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Alterations in Rat Aortic Alpha$_1$-Adrenoceptors and Alpha$_1$-Adrenergic Stimulated Phosphoinositide Hydrolysis in Intraperitoneal Sepsis

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Naval Medical Research Institute, (J.A.C., E.A.S., B.L.R.) and the Armed Forces Radiobiological Research Institute, Bethesda, Maryland (R.Z.L.), and Department of Anesthesiology, Critical Care Medicine, and Child Health and Development, George Washington University School of Medicine, Washington, District of Columbia (J.A.C.)

We investigated the alterations of rat aortic alpha$_1$-adrenoceptors and alpha$_1$-adrenergic stimulated phosphoinositide (PI) metabolism in intraperitoneal sepsis. An analysis of [^{125}I]-hydroxyethylaminotetralone (HEAT) binding to alpha$_1$-adrenoceptors on rat aortic membranes revealed decreased numbers of receptors without changes in affinity. The maximum number of binding sites decreased from 349 ± 35 fmol/mg to 146 ± 16 fmol/mg ($P < 0.05$ vs. control). PI metabolism was similarly attenuated in aortae from septic rats. The norepinephrine-stimulated hydrolysis of [^{32}P]-phosphatidylinositol-4,5-bisphosphate was significantly decreased in aortae from septic rats as was the alpha$_1$-adrenoceptor stimulated accumulation of [^{3}H]-inositol monophosphate. Finally, the basal labeling of [^{32}P]-phosphatidylinositol-4,5-bisphosphate but not of [^{32}P]-phosphatidylinositol or [^{32}P]-phosphatidic acid was significantly diminished. These results imply that signal transduction induced by alpha$_1$-adrenoceptor agonists in rat aorta is significantly altered in intraperitoneal sepsis. These findings may help define the mechanisms of depressed aortic contractility in models of sepsis and endotoxic shock.

Key words: septic shock, phosphoinositide metabolism, vascular contraction, vasoconstriction, signal transduction

INTRODUCTION

Sepsis and septic shock are major causes of death in the United States among critically ill patients. Despite sophisticated and aggressive surgical and medical

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A preliminary account of these findings was presented at the first International Shock Conference and published in abstract form [36].

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interventions, the mortality rate in septic shock remains between 40 and 60% [1]. In dissecting the pathophysiology of sepsis and endotoxemia, numerous investigators have noted diminished peripheral vascular responsiveness to norepinephrine in both humans [2] and in animal models of sepsis and endotoxemia [3,4]. Furthermore, an attenuated response to exogenously applied norepinephrine (NE) has been noted in isolated aorta by several groups of investigators [5,6] using a variety of models of sepsis and endotoxemia. The mechanism of this phenomenon remains unknown [see refs. 3,4 for review], but could reside in part in the signal transduction apparatus.

We recently found that NE-induced contraction of rat aorta, which is mediated by alpha$_1$-adrenoceptors, appears to correlate with the breakdown of phosphoinositides (PI) [7]. Our original observations have now been replicated [8]. We also noted that the potent vasoconstrictor 5-hydroxytryptamine [9] and several vasoactive prostaglandins [10] also appear to induce rat aortic contraction, at least in part, via PI breakdown [for review see refs. 3,4]. According to this scheme, following binding of a ligand to the receptor a phosphoinositide-specific phospholipase C is activated which cleaves phosphatidylinositol-4,5-bisphosphate to release inositol-triphosphate (IP_3) as well as diacylglycerol (DAG). It is proposed that IP_3 mobilizes intracellular calcium [11–15] while DAG activates protein kinase C. Phorbol esters, which mimic the effects of endogenous DAG, are potent inducers of rat aortic contraction. Phorbol esters induce vasoconstriction in part by the mobilization of extracellular calcium in a nitrendipine-sensitive fashion [16].

We previously discovered that hepatic alpha$_1$-adrenoceptors [17], as well as vasopressin receptors [18], were decreased in intraperitoneal sepsis as well as in chronic endotoxin infusion models of sepsis [19]. Spitzer and colleagues [20] found that the alpha$_1$-adrenoceptors and vasopressin receptor-mediated mobilization of intracellular calcium and activation of phosphoinositide hydrolysis were altered in intraperitoneal sepsis and in endotoxemia. These findings suggested to us that vascular PI metabolism and adrenoceptors might be similarly altered in intraperitoneal sepsis.

In this paper we report significant alterations in aortic PI metabolism in rat intraperitoneal sepsis. We also discovered diminution of aortic alpha$_1$-adrenoceptors. These results suggest that experimental sepsis in the rat induces alterations in receptor-coupled signal transduction in the aorta.

MATERIALS AND METHODS

Animals and Their Treatment

Sprague-Dawley rats (Taconic Farms, Baltimore, MD) weighing 200-350 g were used in all experiments. Cecal ligation with two-hole puncture (CLP) was performed as previously detailed [17]. The sham procedure was identical except that the cecum was not devascularized, ligated or punctured. At 18–24 hr post-procedure, surviving animals (60–70% survival at this time point) were sacrificed by decapitation. Surviving animals displayed the signs of sepsis described by Wichterman et al. [21] including piloerection, a bloody discharge from the nose and mucous membranes, bloody diarrhea and lethargy.

Phosphoinositide Metabolism

PI turnover in the rat aorta measuring [^{3}H]-inositol monophosphate accumulation in the presence of 10 mM LiCl was determined by a modification of the

procedures of Berridge et al. [22] as previously described [7,9,23,24] using [^{3}H]-myo-inositol (16 Ci/mmole, New England Nuclear, Boston, MA). We previously showed that this procedure accurately separates inositol mono-, bis- and tris-phosphates.

For measurement of [^{32}P]-phosphoinositide metabolism, rat aortic rings (4 mm length) were prepared [10] and pre-incubated for 15 min in an oxygenated Hepes buffer of the following composition (in mM) at 37°C: 140 NaCl, 10 D-glucose, 5 Hepes, 1.0 $MgCl_2$, 1.5 $CaCl_2$ pH-7.40. The segments were then incubated in [^{32}P]-orthophosphate (carrier free, Amersham) containing Hepes buffer (30μ Ci/ml) for 30 min to label phosphoinositide pools. Preliminary experiments disclosed steady-state labeling of PI pools by this time period. Test agents were then added for various periods of time and the reaction terminated by the addition of 0.9 ml chloroform/methanol/HCl (100:200:0.1) solution followed by the addition of 0.3 ml water and 0.3 ml chloroform. Following lipid extraction, the lower phase was removed, washed twice with upper phase and concentrated in a Speed-Vac. The samples were then applied to oxalate pre-coated high performance thin layer chromatography plates (HPTLC) prepared and run according to Jolles et al. [25]. [^{32}P]-phosphoinositides were identified by autoradiography with Kodak X-OMAT film (XAR-2) and by authentic standards (Sigma Chemical Co., St. Louis, MO). The spots so identified were scraped into scintillation vials and quantified by liquid scintillation spectrometry.

Alpha$_1$-Adrenoceptor Measurements in Rat Aorta

Aortas from 12 rats (sham or CLP) were homogenized in 20 mM Tris-Cl buffer (pH-7.40, 25°C) with a polytron homogenizer and then rehomogenized with a tightly-fitting glass-glass homogenizer. This homogenate was centrifuged at 1,000 $\times$ g for 15 min (4°C) to sediment cellular debris and then a crude plasma membrane fraction was prepared by centrifugation at 35,000 $\times$ g for 45 min. The resulting pellet was resuspended in binding buffer [17] and incubated (0.5 ml t.v.) at 25°C for 60 min in the presence of increasing doses of the selective alpha$_1$-agonist [^{125}I]-hydroxyethylaminotetralone (HEAT) (2,200 Ci/mmole, New England Nuclear, Boston, MA) in the presence and absence of 1μM prazosin to determine specific binding. Membranes were harvested by filtration on GF/B filters (Whatman) and washed by three 5-ml washes of ice-cold binding buffer. Filters were placed into vials and counted in a gamma counter.

Binding data were analyzed and binding parameters (Kd, Bmax) determined using the nonlinear least-squares computerized curve fitting program (LIGAND) as previously detailed [26] using the NIH DEC/10 computer. This iterative procedure constructs models of binding according to the law of mass action for the interaction of multiple ligands with multiple binding sites. The results of three or more experiments were averaged to provide a weighted mean and SEM. Protein concentration was determined as described by Bradford [27].

Materials

Solvents were of reagent grade or better (Fisher Chemical Co., St. Louis. MO); all other materials, unless otherwise specified, were from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Alterations in Rat Aortic PI Metabolism

Recent findings [20] have suggested that alterations might exist in hepatic PI metabolism in various sepsis and endotoxin models. We wished to determine whether similar changes might occur in aorta. Figure 1 shows that the NE-activated PI hydrolysis, as measured by the accumulation of [^{3}H]-inositol monophosphate, (PI) was significantly attenuated in the aortas from septic rats, when compared with sham-operated controls. Dose-response studies for a short time of incubation have disclosed an EC_{50} of 0.93 $\pm$ 0.6 μM for NE-induced [^{3}H]-IP accumulation [see ref. 21]; prazosin, a selective alpha$_1$-antagonist inhibits the response with an IC_{50} of 3nM, while yohimbine (an alpha$_2$-antagonist) has an IC_{50} of 100 nM [7]. Thus, the measurement of NE-induced [^{3}H]-IP accumulation in rat aorta accurately reflects the activity of alpha$_1$-adrenoceptors.

Since phosphatidylinositol–4,5–bisphosphate (PIP_2) is thought to be the primary substrate for PI hydrolysis in rat aorta [28], we sought to measure the accumulation of [^{3}H]-inositol triphosphate (IP_3) using our previously described technique. Because of the very small amounts of IP_3 released which are not degraded into IP we were unable to measure this metabolite (not shown). We were, however, able to measure the NE-stimulated breakdown of [^{32}P]-phosphatidylinositol–4,5–bisphosphate. After a 30 sec exposure to 10 μM NE, [^{32}P]-PIP_2 levels were decreased in aortas from sham-operated rats, but unchanged in aortas from septic rats (Table I). After a 1 min exposure to 10 μM NE [^{32}P]-PIP_2 levels returned to baseline (Table II). This effect is similar to that reported by Rapoport in rat aorta [29].

Because the basal levels of [^{32}P]-PIP_2 as well as basal [^{3}H]-IP accumulation were diminished, we sought to determine whether these alterations represented generalized changes in PI synthesis. Accordingly, we measured the labeling of various PI metabolites in aortas from septic and sham-operated rats (Table II) in the basal state

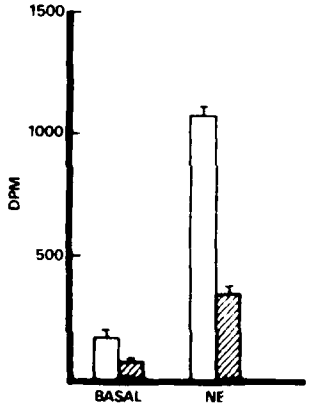

Fig. 1. The effect of intraperitoneal sepsis on norepinephrine-stimulated phosphoinositide hydrolysis in rat aorta. Aortic rings from control (open bars) and septic (hatched bars) were incubated in the presence and absence of 10 μM norepinephrine and [^{3}H]-inositol monophosphate accumulation determined. Data represent mean $\pm$ SEM for 16 individual determinations. The differences in basal and stimulated accumulation are significant ($P < 0.01$).

TABLE I. Altered NE Induced PIP_2 Hydrolysis in Sepsis

	Sham	Septic
Control aorta	700 ± 76 dpm	438 ± 89 dpm[a]
30 sec 10 M NE	567 ± 79 dpm	413 ± 39 dpm[b]

[a]($P < 0.05$) vs. control in sham operated rats.
[b]($P > 0.05$) or no significant change vs. control in septic rats.

TABLE II. Time Course [^{32}P] Incorporation Into Phosphoinositides in the Presence of 10 M NE (dpm/mg) in Rat Aorta

	Sham	
	0 min	1 min
PI	311 ± 510	326 ± 95
PIP	159 ± 56	187 ± 69
PIP_2	294 ± 130	362 ± 160
PA	202 ± 61	295 ± 93
	Septic	
Time	0 min	1 min
PI	311 ± 75	471 ± 182
PIP	110 ± 51	186 ± 40
PIP_2	152 ± 53[a]	157 ± 38[a]
PA	206 ± 27	523 ± 284

[a]$P < 0.05$ vs. sham. Data represent mean ± SEM of six individual determinations.

as well as after a 1 min exposure 10 μM NE. Decreased PIP_2 labeling continues at the 1 min time points as well as at the 30 sec time point in septic aorta.

As is seen (Fig. 2) there was apparently a selective decrease in [^{32}P]-PIP_2 labeling in aortas from septic rats, without changes in the basal levels of [P]-PIP, [^{32}P]-PI or [^{32}P]-PA. Since PIP_2 has recently been shown to be the preferential substrate for the guanine nucleotide activated phospholipase C in rat aorta [28], this decreased substrate availability could account, in part, for the observed decrease in [^{3}H]-IP accumulation. It does not account for the diminished [^{32}P]-PIP_2 breakdown. These results suggested to us that earlier events in the signal transduction pathway for aortic alpha$_1$-adrenoceptors might be perturbed in intraperitoneal sepsis.

Alpha$_1$-Adrenoceptor Alterations in Sepsis

Since we had previously found changes in hepatic alpha$_1$-adrenoceptors, it was reasonable to suggest that aortic receptors might be similarly altered. Figure 3 shows, using [^{125}I]-HEAT as a ligand, a 50% reduction in rat aortic alpha$_1$-adrenoceptors during sepsis. The maximum number of binding sites is decreased without a change in affinity (Table III), suggesting fewer ligand recognition sites. These results suggest that the decrease in number in alpha$_1$-adrenoceptors could *contribute* to the observed alterations in signal transduction.

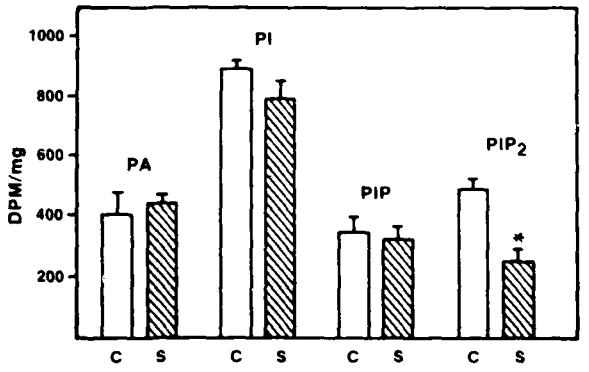

Fig. 2. The effect of intraperitoneal sepsis on basal polyphospoinositide labeling in rat aorta. Rat aortic rings were incubated with [^{32}P]-orthophosphate as described in Methods and phospholipids isolated and quantitated. Data represent mean ± SEM for six individual determinations. (*$P < 0.01$). C = control; S = septic.

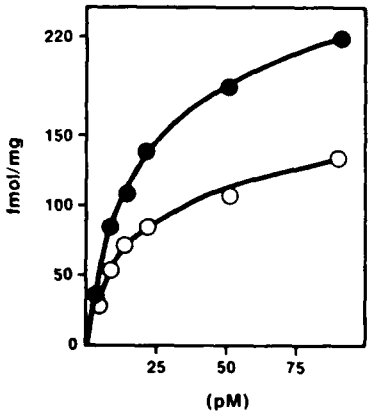

Fig. 3. The effect of intraperitoneal sepsis on rat aortic alpha$_1$-adrenergic receptors. Rat membranes were prepared and incubated with increasing doses of [^{125}I]-HEAT as described in Methods. Data represent mean of duplicate determinations of specific binding for a typical experiment which has been replicated three times. Computer-derived parameter estimates for sham (closed circles) and septic (open circles) rats are shown in Table III.

TABLE III. Alpha$_1$-Adrenoceptor Alteration in Sepsis

	Control		Septic	
Aorta	Kd(nM)	Bmax (Fmol/mg)	Kd(nM)	Bmax (Fmol/mg)
Alpha$_1$-adrenergic receptors	0.016 ± 0.003	349 ± 35	0.013 ± 0.013	146 ± 16[a]

[a]$P < 0.05$ vs. sham operated control rats. Data represent mean ± SEM of computer derived estimates for N = 3-4 separate experiments.

DISCUSSION

Our findings demonstrate that rat aortic alpha$_1$-adrenoceptor mediated PI hydrolysis, as well as alpha$_1$-adrenoceptors, are significantly altered in rat intraperitoneal sepsis. Studies employing rat aortic preparations in vitro have also demonstrated attenuated alpha$_1$-adrenoceptor responsiveness [5]; our findings suggest that at least a part of this change in responsiveness could be related to the signal transduction pathway involving alpha$_1$-adrenoceptors. Our findings further suggest that defects may reside in the synthesis of the substrate (i.e., PIP_2) for the nucleotide-activated phospholipase C. We have recently obtained evidence suggesting that these observed biochemical alterations have further consequences. For example, we discovered that NE-induced bidirectional calcium fluxes [37] as well as NE-induced phosphorylation of contractile proteins is similarly decreased in aortas from septic rats [30].

The mechanism of these observations is unknown and will require further investigation. The absence of changes in receptor affinity argues against the presence of a reversibly bound inhibitor. Such a substance would cause a decrease in affinity without changing the number of binding sites. Generalized receptor down-regulation as well seems unlikely since we have measured several other receptor types in this sepsis model (e.g., opiate, serotonergic, hepatic beta adrenergic) which were unchanged (not shown).

It is significant that we were able to verify the alterations in NE-induced PI breakdown by two independent techniques. The first was the measurement of [^{3}H]-IP accumulation in the presence of LiCl. The main advantage of this technique is its simplicity. This method has certain disadvantages which include the difficulty in measuring the predominant product (IP_3) in rat aorta. The inability to measure this metabolite in rat aorta has been seen by others [29]. We elected to use a second, independent method which entails the measurement of [^{32}P]-PIP_2 breakdown. Although this technique is more laborious, it gave quite similar results. These two techniques showed that the NE-induced PI hydrolysis was significantly decreased in sepsis.

It is conceivable that an endotoxin-derived molecule could elicit the biochemical changes we have discovered. Endotoxin derived molecules have been shown in vitro to activate protein kinase C [31]. It is also known that activation of protein kinase C by phorbol esters induces many of the same changes in PI metabolism in rat aorta as are found in this chronic sepsis model [32]. Thus we showed that phorbol-12,13-dibutyrate as well as phorbol-myristate diacetate attenuated the NE-stimulate PI breakdown in rat aorta [32]. It is also possible that other, unknown, factors contribute to the changes in receptor number and PI metabolism described in this paper.

It is important to note that hepatic alpha$_1$-adrenoceptors and vasopressin receptors are also decreased in experimental sepsis and endotoxemia models [17-20]. These findings have been demonstrated to correlate with the attenuation of alpha$_1$-adrenoceptor and vasopressin-receptor mediated PIP_2 breakdown, intracellular calcium release and phosphorylase a activation in hepatocytes from septic and endotoxin-treated rats [20,33]. Like aorta, the liver shows a marked insensitivity to alpha$_1$-adrenoceptor stimulation in intraperitoneal sepsis [33-36].

In conclusion, we found that rat aortic alpha$_1$-adrenoceptor mediated PI hydrolysis, PIP_2 breakdown and receptor numbers were all decreased in intraperitoneal sepsis. We speculate that such alterations could contribute to the previously observed changes in aortic responsivity to NE in sepsis and endotoxemia.

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The experiments described in this paper were performed in adherence to the NIH guidelines for the use of experimental animals.