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Alterations in the myocardial β-adrenergic system during experimental endotoxemia

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Abstract: In this study to investigate whether β-adrenergic receptor systems in the heart are impaired during endotoxemia, we examined two models of septic shock in rats, each of which has a different time course for the shock state. Male Wistar rats were divided into two groups: (1) the LPS (lipopolysaccharide) iv group (*Escherichia coli* endotoxin 1.0 mg·kg^{-1} iv bolus administration), and (2) the CLP (cecal ligation and puncture model) group. As a control group for each model, a 0.9% saline injection group and a sham-operated group were also prepared. At 3, 12, and 24 h after treatment, the rats were killed and their hearts were removed as rapidly as possible. In the LPS iv and CLP groups, an increase in the plasma epinephrine (E) and norepinephrine (NE) levels compared with the control and sham-operated groups was observed at both 3 and 12 h after treatment ($P < 0.05$). There was a decrease in myocardial tissue NE concentration at 3, 12 and 24 h in the CLP group. This decrease was especially marked at 24 h. In the LPS iv group, a decrease in β-receptor density was observed at 3 h (control, 87.07 ± 4.59 fmol·mg^{-1} protein; LPS iv, 60.73 ± 3.51 fmol·mg^{-1} protein), but was not observed at 24 h. In contrast, a decrease in β-receptor density in the CLP group was observed at 24 h (sham-operated, 80.9 ± 3.65 fmol·mg^{-1} protein; CLP, 66.1 ± 4.08 fmol·mg^{-1} protein), but was not observed at 3 h. The β-receptor density in the hearts of LPS iv rats and the altered hemodynamics recovered in line with the decrease in plasma catecholamines (CA). However, in the CLP group the alteration in hemodynamics was not in line with plasma CA. The alteration in hemodynamics of septic-shock rats observed in this study was linked to the change in heart β-receptor density rather than the change in plasma CA. These observations suggested that the alterations which occur in the β-receptor system during endotoxemia depend upon the model of animal sepsis that is employed, and the time course of the septic-shock state. These alterations in the β-adrenergic system are thought to cause myocardial dysfunction during endotoxemia.

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Introduction

Septic shock induced by invading gram-negative bacteria is at present one of the most common causes of death in intensive care units. This state is clinically characterized by cardiovascular collapse, impaired perfusion of peripheral tissues, and pulmonary dysfunction [1]. However, the molecular and cellular events that lead to the development and progression of this syndrome have not been clearly defined [1,2].

Myocardial dysfunction often occurs during sepsis and endotoxicosis, but until now the time course and mechanism of cardiac failure have not been clarified [3,4]. There have been several reports based on clinical and experimental studies which describe the activation of sympathetic nerves during endotoxicosis [5–7], and the increase in cardiac norepinephrine (NE) turnover rates [8,9]. In addition, the elevation in plasma catecholamines (CA) and reduced responsiveness to CA have been described by a number of investigators. Parratt [10] has reported that the effects of CA infusion on blood pressure, heart rate, cardiac output, and the left ventricular maximal rate of pressure development $(dP/dt)_{max}$ were reduced at 2–3 h after endotoxin administration. Therefore it is considered that the β-adrenergic system was impaired during endotoxemia.

Although some controversy remains, there are several reports suggesting that endotoxin causes an alteration in the β-adrenergic receptor system of the heart [11,12]. Shepherd et al. [11] suggested that during septic shock there was a down-regulation in β-receptor density in the rat heart 3 h after endotoxin administration. However, Jones and Romano [12] reported that no alteration in the total number of β-adrenergic receptors

could be observed in either the endotoxin administration, or in the cecal ligation and puncture models. In this study, we have examined the influence of endotoxemia on the β-adrenergic receptor system of the heart using two different models of septic shock in the rat.

The most commonly used septic-shock model is the endotoxin single bolus injection. This is because this endotoxin model is simple to prepare and is reproducible. However, owing to a rapid death rate, this is not a clinical model [7]. In contrast, the cecal ligation and puncture (CLP) model, as described by Wichterman et al. [13], has a prolonged time course for the development of the shock state. In this respect, the CLP model closely resembles the septic-shock state clinically observed in humans. We therefore used these two septic shock models to study the changes that occur in the β-adrenergic receptor system of the heart.

Materials and methods

Animals and endotoxin

Male Wistar rats weighing between 180 and 250 g were used in all experiments. *Escherichia coli* endotoxin (lipopolysaccharide (LPS) B; 055:B5) was obtained from Difco Laboratories (Detroit, MI, USA). Prior to the experiment, the rats were maintained in wire-mesh cages with standard laboratory feed and water ad libitum under a 12 h light/dark cycle at 22°C.

Models of experimental sepsis

After obtaining the approval of the local ethical committee, two models for sepsis in the rat were prepared: (1) endotoxicosis (using a bolus injection) and (2) cecal ligation and puncture (CLP) peritonitis. As a control group for each model, a saline injection group and a sham-operated group were also prepared.

Endotoxicosis. *E. coli* endotoxin was administered at a 1.0 $mg \cdot kg^{-1}$ dose to the rats as a single bolus injection. under light ether anesthesia, via the dorsal penile vein. This dosage provides an approximately sublethal dose [14]. Yoshikawa and Goto [2] have described the relationship between LPS iv dose and mortality. Control rats received an identical volume of sterile 0.9% saline. At 3, 12, or 24 h after the administration of endotoxin or 0.9% saline, the heart was excised as rapidly as possible.

Peritonitis. The CLP model of sepsis was produced using the technique described and characterized by Wichterman et al. [13]. Under light ether anesthesia, a 2-cm midline abdominal incision was made at the level of the cecum. The cecum was extracted through the incision and ligated with 5-0 silk, just below the ileocecal valve. The cecum was punctured twice with a 20-gauge needle and a small amount of the cecal contents was expressed. The perforated cecum was returned to the abdominal cavity and the abdomen was closed in 2 layers with 3-0 silk. This experimental CLP model resulted in approximately 20% mortality at 24 h. In the sham operation, the ligation and puncture of the cecum were omitted.

Following the surgical procedure, all animals were kept in cages with access to food and water ad lib. At 3, 12, or 24 h after the surgical procedure, the heart was excised a described above.

The LPS iv, the control, and the sham-operated groups each consisted of seven Wistar rats. All rats in these three groups survived for 24 h. The CLP group consisted of nine rats. Two of the nine rats died within 24 h. The seven rats that survived CLP treatment were used in this study. To exclude the effects of blood-sampling on physiological measurements, another set of animals was used for systemic physiological measurements. These animals were sub-grouped and treated as the four groups described above. After identical treatments, the rats were cannulated with a 24-gauge teflon catheter through the femoral artery. At 3, 12, and 24 h after treatments, the blood pressure and heart rate were measured using an arterial blood pressure monitoring system (AP-601 G; Nihon Koden, Tokyo, Japan) cannulated to the femoral artery. The plasma epinephrine (E) concentration and NE concentration were measured at 3, 12, and 24 h after treatment using blood collected through the femoral artery. Each group used for physiological measurements contained the same number of rats as the groups used for biochemical measurements. Both sets of rats had the same survival rate.

Membrane preparation

Membranes were prepared as previously described by Williams and Lefkowitz [15], with minor modifications. Briefly, the heart was rinsed in 10 volumes of 0.25 M sucrose, 5 mM Tris-HCl (pH 7.4), and 1 mM $MgCl_2$ buffer, and homogenized at 4°C using a Potter homogenizer. The homogenate was filtered through a single layer of cheesecloth, and centrifuged at 480 *g* for 10 min at 4°C. The supernatant was then centrifuged at 30000 *g* for 10 min. The pellet was suspended in 50 mM Tris-HCl (pH 7.5) and 10 mM $MgCl_2$ buffer. This suspension was centrifuged and washed twice in the same buffer. The final pellet was suspended in 5 ml of 50 mM Tris-buffer. The protein concentration was determined by the Bradford method using γ-globulin as a standard [16]. The volume of the membrane suspension was adjusted to produce a final concentration of 3–4 mg protein$\cdot ml^{-1}$.

Radioligand binding assays

Binding assays were performed as described by Lurie et al. [17] with minor modifications. Briefly, an aliquot of membrane solution was incubated in a final volume of 2 ml containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ buffer, and (−)^{3}H-dihydroalprenolol (^{3}H-DHA; 60 Ci·mmol^{-1}; New England Nuclear, Boston, MA, USA). Approximately 300 μg protein was used in each incubation tube. Non-specific binding was determined by the presence of 10^{-3} M (−)isoproterenol. Non-specific binding accounted for 30%–40% of the total binding.

Samples were incubated at 30°C for 30 min. The reaction was stopped by rapid filtration through Whatman GF/C filters. Each filter was washed three times with 5 ml of the same buffer solution as described above. The filters were immediately placed in scintillation vials and dried at 100°C for 60 min, then 3 ml of the scintillation cocktail Reaflor (Sigma Chemical, St Louis, MO, USA) was added. The radioactivity trapped on the filters was measured using an Aloca 650 liquid scintillation counter (Aloca 650; Aloca Co. Ltd., Japan).

Other methods

The plasma E and NE concentrations were measured by the high-performance liquid chromatography (HPLC method) [18]. After extraction, the myocardial NE concentration was also measured by the HPLC method. Briefly, a sample of heart, dissected from the same animal heart used for the receptor sample preparation, was weighed and homogenized in 10 volumes of homogenizing buffer containing 0.1 M EDTA(2Na), 1 M $NaHSO_3$, and 0.05 M $HClO_4$. After centrifugation, the deproteinized supernatants were assayed (after extraction on alumina columns as previous described [19]) for E and NE using an ion-pair high-performance liquid chromatography system (TOSOH, HLC-8030 system).

Binding assay data analysis

The specific binding was calculated by subtracting the value for non-specific binding, as determined in the presence of an excess of unlabelled ligand, from the value for total binding. The dissociation constant and the maximum number of binding sites were determined by Scatchard analysis of the data obtained from saturation curves. The Scatchard plots of the binding data were prepared using the graphic method described by Rosenthal [20].

Statistical analysis

All data are presented as arithmetic means ± SEM. ANOVA was carried out for multiple comparison and Scheffe's method was used for comparison of means. Statistical significance was set at $P < 0.05$.

Results

LPS iv group

The systolic, diastolic, and mean blood pressures were lower than the control values at 3 h, but blood pressure had recovered to the control values by 24 h after LPS administration (Table 1). Heart rate at 3 h was elevated compared with the control value, but had recovered to the control value by 12 h after LPS administration (Table 1). Plasma E and NE concentrations at 3 and 12 h were elevated ($P < 0.05$) compared with the control values, but these values also recovered to the control level by 24 h (Table 1). Compared with the control values, there was a decrease in the tissue NE concentration in the LPS group at 3, 12, and 24 h (Table 2). There was a decrease in the β-receptor density (Bmax value) at 3 h (control, 87.07 ± 4.59 fmol·mg^{-1} protein; LPS iv, 60.73 ± 3.51 fmol·mg^{-1} protein), but this had recovered by 24 h after LPS administration (Fig. 1 and Table 3). The affinity (Kd value) did not change at 3, 12, or 24 h (Table 4).

CLP group

There was no significant difference in blood pressures at 3 h compared with the sham-operated group, but a decrease in blood pressures was observed at 12 and 24 h compared with the sham-operated group (Table 1).

Table 1. Physiological variables of four groups at 3, 12, and 24 h after treatment

	3 h				12 h				24 h			
	Control	LPS iv	Sham	CLP	Control	LPS iv	Sham	CLP	Control	LPS iv	Sham	CLP
Blood pressure (mmHg)												
Systolic	106 ± 2	90 ± 5*	122 ± 5*	134 ± 4*	109 ± 3	92 ± 8*	121 ± 6	96 ± 7*#	112 ± 4	106 ± 8	126 ± 4	89 ± 4#
Diastolic	71 ± 3	54 ± 5*	86 ± 6	94 ± 4	75 ± 3	61 ± 6*	85 ± 3	59 ± 5*#	75 ± 6	74 ± 2	70 ± 4	64 ± 7
Mean	79 ± 3	73 ± 5*	104 ± 3*	110 ± 3*	85 ± 3	73 ± 6	98 ± 6	67 ± 3*#	89 ± 8	87 ± 6	90 ± 4	73 ± 7#
Heart rate (beat·min^{-1})	356 ± 8	398 ± 10*	360 ± 7	360 ± 7	358 ± 8	380 ± 14	358 ± 6	388 ± 8*#	348 ± 8	350 ± 10	352 ± 8	394 ± 5*#
Plasma catecholamines												
Epinephrine (ng·ml^{-1})	2.7 ± 0.4	5.6 ± 1.3*	4.4 ± 1.2*	5.6 ± 1.2*	2.4 ± 0.2	4.2 ± 1.2*	3.7 ± 0.8	5.4 ± 0.9*#	2.5 ± 0.2	3.5 ± 1.1	3.2 ± 0.6	5.6 ± 0.8*#
Norepinephrine (ng·ml^{-1})	0.7 ± 0.1	4.6 ± 0.9*	4.5 ± 0.7*	4.9 ± 0.5*	0.9 ± 0.2	4.2 ± 0.7*	3.6 ± 0.8*	4.9 ± 0.9*#	0.9 ± 0.4	1.9 ± 1.1	2.1 ± 0.7	5.1 ± 0.8*#

Values are means ± SEM. LPS, lipopolysaccharide; CLP, cecal ligation and puncture.
* $P < 0.05$ compared with control group; # $P < 0.05$ compared with sham-operated group.

There was no significant difference in the heart rate at 3h compared with the sham-operated group, but the heart rate was elevated by 12h after the treatment (Table 1). The plasma E concentration was elevated at 3, 12, and 24h after the operation compared with the sham-operated group, and the plasma NE level was elevated at 24h after the operation (Table 1).

A decrease in the tissue NE concentration was observed at 3, 12, and 24h after the operation. There was a marked decrease at 24h, and at this point tissue NE was approximately 50% of the concentration observed in the sham-operated group (Table 2).

The β-receptor density did not change at 3 or 12h after the operation, but at 24h there was a decrease of approximately 20% in density (66.1 ± 4.08 fmol·mg^{-1} protein) compared with the sham-operated group (80.9 ± 3.65 fmol·mg^{-1} protein) ($P < 0.05$) (Table 3). There was no significant difference in the Kd values of the sham-operated and CLP groups (Table 4).

Sham-operated group

The plasma E and NE concentrations were elevated at 3h after the operation, but these values had decreased by 24h (Table 1). No significant alteration in B_{max} or Kd values at 3, 12, or 24h was observed (Tables 3 and 4).

Discussion

Two septic-shock models

We chose two experimental sepsis models. One type was a single bolus injection model, and the other was the CLP model. The single bolus injection model is often used as a septic-shock model owing to its simple preparation and reproducibility. It is possible to induce shock of varying severity with this model by using an appropriate dose of endotoxin. For example, a high dose of endotoxin can be used to create an experimental model of rapid septic death, but this is not commonly observed in the clinical scene. Clinical septic shock has a prolonged time course for the development of the shock state [7]. Therefore, for this study we chose a sublethal dose (1 mg·kg^{-1}) for the bolus endotoxin injection model [6,14]. The other septic-shock model we used was the CLP model. The CLP model, as described by Wichterman et al. [13], has a prolonged time course for the development of the shock state. In this regard, the CLP model resembles the development of septic shock as observed clinically [7].

In a comparison of the bolus injection and CLP models, Yelich [21] reported that these two models displayed similar metabolic and hormonal responses after endotoxemic induction. The fundamental differences observed in these two sepsis models is the time course

Table 2. Myocardial tissue norepinephrine concentration (ng·g^{-1} wet weight) of four groups at 3, 12, and 24h after treatment

	3h	12h	24h
Control	55.8 ± 3.2	50.9 ± 4.2	51.6 ± 2.1
LPS iv	42.4 ± 2.5*	43.8 ± 2.3*	41.3 ± 0.5*
Sham-operated	62.7 ± 6.8	56.4 ± 3.2	51.2 ± 0.2
CLP	45.4 ± 0.6*	33.2 ± 2.8*	25.9 ± 3.3*

Values are means ± SEM.
* $P < 0.05$ compared with control group and compared with sham-operated group.

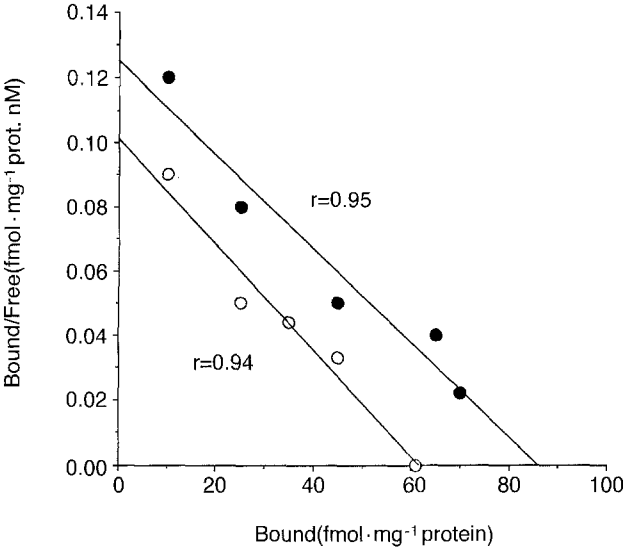

Fig. 1. An example of a Scatchard plot for [^{3}H]-dibydroalprenolol DHA binding to β-adrenergic receptors performed on crude cardiac membrane. *Closed circles*, control group; *open circles*, lipopoly saccharides iv. group

Table 3. Myocardial β-adrenergic receptor densities (B_{max}) (fmol·mg^{-1} protein) of four groups at 3, 12, and 24h after treatment

	3h	12h	24h
Control	87.07 ± 4.59	85.16 ± 5.71	84.56 ± 3.58
LPS iv	60.73 ± 3.51*	78.48 ± 6.24	88.50 ± 6.62
Sham-operated	86.87 ± 3.68	82.88 ± 5.54	80.9 ± 3.65
CLP	78.36 ± 3.59	72.94 ± 6.14	66.1 ± 4.08*

Values are means ± SEM.
* $P < 0.05$ compared with control group and compared with sham-operated group.

Table 4. β-adrenergic receptor Kd (affinity) value (nM) of four groups at 3, 12, and 24 h after treatment

	3 h	12 h	24 h
Control	1.53 ± 0.09	1.53 ± 0.10	1.55 ± 0.08
LPS iv	1.58 ± 0.07	1.45 ± 0.06	1.45 ± 0.05
Sham-operated	1.40 ± 0.05	1.55 ± 0.15	1.42 ± 0.07
CLP	1.45 ± 0.06	1.48 ± 0.10	1.49 ± 0.07

Values are means ± SEM.
No significant difference between groups.

for the alterations in plasma E, NE, and mortality [7]. Jones and Romano [6] reported that there was a rapid increase in plasma CA by 30 min after the administration of the endotoxin injection. This is in contrast to the CLP model, where plasma CA shows a gradually increase 5–6 h after the operation.

Change in plasma catecholamines and tissue norepinephrine concentrations

In our experiments, plasma E and NE concentrations were elevated in both the LPS iv group and the CLP group, although the time course differed. In the LPS iv group, plasma E and NE concentrations were elevated at 3 h after *E. coli* endotoxin administration, but they had recovered by 24 h after administration. Jones and Romano [6] suggested that this elevation in catecholamines was induced by the endotoxin.

On the other hand, plasma CA concentration in the CLP group was elevated at 3, 12, and 24 h after the operation. The elevation in CA observed at 3 h may be partly induced by the operation itself. In our experiments the plasma CA was also elevated in the sham-operated rats at 3 h, although to a lesser degree than that observed for the CLP group. Kovarik et al. [7] also observed a CA elevation in sham-operated rats due to the operation at 5 h after the operation. According to the Jones and Romano [6] report, the elevation of CA observed at 24 h was due to the action of the endotoxin.

At 3 h, blood pressure was decreased in the LPS iv group despite the elevation in plasma CA. In contrast, blood pressure was elevated in the sham-operated and CLP groups at 3 h, together with plasma CA. This difference could be caused by an alteration in the β-receptor density in the heart. In the LPS iv group, blood pressure recovered along with the recovery of heart β-receptor density. In the CLP group, blood pressure decreased along with the decrease in β-receptor density in the heart. These observations suggest that during septic shock, blood pressure was dependent upon the β-receptor density in the heart rather than plasma CA.

It has previously been reported that there is an increase in NE turnover in endotoxic and peritonitic rats [8,9], and that peripheral tissue depletion of NE occurs in a variety of endotoxic models [22,23]. In our experiments, myocardial NE depletion occurred in both the LPS iv group and the CLP group at 3 and 24 h. The depletion of NE in the CLP group at 24 h was especially marked. Jones and co-workers [8,9] reported that the NE turnover rate increased in both the endotoxin administration and CLP rat models, and that although there was no change in NE concentration in the heart, NE release by the peripheral sympathetic nerve increased. They also suggested that the depletion in NE concentration from the peripheral organs during endotoxemia was related to the endotoxin dose and time course of septic shock [8,9]. However, Pohorecky et al. [24] reported that monoamine synthesis was depressed in the endotoxin iv rats. Therefore, it is possible that the depletion of NE concentration in the heart observed in our study was induced by the increased release of NE by the peripheral sympathetic nerves and by decreased synthesis in the nerve terminal, particularly in the CLP group.

Alteration in β-receptor densities in the two septic-shock rat models

In this study, we observed that β-adrenergic receptor density decreased in the LPS iv group at 3 h and in the CLP group at 24 h after treatment. In the LPS iv group, β-receptor density at 3 h had decreased, but it recovered at 24 h. On the other hand, β-receptor density in the CLP group only decreased at 24 h. There is some controversy regarding β-receptor density during endotoxic shock. Shepherd et al. [11] reported that β-receptor density decreased in the rat heart at 3 h after endotoxin administration. Silverman et al. [25] also reported that 5 h after endotoxin administration, β-receptor density in rat heart decreased by approximately 30% compared with the control. However, Jones and co-workers [12,26] claimed that there was no change in β-receptor density in the rat heart any time in either the endotoxin administration or CLP models. Jones and Romano [12] also reported that Shepherd et al. [11] did not measure total β-receptor density, but had counted density using the sarcolemmal fraction. Jones and Romano [12] further reported that the elevation in plasma catecholamine caused the transfer of β-receptors from the sarcolemmal fraction to the cytosolic fraction, refered to as "internalization," and there was no decrease in the total β-receptor density when both the sarcolemmal (SL) and cytosolic (CY) vesicular membrane fraction were used in measurements. In our experiment using crude membranes (comparable to SL in ref. [12]), it is possible that the decrease in β-receptor density in the LPS iv group at 3 h and in the CLP group at 24 h may result from internalization, and the recovery of β-receptor density at 24 h may be due to transference from the CY to the SL. However, our study indicated that the

most important factor maintaining the hemodynamics during septic shock was the β-receptor density of the SL, not of the CY.

Mechanism of β-receptor down-regulation

We considered that the observed alteration in β-receptor densities was dependent on the model of rat septic shock used, and the time course of the septic-shock state. In the CLP group, the CA concentration was elevated both at 3 and 24 h after the operation. However this value was thought to be a point in the increasing process of CA induced by a gradually increasing LPS. In contrast, in the LPS iv group the value observed was thought to be a point in the decreasing process of CA concentration, which had reached its maximum point of increase at 30–60 min after the LPS injection. The β-receptor down-regulation was only observed 24 h after the operation. Considering the time course of the CA increase in the CLP group, it is considered that the exposure of β-receptor to CA was not sufficient to induce a down-regulation at 3 h after the operation.

Conclusion

Myocardial dysfunction often occurs during endotoxemia, but the mechanisms involved have not yet been clarified. In our experiments, an alteration in β-adrenergic receptor densities was observed in different animal models, and at different time courses. Our investigations indicated that the change in hemodynamics during septic shock is caused by the change in β-adrenergic receptor density in the heart rather than the plasma CA. There alterations may cause myocardial dysfunction during endotoxemia.

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