Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension

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ABSTRACT **Macrophages are the primary cellular targets of bacterial lipopolysaccharide (LPS), but the role of macrophage-derived cytokines in LPS-induced septic shock is uncertain. Recent evidence indicates that activation of peripheral CB1 cannabinoid receptors contributes to hemorrhagic hypotension and that macrophage-derived anandamide as well as unidentified platelet-derived substances may be contributing factors. Here we demonstrate that rat platelets contain the endogenous cannabinoid 2-arachidonyl glyceride (2-AG), as identified by reverse phase high-performance liquid chromatography, gas chromatography, and mass spectrometry, and that in vitro exposure of platelets to LPS (200 μg/ml) markedly increases 2-AG levels. LPS-stimulated, but not control, macrophages contain anandamide, which is undetectable in either control or LPS-stimulated platelets. Prolonged hypotension and tachycardia are elicited in urethane-anesthetized rats treated *1)* with LPS (15 mg/kg i.v.); *2)* with macrophages plus platelets isolated from 3 ml of blood from an LPS-treated donor rat; or *3)* with rat macrophages or *4)* platelets preincubated in vitro with LPS (200 μg/ml). In all four cases, the hypotension but not the tachycardia is prevented by pretreatment of the recipient rat with the CB1 receptor antagonist SR141716A (3 mg/kg i.v.), which also inhibits the hypotensive response to anandamide or 2-AG. The hypotension elicited by LPS-treated macrophages or platelets remains unchanged in the absence of sympathetic tone or after blockade of nitric oxide synthase. These findings indicate that platelets and macrophages generate different endogenous cannabinoids, and that both 2-AG and anandamide may be paracrine mediators of endotoxin-induced hypotension via activation of vascular CB1 receptors.—Varga, K., Wagner, J. A., Bridgen, D. T., Kunos, G. Platelet- and macrophage-derived endogenous cannabinoids are involved in endotoxin-induced hypotension. *FASEB J.* 12, 1035–1044 (1998)**

Key Words: anandamide · 2-arachidonyl glyceride · CB1 cannabinoid receptors · vasodilation

ENDOTOXIC SHOCK is a potentially lethal failure of multiple organs that is initiated by lipopolysaccharide (LPS)[2] present in the outer membrane of gram-negative bacteria. The primary cellular target of LPS are macrophages, which are activated by LPS via the CD14 protein expressed on their membranes to generate various cytokines, including tumor necrosis factor α and interleukin 1β (1, 2). Although various symptoms of septic shock have been attributed to the LPS-induced release of cytokines from circulating macrophages (1–3), pharmacological antagonism of cytokine effects failed to provide protection from the hypotension of septic shock (4, 5), the mechanism of which remains to be clarified. In addition to their well-known neurobehavioral effects, cannabinoids, including the endogenous ligand arachidonyl ethanolamide (anandamide) (6), can also elicit hypotension mediated via peripherally located CB1 cannabinoid receptors (7–10). Anandamide, originally discovered in brain (6, 11–13), is also present in a macrophage cell line (14); a second endogenous ligand, 2-arachidonyl glyceride (2-AG), has been identified in the gut (15) as well as in brain (16–18). We recently reported that activation of peripheral CB1 receptors in rats by macrophage- and platelet-derived substances contributes to the hypotension in hemorrhagic shock, and that anandamide is generated in activated circulating macrophages but not in platelets (19). The purpose of this study was to test the hypothesis that a similar mechanism may be involved in endotoxic shock and to identify the nature of the platelet-derived factor responsible for eliciting CB1 receptor-mediated hypotension. We report here that

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[2] Abbreviations: HPLC, high-performance liquid chromatography; LPS, lipopolysaccharide; 2-AG, 2-arachidonyl glyceride; THC, Δ^9-tetrahydrocannabinol; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; GC, gas chromatography; BSTFA, bis[trimethylsilyl]trifluoroacetamide; anandamide, arachidonyl ethanolamide; SR141716A, N-[piperidine-1-yl]-5-[4-chloro-phenyl]-1-[2,4-di-chloro-phenyl]-4-methyl-1H-pyrazole-3-carboxamide HCl.

rat platelets generate 2-AG, and that LPS stimulates the production of 2-AG in platelets and induces the production of ananamide in macrophages. Furthermore, rat platelets and macrophages obtained from LPS-treated rats or exposed to LPS in vitro trigger CB1 receptor-mediated hypotension in normal recipient rats, which can be also elicited by the administration of synthetic anandamide or 2-AG.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats weighing 300–350 g were housed under a 14:10 h light/dark cycle, fed normal rat chow, and given access to drinking water ad libitum. The animals were anesthetized with urethane, 0.7 g/kg i.v. + 0.3 g/kg i.p., and were heparinized (500 I.U./kg i.v.). Drugs or isolated blood cell preparations were injected via a polyethylene cannula in the femoral vein. Arterial blood pressure and heart rate were monitored via a cannula inserted into the femoral artery and connected to a pressure transducer and physiograph. Heart rate was derived from the pressure pulse via a tachograph preamplifier.

To induce septic shock in anesthetized rats, the animals received an i.v. injection of 15 mg/kg *Escherichia coli* endotoxin (LPS). This submaximal hypotensive dose was selected because it consistently produced gradually developing, prolonged hypotension associated with tachycardia. Ten minutes before injection of LPS, these rats received an i.v. injection of either vehicle or 3 mg/kg SR141716A, a selective CB1 receptor antagonist (20).

For survival studies, three groups of unanesthetized rats received an i.p. injection of 45 mg/kg LPS. The animals also received two i.p. injections of vehicle (group #1), 3 mg/kg SR141716A (group #2), or 1 mg/kg Δ^9-tetrahydrocannabinol (THC, group #3) 30 min before and 6 h after injection of LPS. The number of surviving animals was established at 12, 24, 36, and 48 h after LPS treatment.

Preparation of isolated blood cells

Heparinized rat blood was removed via an intraarterial cannula. Mononuclear cells were isolated from whole blood by the method of Böyum (21), as described previously (19). Briefly, 5 ml of heparinized whole blood was carefully layered over 5 ml of Histopaque −1077 solution (Sigma Chemical Co., St. Louis, Mo.) and centrifuged at 400 *g* for 20 min. The mononuclear fraction formed a white layer at the interface, whereas erythrocytes and granulocytes sedimented and formed a pellet. The mononuclear fraction was aspirated, resuspended in 2 ml of phosphate-buffered saline (PBS), and centrifuged at 800 *g* for 20 min. The resulting pellet was resuspended in 0.5 ml PBS and used for subsequent experiments. The mononuclear cell fraction prepared from 5 ml of heparinized rat blood contained 1–2 $\times$ 10^7 lymphocytes, $\sim 10^6$ macrophages, and 15–20 platelets per macrophage.

For preparing separate platelet and macrophage cell fractions for in vivo injection into recipients, platelet-rich plasma was removed from 5 ml heparinized blood of a normal control rat by centrifugation at 64 *g* for 15 min. The mononuclear cell fraction was prepared from the remaining cellular pellet, as described above, after replacing the plasma with PBS. By first removing platelet-rich plasma, the number of contaminating platelets in these preparations was reduced to 3–5 platelets per macrophage. Both preparations were then incubated for 90 min with 200 μg/ml LPS. At the end of the incubation, the preparations were centrifuged (400 *g*, 15 min), washed twice, and then resuspended in 0.5 ml Krebs' buffer in siliconized glass test tubes for i.v. injection into recipient rats.

For biochemical analyses, platelet-rich plasma and the platelet-depleted mononuclear cell preparation were separately plated in 10 cm plastic culture dishes. Nonadherent cells were removed after 1 h by aspiration, and the remaining adherent cells were maintained in RPMI 1640 medium containing 10% fetal calf serum under standard cell culture conditions (19).

Biochemical analysis of anandamide and 2-arachidonyl glyceride

For the identification of anandamide, platelets and macrophages were isolated from a total of 100 ml of rat blood. The cells (2–3$\times$$10^{10}$ pure platelets or 2–3$\times$$10^7$ macrophages contaminated with $\sim 10^8$ platelets, respectively) were incubated for 90 min with 200 μg/ml LPS or vehicle. The medium was then replaced with serum-free RPMI 1640 medium containing 200 μM phenylmethylsulfonylfluoride (PMSF) to prevent the breakdown of anandamide (22). Thirty minutes later, the cells + medium were extracted three times with 2 volumes of ethylacetate; the organic phase was dried under nitrogen and resuspended in ethanol. The extract was fractionated by reverse phase high-performance liquid chromatography (HPLC), using a Waters Guard-pak precolumn module, a RCM-100 radial compression module fitted with an 8 $\times$ 100 mm Radial Pak A cartridge containing 4 μm C-18 packing, a PM-80 solvent delivery system (Bioanalytical Systems, W. Lafayette, Ind.), and an acidified methanol:water gradient, as described elsewhere (23). The fractions corresponding to the elution time of authentic anandamide (95 to 103 min) were pooled, dried under nitrogen, and derivatized with bis[trimethylsilyl]trifluoroacetamide (BSTFA) for 1 h at 70°C. Samples were dried again and resuspended in n-hexane. The trimethylsilylether derivatives were subjected to gas chromatography/mass spectrometry as described previously (19). Briefly, the samples were injected in 'spitless' mode into a gas chromatograph (GC, Hewlett-Packard 5890) equipped with an HP-1 capillary column (25 m$\times$0.2 mm$\times$0.33 μm) and interfaced with a mass spectrometer (Hewlett-Packard 5988A). Four minutes after injection, the oven temperature was ramped from 90°C to 300°C at the rate of 10°C/min. Helium was used as carrier gas. The injector and detector temperatures were 260°C, and the source temperature was 200°C. Anandamide eluted from the GC column at ~23.2 min. Based on the electron impact spectrum of anandamide, selected ion monitoring analyses were performed on m/z 85, m/z 116, and m/z 404.

For analysis of 2-AG, macrophages and platelets were obtained from 30 ml rat blood. Incubation of the cells with LPS and their subsequent processing were as described above, including the use of PMSF to inhibit the degradation of 2-AG (24). Using the same reverse phase HPLC system as for anandamide, authentic 2-AG eluted between 105 and 115 min. These fractions were pooled, dried under nitrogen, and derivatized with BSTFA, as described above. GC/MS analysis of 2-AG was done under the same conditions, except a different GC column (SPB-5, 30m$\times$0.25 mm$\times$0.25 μm) was used. 2-AG eluted from this GC column at ~26.9–27.0 min. Based on the electron impact spectrum of authentic 2-AG, selected ion monitoring analyses were performed on m/z 103, m/z 129, m/z 147, m/z 432, m/z 451, and m/z 507.

Drugs

Anandamide (arachidonyl ethanolamide) was synthesized by Dr. Raj Razdan (Organix Inc., Woburn, Mass.); SR141716A

(N-[piperidine-1-yl]-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-1H-pyrazole-3-carboxamide HCl) was provided by Dr. John Lowe (Pfizer Central Research). 2-AG was obtained from Deva Biotech Inc. (Hatboro, Pa). THC was obtained from the National Institute on Drug Abuse. *E. coli* lipopolysaccharide (0127:B8) was from Sigma Chemical Co. The vehicle for anandamide, THC, and SR141716A was emulphor:ethanol:saline 1:1:18. Emulphor is a polyoxyphenylated vegetable oil. 2-AG was originally received in n-hexane, which was evaporated; the drug was redissolved in ethanol and diluted in emulphor:ethanol:saline 1:1:8 immediately before its i.v. administration.

Statistical analyses

Time-dependent changes in mean blood pressure and heart rate in the different treatment groups were compared by analysis of variance, followed by Bonferroni's post hoc test. Survival data were analyzed by using the chi square test.

RESULTS

A CB1 receptor antagonist protects against LPS-induced hypotension

In urethane-anesthetized rats, the bolus i.v. injection of 15 mg/kg LPS caused gradually developing, profound, and long-lasting hypotension and tachycardia. Pretreatment of the rats with 3 mg/kg SR141716A, a selective CB1 receptor antagonist (20), completely prevented the hypotension, but not the tachycardia, caused by LPS (**Fig. 1**). This dose of SR141716A causes similar, near-maximal inhibition of the hypotensive (7, 8) as well as the neurobehavioral effects of both THC and anandamide (20), which are thought to be mediated by CB1 receptors (8, 20). In contrast, similar doses of SR141716A did not antagonize the ability of the cannabinoid agonist CP-55,940 to suppress mitogen-induced splenocyte proliferation and natural killer activity (25), thought to be mediated by CB2 receptors (26). In agreement with our earlier findings (7, 27), 3 mg/kg SR141716A alone caused no consistent change in basal blood pressure (−4±3 mmHg) and no change in heart rate. In other animals, the maximal hypotensive response to the same dose of LPS was unaffected by pretreatment with intracisternally administered SR141716A, 300 μg/kg (ΔBP: −29±5 mmHg, n=4, compared to −22±2 mmHg in the absence of SR141716A, n=26, $P>0.1$). This suggests that the hypotensive but not the tachycardic response to LPS is due to activation of peripherally located CB1 receptors, possibly by an endogenous cannabinoid.

Since circulating macrophages are targets for LPS (1, 2) and can generate anandamide (19), it is plausible that they are the source of the endogenous cannabinoid mediating the hypotensive response to LPS. To test this, 5 ml of arterial blood was withdrawn from LPS-treated rats at the peak of their hypotensive response for preparation of the mononuclear cell fraction. These cells caused prolonged hypotension and tachycardia when injected into untreated control rats, but only tachycardia when injected into recipient rats pretreated with SR141716A (**Fig. 2**). Mononuclear cells from untreated donors caused only minimal changes in blood pressure (−7±1 mmHg) or heart rate (+24±9 beats/min). The mononuclear cell preparation contained macrophages, lymphocytes, and contaminating platelets. Lymphocytes from LPS-treated rats, separated by aspiration after allowing the macrophages and platelets to adhere to a plastic culture dish, did not cause any change in blood pressure or heart rate in recipient rats. Therefore, macrophages and/or platelets appear to be responsible for the CB1 receptor-mediated hypotension in response to LPS treatment.

To examine the respective roles of macrophages and platelets in LPS-induced hypotension, platelet-rich plasma was removed from 5 ml of control rat

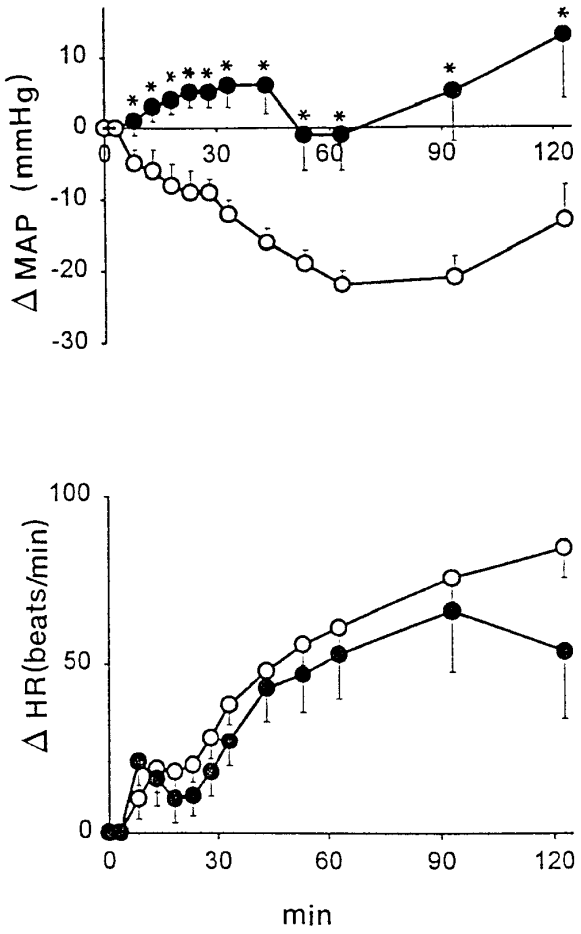

Figure 1. The effect of LPS (15 mg/kg i.v.) on mean arterial pressure (MAP) and heart rate (HR) in urethane-anesthetized control (○, n=26) or SR141716A-pretreated rats (●, n=9). SR141716A (3 mg/kg i.v.) was given 10 min prior to LPS, which was injected at 0 min. Vertical bars represent SEM. *Significant difference ($P<0.05$) from corresponding control value.

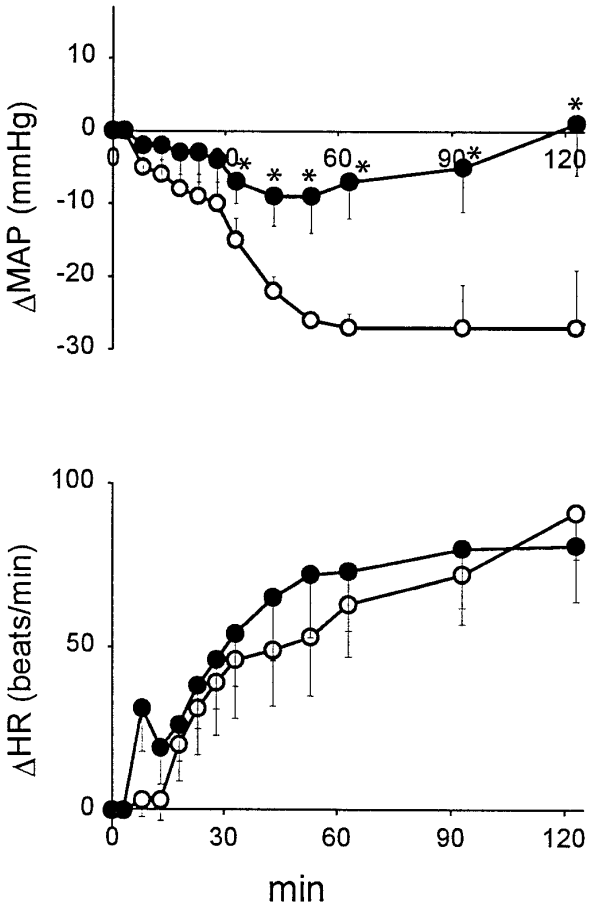

Figure 2. Changes in blood pressure and heart rate of recipient rats in response to mononuclear cells isolated from LPS-treated rats. Mononuclear cells (contaminated with platelets) were isolated from 10 ml of heparinized blood removed from a donor at the peak of the hypotensive response to 15 mg/kg LPS. Cells were resuspended in 1 ml saline, divided into two equal aliquots, and injected into urethane-anesthetized control (○, n=5) and SR141716A-pretreated rats (●, n=4) at 0 min. Vertical bars represent SEM. *Significant difference (P<0.05) from corresponding control value.

blood before isolating the mononuclear cells, and both fractions were then incubated in vitro with LPS. LPS is known to be able to directly activate both macrophages (1, 2) and platelets (28), and such direct activation also provides the advantage of avoiding possible indirect effects of in vivo treatment with LPS. Injection of either the LPS-treated platelets or mononuclear cells into normal recipients caused hypotension and tachycardia (**Fig. 3**). In both cases hypotension was prevented by SR141716A pretreatment, whereas the tachycardia was unaffected (platelets) or partially reduced (macrophages).

LPS induces the production of anandamide in circulating macrophages

We examined the ability of LPS to induce the production of anandamide in isolated macrophages and platelets. Each treatment group consisted of cells isolated from a total of 100 ml rat blood. The cells were plated, treated in vitro with LPS or vehicle, and extracted with ethylacetate, as described in Materials and Methods. The extract was then fractionated by reverse phase HPLC, and the fractions corresponding to the elution profile of authentic anandamide were pooled and subjected to GC/MS analysis to test for the presence of anandamide. As illustrated in **Fig. 4*A, C***, anandamide could be clearly identified by its GC elution time and m/z ratios in macrophages treated in vitro with LPS. To estimate the amount of anandamide in cells, a calibration curve was generated from the areas under the GC elution curves obtained with different amounts of authentic anandamide. Using this curve, the amount of anandamide in macrophages was estimated to be 130 ng, or about 20 amol/cell. There was no detectable anandamide (<1 ng) in the same number of macrophages treated with vehicle (Fig. 4*B*), nor was there detectable anandamide in either LPS-treated (Fig. 4*D*) or control platelets (not shown).

Cardiovascular effects of 2-arachidonyl glyceride

2-AG has slightly lower affinity for CB1 receptors than anandamide (15, 16), but its cardiovascular actions are unknown. We tested the effects of 2-AG on the blood pressure and heart rate of eight urethane-an-

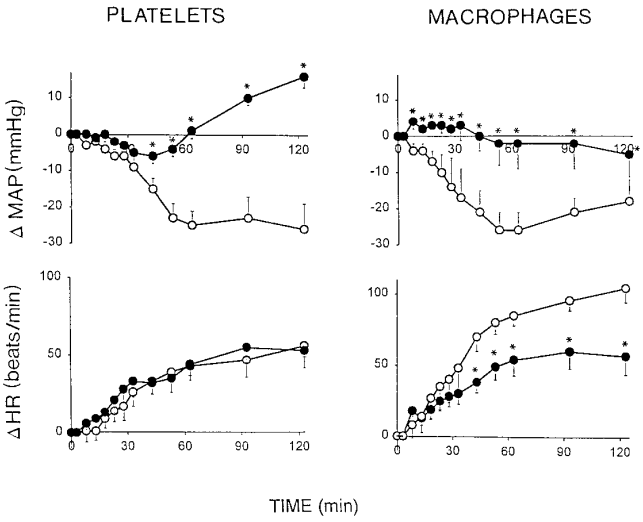

Figure 3. The effects of platelets and macrophages incubated in vitro with LPS on mean arterial pressure (top) and heart rate (bottom) in control (○, n=5–6) or SR141716A-pretreated recipient rats (●, n=5–6). Cells were isolated and incubated with 200 μg/ml LPS or vehicle for 90 min, as described in Materials and Methods. Basal MAP and HR were 104 ± 9 mmHg and 356 ± 14 beats/min before and 102 ± 8 mmHg and 365 ± 18 beats/min after SR141716A (3 mg/kg i.v.). Cells were injected at 0 min. Vertical bars represent SEM. *Significant difference (P<0.05) from corresponding control values.

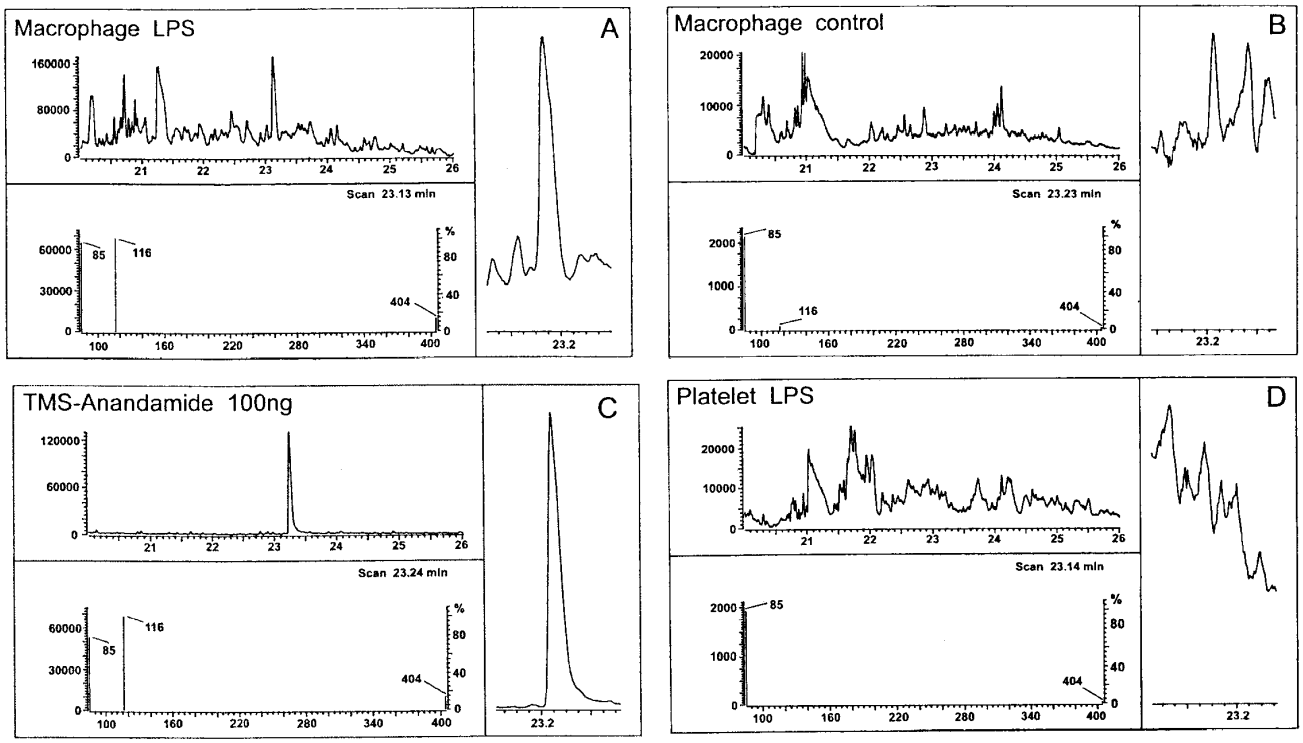

Figure 4. GC/MS analysis of anandamide in rat macrophages and platelets. Macrophages (*A*, *B*) and platelets (*D*) isolated from 100 ml of normal rat blood were incubated in vitro with 200 μg/ml LPS (*A*, *D*) or vehicle (*B*) for 90 min, as detailed in Materials and Methods. Ethyl acetate extracts of cells plus medium were prepurified by reverse phase HPLC and the anandamide fractions were subjected to GC/MS analysis. GC/MS analysis of 100 ng of authentic anandamide is shown in panel *C*. Upper left panels (*A–D*): combined currents obtained by selected ion monitoring on m/z 85, m/z 116, and m/z 404; Lower left panels: ratio of the monitored ions at the apex of the anandamide peak; Right panels: expanded view of the anandamide region of the ion chromatogram. The amount of anandamide in the GC elution peak in LPS-stimulated macrophages (*A*, 130 ng) was estimated from a calibration curve generated with 50, 100, and 150 ng anandamide. The limit of sensitivity for anandamide is 1 ng in this GC/MS system.

esthetized rats. Bolus injection of 2-AG (1 and 3 mg/kg i.v.) caused dose-dependent hypotension (peak change: −12±2 and −34±5 mmHg, respectively) and tachycardia (+22±4 and +39±9 beats/min, respectively). The hypotensive response to both doses of 2-AG was significantly reduced when tested 10 min after treatment of the rats with 3 mg/kg SR141716A (−6±2 mmHg, $P<0.03$, and −19±3 mmHg, $P<0.001$, respectively), whereas the tachycardia remained unchanged (+20±3 and +37±9 beats/min, respectively). As this response pattern is similar to that caused by LPS-activated macrophages and platelets, we tested whether 2-AG is present in these cells.

LPS stimulates the production of 2-arachidonyl glyceride in platelets

For identifying 2-AG in platelets and macrophages, cells from a total of 30 ml rat blood were used. Cells were prepared, treated with LPS, then incubated in the presence of PMSF, extracted with ethylacetate, and fractionated by reverse phase HPLC in the same way as for anandamide assays. Using the same solvent gradient, 2-AG is eluted immediately after anandamide in this reverse phase HPLC system (between 105 and 115 min). The pooled HPLC fractions were then subjected to GC/MS analysis. As seen in **Fig. 5**, 2-AG could be clearly identified both in control and LPS-treated platelets, the amount in the latter being about threefold higher than in controls. 2-AG was also identified in control and LPS-treated macrophage preparations (not shown). However, since contaminating platelets cannot be removed from the mononuclear cell fraction and their numbers are variable, the cellular source of 2-AG cannot be clearly identified in these latter preparations.

Macrophage-induced, CB1 receptor-mediated hypotension is independent of sympathetic tone or nitric oxide

These findings support the hypothesis that LPS-induced hypotension is mediated, at least in part, by macrophage- and platelet-derived cannabinoids acting at peripheral CB1 receptors, but do not indicate the location of these receptors. Exogenous anandamide can reduce sympathetic vasoconstrictor tone by acting at presynaptic CB1 cannabinoid

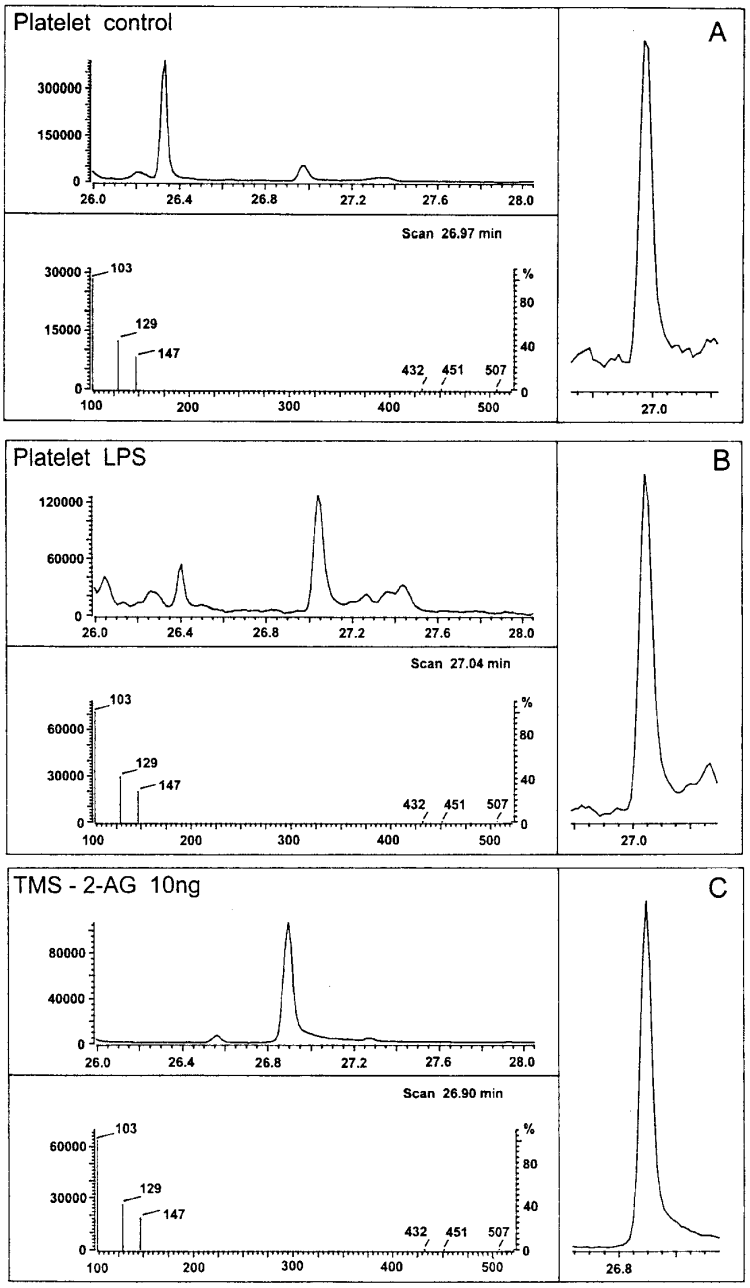

Figure 5. GC/MS analysis of 2-AG in rat platelets. Platelets isolated from 30 ml of rat blood ($\sim 10^{10}$ pure platelets) were incubated with vehicle (*A*) or LPS (*B*), extracted with ethyl acetate, prepurified with RP-HPLC, and the 2-AG containing fractions were subjected to GC/MS, as detailed in Materials and Methods. GC/MS analysis of 10 ng authentic 2-AG is illustrated in panel *C*. Based on the electron impact spectrum of authentic 2-AG, selected ion monitoring analyses were performed on m/z 103, m/z 129, m/z 147, m/z 432, m/z 451, and m/z 507. The amount of 2-AG in the GC elution peaks was estimated as 3.4 ng (control) and 9.3 ng (LPS), using a calibration curve generated with 0.5, 2, 5, 10, and 20 ng 2-AG. The limit of sensitivity for 2-AG was 100 pg.

receptors located on peripheral sympathetic nerve terminals (27, 29, 30), but can also cause vasodilation by a direct action on vascular smooth muscle (31–33). To determine which of these two mechanisms is involved in the effect of macrophage- and platelet-derived cannabinoids, we compared the hypotensive action of LPS-treated mononuclear cells in control rats vs. in three rats pretreated with phentolamine (2 mg/kg i.v.) to remove sympathetic vasoconstrictor tone, then continuously infused with vasopressin (2 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.v.) to restore basal blood pressure to control levels. The hypotensive effect of LPS-treated cells in the latter group (-33 ± 4 mmHg) was not different from that in controls (-26 ± 5 mmHg), which suggests that the vasodilator effect of macrophage-derived cannabinoids is independent of sympathetic tone. We also tested whether endothelial NO is involved in this vasodilator effect. In six rats, the i.v. injection of the irreversible nitric oxide synthase inhibitor, L-NAME (20 mg/kg), caused a sustained increase in basal blood pressure ($+24 \pm 3$ mmHg), but the hypotensive and tachycardic responses to the subsequent administration of LPS-treated mononuclear cells (-18 ± 4 mmHg, $+81 \pm 8$ beats/min) were not significantly different from the same responses in untreated controls (see Fig. 3). This indicates that, as in hemorrhagic shock (19), the hypotension elicited by activated macrophages/platelets is independent of vascular NO.

Effects of SR141716A and THC on survival from endotoxic shock

Since SR141716A was able to prevent LPS-induced hypotension, we tested its ability to modify the rate of survival from LPS-induced shock. As illustrated in **Fig. 6**, treatment with SR141716A (see Materials and Methods) significantly increased the survival rate at all four time points tested. Treatment with the cannabinoid agonist THC also increased the rate of survival during the first 24 h after LPS administration.

DISCUSSION

The results presented indicate that systemic, but not central, administration of a selective CB1 receptor antagonist, SR141716A, protects rats against hypotension induced by bacterial LPS, whereas the tachycardic response to LPS remains unchanged. A remarkably similar pattern of cardiovascular response was observed in control rats in response to isolated macrophages and platelets previously exposed to LPS in vivo or in vitro. This strongly suggests that the cardiovascular responses to in vivo treatment with endotoxin are likely mediated by macrophage- and platelet-derived substances acting via CB1 cannabinoid receptors. A similar mechanism has recently been proposed to contribute to hemorrhagic hypotension (19); macrophage-derived anandamide as well as unidentified, platelet-derived substances have been implicated as possible paracrine mediators. The present findings significantly extend this hypothesis not only by applying it to another model of shock, but also by identifying, for the first time, the presence in platelets of a second endogenous cannabinoid, 2-AG, and stimulation of its production by LPS.

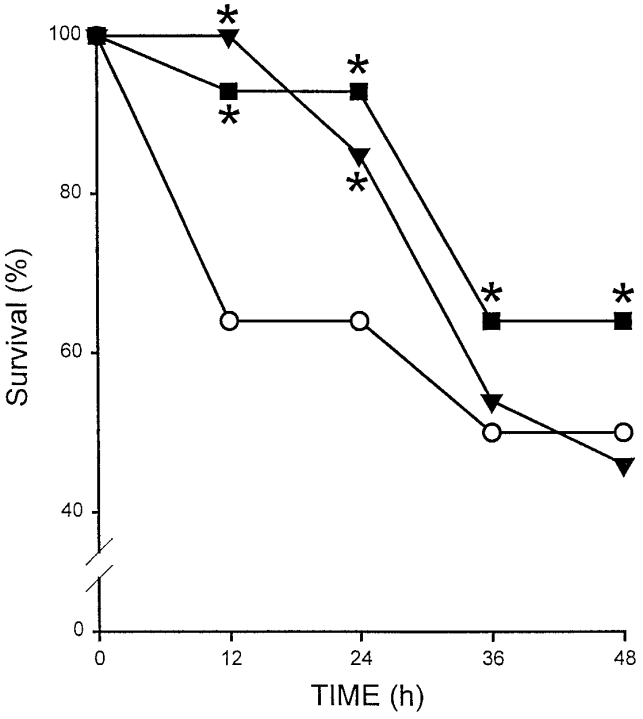

Figure 6. The effects of SR141716A and Δ^9-tetrahydrocannabinol (THC) on survival from LPS-induced shock in unanesthetized rats. For details on doses and treatment protocols, see Materials and Methods. The number of animals in the three groups was 14 (○, LPS only), 14 (■, SR141716A+LPS), or 13 (▼, THC+LPS). *Significant difference from corresponding value in rats treated with LPS only.

Previous studies have demonstrated that anandamide can elicit CB1 receptor-mediated hypotension in rats (7–10). Anandamide was undetectable in control macrophages (Fig. 4*B*), whereas substantial amounts could be identified in LPS-treated cells (Fig. 4*A*), which makes it a plausible mediator of the CB1 receptor-mediated hypotension induced by activated macrophages. 2-AG has long been postulated as a transient byproduct of the generation of arachidonic acid from phosphatidylinositol in platelets, although its presence has not been documented (34). Here we clearly identify, for the first time, the presence of 2-AG in platelets and show that it is substantially increased in response to LPS. Since platelets do not contain detectable levels of anandamide and the hypotensive action of 2-AG is attenuated by SR141716A, it is likely that 2-AG is responsible for the ability of LPS-treated platelets to induce CB1 receptor-mediated hypotension. Although platelet contamination of 2-AG precluded its unequivocal identification in circulating rat macrophages (see Results), the presence of 2-AG in the J774 macrophage cell line (V. Di Marzo, personal communication) makes it likely that macrophages may produce both of these endogenous cannabinoids.

Both 2-AG and LPS-treatment also elicit tachycardia resistant to SR141716A, which suggests that 2-AG could be a mediator of this effect. The resistance of the tachycardia to blockade by SR141716A may suggest the involvement of CB2 receptors, which could be tested by using a novel, selective CB2 receptor antagonist (35). Alternatively, both anandamide and 2-AG are rapidly degraded to yield arachidonic acid (18, 22), and arachidonic acid elicits tachycardia in rats by a mechanism not involving cannabinoid receptors (K. Varga and G. Kunos, unpublished observations). Additional studies are in progress to determine the mechanism of the tachycardia in response to LPS treatment.

Although 2-AG could be detected in control platelets, these cells do not trigger hypotension in recipient rats, and there is no evidence for tonic activation of hypotensive CB1 receptors under control conditions (7, 8, 10, 19). Attachment of macrophages and platelets to the vascular wall is known to increase in both hemorrhagic (36) and septic shock (37) through the increased expression of selectins (38,

39) and other cell adhesion molecules. Cannabinoids released from these adherent cells may act as 'juxtacrine' mediators at CB1 receptors in the vasculature. It is plausible that 2-AG released from nonadherent platelets is diluted in plasma, whereas activation of platelets by LPS not only increases the cellular levels of 2-AG (Fig. 5), but the adherence of platelets to the vascular wall (38) greatly enhances the local concentration of platelet-derived 2-AG at vascular receptors. A high-affinity uptake system for 2-AG in platelets, similar to that documented for anandamide in neurons (40, 41) and macrophages (42), could further limit the free concentration of 2-AG in plasma. However, no evidence for facilitated diffusion of 2-AG was found in a recent study in rat basophilic leukemia and mouse neuroblastoma cells (43).

The presence of anandamide and 2-AG in circulating blood cells from animals in different forms of shock provides a plausible explanation for the CB1 receptor-mediated hypotension under these conditions. However, one cannot exclude the alternative (or additional) possibility that these activated cells trigger hypotension indirectly by releasing endogenous cannabinoids from another source, such as the vascular endothelium. Such a mechanism has recently been proposed to account for the ability of SR141716A to inhibit the NO-independent component of carbachol-induced vasodilation in an isolated, buffer-perfused mesenteric vascular bed preparation (33), although this mechanism has been questioned (44, 45). In either case, a likely target of the released cannabinoid is the vascular smooth muscle cell, which has been shown to contain functional CB1 receptors (32). The message for CB1 cannabinoid receptors is also present in postganglionic sympathetic neurons (29), and activation of presynaptic CB1 receptors on such neurons has been shown to inhibit stimulation-induced norepinephrine release both in vitro (29) and in vivo (30). However, the present finding that the hypotension induced by activated macrophages and platelets is unchanged in the absence of sympathetic tone indicates that presynaptic CB1 receptors are not the targets of macrophage- and platelet-derived cannabinoids.

It is possible that macrophage-and platelet-derived cannabinoids also subserve noncardiovascular functions in shock. The immunosuppressive action of cannabinoids is thought to be mediated by CB2 receptors located primarily on macrophages (26, 46). 2-AG is known to be able to activate such receptors (47) and may contribute to the depressed immune response present in both hemorrhagic (48) and septic shock (49).

Pretreatment of animals with the CB1 antagonist SR141716A not only prevented LPS-induced hypotension, but also improved survival. This observation opposes recent findings about hemorrhagic shock where pretreatment with SR141716A shortened survival (19). In this latter model, the same degree of hypotension was induced by controlled bleeding in the absence or presence of SR141716A, and it is possible that removal of cannabinoid-mediated vasodilation further compromised tissue perfusion. In contrast, prevention of LPS-induced hypotension by SR141716A would help to maintain adequate tissue perfusion. Pretreatment with the cannabinoid agonist THC improved survival both in hemorrhagic (19) and endotoxic shock (Fig. 6). By stimulating vascular CB1 receptors, THC may help to counteract excessive sympathetic vasoconstriction triggered by the hypotension. THC will also stimulate CB2 receptors on macrophages, which could suppress the LPS-induced release of cytokines known to contribute to the multiorgan failure of septic shock.

In summary, the present findings document a novel paracrine mechanism of vasodilation in endotoxic shock, where macrophage-derived anandamide and platelet-derived 2-AG are likely responsible for the activation of vascular CB1 cannabinoid receptors. Unlike the tonically active vascular NO system, the vasodilator activity of endogenous cannabinoids is evident only under certain pathological conditions, and it appears to be independent of NO. The activation of this mechanism in hemorrhagic (19) and septic shock represents a novel physiological function of the endogenous cannabinoid system. FJ

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