also seen in heart and thoracic aorta following endotoxemia in the rat [23,44]. We speculate that depression in eNOS expression in 4-wk diabetes and sepsis during 2-wk and 4-wk diabetes could be due to excessive NO generation by iNOS induction. Hyperglycemia itself has been shown to possess a variable response on eNOS in different cell types [45–47]. Since in the present study both ET-1 and iNOS activations exhibit a similar course during sepsis in diabetic rats, we speculate that iNOS stimulation could be due to activation of ETB receptors via elevated ET-1 mechanisms [48].

6.1. Signaling mechanisms following sepsis in diabetics

The present study provides evidence for the involvement of LV MAPK cascade in sepsis following STZ-induced diabetes. MAPKs can be activated by stress stimulation of Gq-protein-coupled-receptors by ET-1 [11]. Hyperglycemia has been shown to phosphorylate ERK1/2 in rat glomerular and mesangial cells [9] and p38-MAPK in vascular smooth muscle cells and aorta derived from diabetic rats [49]. In the present study we observed that 2-wk diabetes up-regulated LV ERK1/2 but did not affect p38-MAPK phosphorylation. However, 4-wk diabetes activated both LV p38-MAPK and ERK1/2. These findings suggest that protective mechanisms mediated by ERK1/2 phosphorylation predominate during 2-wk diabetes. Sepsis also up-regulated LV p38-MAPK phosphorylation during both 2-wk and 4-wk diabetes. It was further observed that LV p38-MAPK phosphorylation was more pronounced following sepsis in 4-wk than in 2-wk diabetes. These data were supported by the observation that sepsis in 4-wk diabetic rats up-regulates the phosphorylation of Hsp27, a downstream effector of the p38-MAPK cascade.
However, unlike phosphorylated p38-MAPK, ERK1/2 phosphorylation was not altered following sepsis in 4-wk diabetes as opposed to sepsis during 2-wk diabetes. The data suggest that sepsis activates p38-MAPK but not ERK1/2 phosphorylation, which correlates with LVEDP alterations depending upon the progression of diabetes from 2 wk to 4 wk. Although we did not find NOS activation during 2-wk diabetes, we observed a profound increase in NOx levels and iNOS expression along with a corresponding increase in p38-MAPK activation during 4 wk. We propose that p38-MAPK activation could be an important signaling mechanism that causes iNOS activation as was reported by Liu et al. [14]. Similar findings were also reported by Song et al. [8] where they demonstrated that iNOS regulates immune dysfunction by p38-MAPK activation during sepsis. These findings suggest that in diabetes phosphorylation of p38-MAPK and not ERK1/2 is associated with iNOS activation and LVEDP dysfunction following sepsis. We also speculate that activated p38-MAPK and iNOS during 4-wk diabetes and diabetes + sepsis outweigh myocardial ERK1/2 mechanisms. A similar finding, though not directly related to the present study, was shown by Purves et al. [52] where they demonstrated using cultured sensory neurons that cotreatment with high glucose and oxidative stress results in an additive effect on p38-MAPK phosphorylation without any additional effect on ERK1/2 activation. However, studies are required to further delineate ERK1/2 and p38-MAPK pathways during sepsis in diabetes.

The findings of the present study suggest that sepsis caused a factorial increase in LV p38-MAPK phosphorylation along with iNOS and Hsp27 up-regulation following progression from 2-wk to 4-wk diabetes. The study provides evidence that sepsis up-regulated LV p38-MAPK phosphorylation and elevated LVEDP during 4-wk diabetes. We concluded that sepsis contributes in the development of LVEDP dysfunction and alteration in signaling mechanisms depending upon the progression from 2-wk to 4-wk diabetes in the rat. However, more studies will be required to determine the inherent link or interaction between p38-MAPK up-regulation, ET-1 and NO mechanisms in the development of CHF during sepsis in diabetic subjects.

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