

Lipopolysaccharide Induces Anandamide Synthesis in Macrophages via CD14/MAPK/Phosphoinositide 3-Kinase/NF- κ B Independently of Platelet-activating Factor*

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Macrophage-derived endocannabinoids have been implicated in endotoxin (lipopolysaccharide (LPS))-induced hypotension, but the endocannabinoid involved and the mechanism of its regulation by LPS are unknown. In RAW264.7 mouse macrophages, LPS (10 ng/ml) increases anandamide (AEA) levels >10-fold via CD14-, NF- κ B-, and p44/42-dependent, platelet-activating factor-independent activation of the AEA biosynthetic enzymes, *N*-acyltransferase and phospholipase D. LPS also induces the AEA-degrading enzyme fatty acid amidohydrolase (FAAH), and inhibition of FAAH activity potentiates, whereas actinomycin D or cycloheximide blocks the LPS-induced increase in AEA levels and *N*-acyltransferase and phospholipase D activities. In contrast, cellular levels of the endocannabinoid 2-arachidonoylglycerol (2-AG) are unaffected by LPS but increased by platelet-activating factor. LPS similarly induces AEA, but not 2-AG, in mouse peritoneal macrophages where basal AEA levels are higher, and the LPS-stimulated increase in AEA is potentiated in cells from FAAH^{-/-} as compared with FAAH^{+/+} mice. Intravenous administration of 10⁷ LPS-treated mouse macrophages to anesthetized rats elicits hypotension, which is much greater in response to FAAH^{-/-} than FAAH^{+/+} cells and is susceptible to inhibition by SR141716, a cannabinoid CB₁ receptor antagonist. We conclude that AEA and 2-AG synthesis are differentially regulated in macrophages, and AEA rather than 2-AG is a major contributor to LPS-induced hypotension.

Anandamide (arachidonylethanolamide, AEA)¹ and 2-arachidonoylglycerol (2-AG), recently discovered endogenous ligands of cannabinoid receptors (1), are synthesized by neurons (2) as well as macrophages (3–5) in a calcium-dependent manner. The effects of

AEA at the receptor are terminated by its rapid uptake through a high affinity membrane transporter (6) and subsequent intracellular degradation by fatty acid amidohydrolase (FAAH) (7). Although 2-AG is also a substrate for FAAH *in vitro*, it is metabolized *in vivo* by a monoglyceride lipase (8). AEA and 2-AG display many of the pharmacological properties of Δ^9 -tetrahydrocannabinol, including the ability to elicit hypotension mediated by peripheral CB₁ receptors (9, 10), and macrophage-derived endocannabinoids have been shown to contribute to the hypotension in various forms of shock (4, 5, 11).

Lipopolysaccharide (LPS), an integral part of the outer membrane of Gram-negative bacteria, is a major pathogenic factor in septic shock. Macrophages are the primary target of LPS, which interacts with the CD14 protein/toll-like receptor-4 complex (12, 13) to activate multiple signaling pathways (14), which, in turn, activate a variety of transcription factors that regulate gene expression (15). LPS induces the expression of the cytokines TNF α , interleukin-1 (IL-1), IL-6, and IL-8, which have been implicated in the pathomechanism of septic shock (12). LPS also induces the production of lipid mediators in macrophages, such as prostaglandins (16), leukotrienes (17), and platelet-activating factor (PAF) (18). PAF acts at specific PAF receptors on macrophages and plays a key role as an autocrine mediator of the macrophage response to LPS stimulation (19, 20). PAF also stimulates the synthesis of 2-AG in mouse P388D1 macrophages without affecting AEA levels (21). Here we used the RAW264.7 mouse macrophage cell line as well as peritoneal macrophages from FAAH knockout mice and their wild-type littermates to define the cellular mechanisms by which LPS induces the production of endocannabinoids in macrophages and to determine the relative role of AEA and 2-AG in LPS-induced hypotension.

EXPERIMENTAL PROCEDURES

Materials—CD14 antibodies (N and C terminus) were from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059, JNK inhibitor, SB203580, wortmannin, MG132 were from Calbiochem. LPS (*Escherichia coli*, 0127:B8), actinomycin D, fatty acid-free bovine serum albumin, L- α -phosphatidylcholine, β -acetyl- γ -O-alkyl (PAF), *N*-palmitoyl-L- α -phosphatidylethanolamine, *Streptomyces chromofuscus* phospholipase D, GF109203X, cycloheximide, α -amanitin, and BTP were from Sigma. AEA and 2-AG were from Cayman (Ann Arbor, MI). AEA [1-³H]ethanolamine and [1-¹⁴C]1,2-phosphatidylcholine-L- α -diarachidonoyl were from American Radiolabeled Chemicals (St. Louis, MO). SR27417A was a gift from Sanofi-Synthelabo (Montpellier, France). SR141716 was obtained from the National Institute on Drug Abuse. ²H₄-labeled AEA was synthesized by the reaction of arachidonyl chloride with [²H₄]ethanolamine (22). [¹⁴C]N-arachidonyl phosphatidylethanolamine was synthesized as described (23).

Animals—FAAH^{-/-} and FAAH^{+/+} mice were obtained from heterozygote breeding pairs (24). Male Sprague-Dawley rats (100–150 g)

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¹ The abbreviations used are: AEA, arachidonoyl ethanolamide (anandamide); 2-AG, 2-arachidonoyl glycerol; FAAH, fatty acid amidohydrolase; NAPE, *N*-arachidonoyl phosphatidylethanolamine; NAT, *N*-acyltransferase; PAF, platelet-activating factor; MAPK, mitogen activated protein kinase; JNK, c-Jun amino-terminal kinase; PLD, phospholipase D; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane; TNF α , tumor necrosis factor- α ; LC/MS, liquid chromatography/mass spectrometry.

anesthetized with urethane, 0.7 g/kg of intravenous plus 0.3 g/kg of intraperitoneal, were used to monitor blood pressure responses to macrophages. Drugs or cell preparations were injected via a polyethylene cannula in the femoral vein. Mean arterial pressure was monitored via a cannula in the femoral artery, connected to a pressure transducer and computerized data acquisition system (EMKA Technologies, Arlington, VA).

Cell Culture and Extraction—Mouse RAW264.7 macrophages (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under standard conditions. To obtain primary macrophages, FAAH^{+/+} and FAAH^{-/-} mice were injected intraperitoneally with 4.0 ml of 2% thioglycolate, and peritoneal cells were harvested 3 days later. Macrophages isolated from four to six mice in each group were pooled, plated onto 75-cm² plastic flasks, and preincubated in 20 ml of RPMI 1640 medium containing 5% fetal bovine serum for 2 h at 37 °C in a humidified 5% CO₂ incubator. Nonadherent cells were removed by washing with 5% dextrose in phosphate-buffered saline. Macrophages were treated for 90 min with vehicle or a maximally effective concentration of LPS (10 ng/ml for RAW264.7 cells or 5 μg/ml for peritoneal macrophages) and washed, and aliquots of 10⁷ cells in 0.1 ml of phosphate-buffered saline were injected intravenously into urethane-anesthetized rats to test their effect on blood pressure. For measuring endocannabinoid levels, cells were washed twice with serum-free medium, and the cells plus medium were harvested and extracted with 2 volumes of chloroform/methanol (2:1) containing 7 nmol of [²H₄]AEA as internal standard. The upper layer was extracted two more times with ice-cold chloroform and deproteinized with 10 volumes of ice-cold acetone. The extract was dried under nitrogen and reconstituted in 50 μl of methanol for analysis by liquid chromatography/in-line mass spectrometry, using an Agilent 1100 series LC-MSD, equipped with a thermostatted autosampler and column compartment.

Endocannabinoid Levels—AEA and 2-AG in cellular extracts were quantified by LC/MS, as described (25). The mass-sensitive detector (model LS) was set for atmospheric pressure chemical ionization, positive polarity, and selected ion monitoring to monitor ions *m/z* 348 for AEA, 352 for [²H₄]AEA, and 379 for 2-AG. The amounts of AEA and 2-AG were determined using inverse linear regression of standard curves, produced using synthetic AEA and 2-AG. Values are expressed as pmol/10⁸ cells.

***N*-Arachidonoyl-phosphatidylethanolamine (NAPE) Levels**—NAPE in lipid extracts of cells was isolated by TLC and quantified by the amount of AEA released through its digestion with *S. chromofuscus* PLD, as described (25).

***N*-Acyltransferase Activity**—RAW264.7 cells were homogenized in 50 mM Tris buffer, pH 8.0, containing 0.32 M sucrose. Cell debris was removed by centrifugation at 1,000 × *g* for 1 min, and aliquots of the supernatant containing 2 mg of total cellular protein were incubated with 10⁶ dpm of 1,2-phosphatidylcholine-*L*-α-[1-¹⁴C]diarachidonoyl in a volume of 0.5 ml containing 0.01% Triton X-100 for 1 h at 37 °C. The reaction was stopped by the addition of 1 ml of chloroform/methanol (2:1, v/v), and the mixture was reextracted by 1 ml of methanol. Lipids were separated by TLC with chloroform/methanol/NH₄OH (80:20:1) as the mobile phase. The radioactivities of the lipid spots were quantified by a phosphorimaging device (Typhoon 8600). *N*-Acyltransferase activity was expressed as *N*-[¹⁴C]arachidonoyl NAPE formed per minute per mg of protein (26).

Activity of NAPE-specific PLD—Extracts of RAW264.7 cells were centrifuged at 1,000 × *g* to remove cell debris and then at 105,000 × *g* for 1 h. The pellet was solubilized for 1 h at 4 °C in 50 mM Tris buffer, pH 8.0, containing 0.5% Nonidet P-40, and then centrifuged again at 105,000 × *g* for 1 h. The pellets were resuspended in 200 μl of BTP buffer (pH 8.0). NAPE-PLD activity was measured as described (27), except that 5 mM CaCl₂ was added to the reaction mixture. Enzyme activity was expressed as the amount of [¹⁴C]AEA formed per min per mg of protein.

FAAH mRNA and FAAH Activity—FAAH and β-actin mRNA were quantified by reverse transcriptase-PCR (28), using appropriate primers. FAAH activity was quantified by the amount of the [³H]ethanolamine released from [³H]AEA labeled on the ethanolamine moiety (29).

RESULTS

LPS Induces AEA but Not 2-AG in RAW264.7 Cells—Incubation of RAW264.7 cells with LPS induced a time- and concentration-dependent increase in the cellular levels of AEA, with a maximal, ~10-fold increase observed at 90 min and 10 ng/ml LPS, whereas 2-AG levels remained unaffected (Fig. 1).

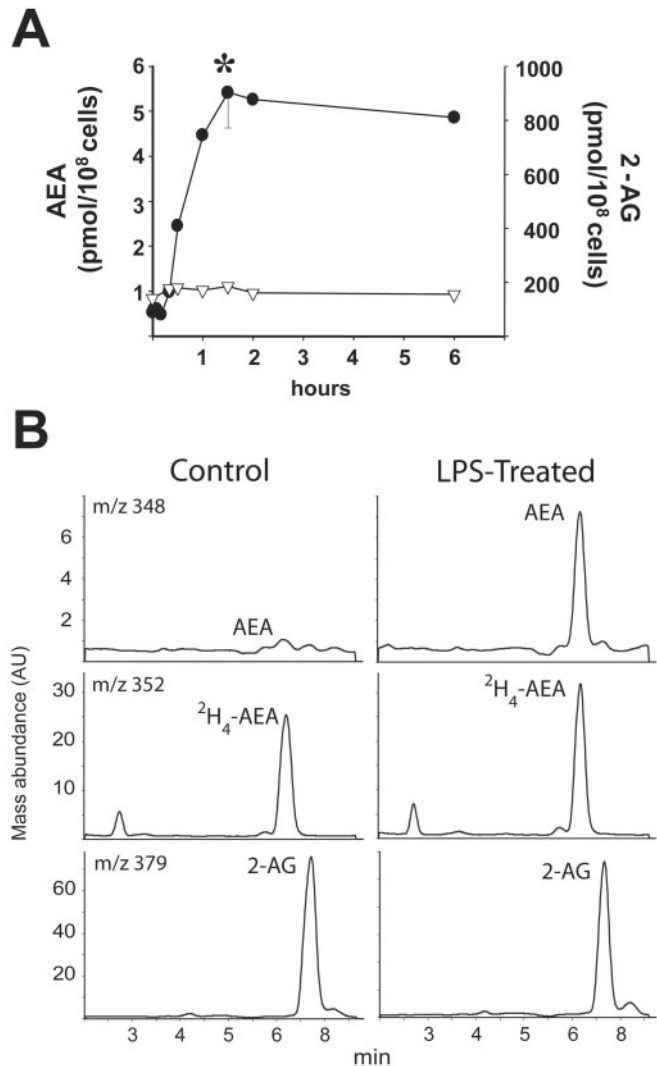


FIG. 1. Time-dependent increase in AEA, but not 2-AG, levels by LPS in RAW264.7 cells. A, time-dependent effect of LPS on AEA (●) and 2-AG (▽) levels in RAW264.7 cells. *, *p* < 0.05 from 0-min control value. *n* = 8 for 0- and 90-min time points; other values represent the mean of two experiments. B, ion chromatograms of AEA, [²H₄]AEA (internal control) and 2-AG.

These conditions were therefore used in subsequent experiments. The LPS-induced increase in AEA was largely prevented in the presence of 6 μg/ml actinomycin D or 10 μg/ml cycloheximide (Fig. 2). Actinomycin D was similarly effective at a lower concentration of 1 μg/ml (0.44 versus 0.95 pmol AEA/10⁸ cells without or with LPS, respectively), and another inhibitor of RNA polymerase II, α-amanitin (75 μg/ml, Ref. 30), also inhibited the LPS-induced increase in AEA levels (0.30 versus 0.60 pmol/10⁸ cells).

LPS Induction of AEA Involves CD14, p44/42 MAP Kinase, and NF-κB—Preincubation of RAW264.7 cells with anti-CD14 antibodies (1:100) overnight or with the combination of the p44/42 kinase inhibitor PD98059 (50 μM) and NF-κB inhibitor MG132 (20 μM) for 30 min almost completely blocked the effect of LPS, whereas incubation with PD98059, the phosphoinositide 3-kinase inhibitor wortmannin (100 nM), or MG132 alone partially reduced the increase in AEA caused by LPS. In contrast, inhibitors of protein kinase C (GF109203X, 2 μM), p38 kinase (SB203580, 1 μM), or Jun terminal kinase (JNK, 10 μM) did not affect the LPS-induced increase in AEA (Fig. 3).

LPS Up-regulates FAAH Expression and FAAH Activity in RAW264.7 Cells—The LPS-induced increase in AEA levels may

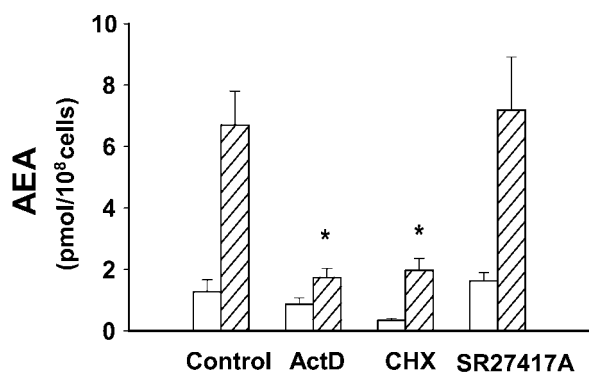


FIG. 2. The LPS-induced increase in AEA in RAW264.7 cells is blocked by actinomycin D (ActD) or cycloheximide (CHX) but not by the PAF receptor antagonist SR27147A. Cells were incubated with vehicle (open bars) or 10 ng/ml LPS for 90 min (hatched bars) in the presence of the indicated treatment. Columns and bars represent means \pm S.E. from six to eight experiments, and * indicates significant difference ($p < 0.05$) from value in LPS-stimulated control cells.

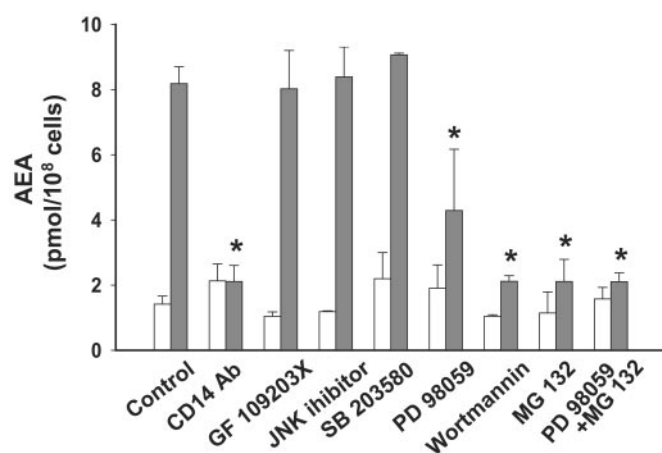


FIG. 3. Signaling pathways involved in the LPS induction of AEA synthesis. Confluent RAW264.7 cells were incubated with vehicle (open columns) or LPS (shaded columns) in the presence of the indicated agents. Columns and bars represent means \pm S.E. from five to eight experiments. * indicates significant difference from value in LPS-treated control cells ($p < 0.05$).

be due to increased synthesis, decreased degradation by FAAH, or both. Treatment of RAW264.7 cells with 10 ng/ml LPS resulted in a significant increase FAAH mRNA levels (Fig. 4A). A 90-min incubation with 10 ng/ml LPS also increased the enzymatic activity of FAAH, from 0.24 ± 0.02 to 0.33 ± 0.02 nmol/min/mg of protein ($p < 0.05$). This rules out decreased degradation of AEA being responsible for its increased levels in LPS-treated cells. Indeed, the LPS-induced increase in AEA levels was further enhanced in the presence of the FAAH inhibitor phenylmethylsulfonyl fluoride ($200 \mu\text{M}$, Fig. 4B).

LPS Increases NAPE Levels in RAW264.7 Cells—In untreated cells, the level of NAPE, the immediate precursor of AEA, was 2.2 ± 0.2 pmol/ 10^8 cells, which corresponds to ~ 8 times the cellular levels of AEA measured in the same cells (Fig. 5A). Treatment with 10 ng/ml LPS caused a ~ 3 -fold increase in NAPE, which was less than the ~ 10 -fold increase in AEA levels in the same cells, resulting in a decrease in the NAPE:AEA ratio to ~ 2.7 . This suggests that LPS increases both the generation of NAPE by *N*-acyltransferase and its subsequent metabolism through its PLD-catalyzed cleavage, yielding AEA. We therefore measured the activities of both *N*-acyltransferase (NAT)- and NAPE-specific PLD in homogenates of vehicle- and LPS-treated cells.

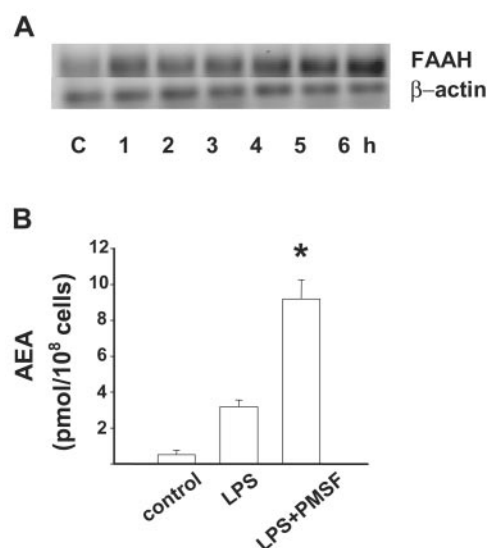


FIG. 4. LPS induces FAAH gene expression (A) and enzyme activity (B) in RAW264.7 cells. As shown in A, cells were incubated with vehicle (c) or with 10 ng/ml LPS. Total RNA was then isolated for reverse transcriptase-PCR. Reverse transcriptase-PCR for β -actin was done in the same samples as a loading control. As shown in B, cells were incubated for 90 min with vehicle or 10 ng/ml LPS. In a third group of cells incubated with LPS, $200 \mu\text{M}$ phenylmethylsulfonyl fluoride (PMSF) was added to the medium for the last 30 min of incubation before harvesting and extracting the cells for measurement of AEA by LC/MS. Columns and bars represent means \pm S.E. from three to five experiments, and * indicates significant difference ($p < 0.05$) from value with LPS alone.

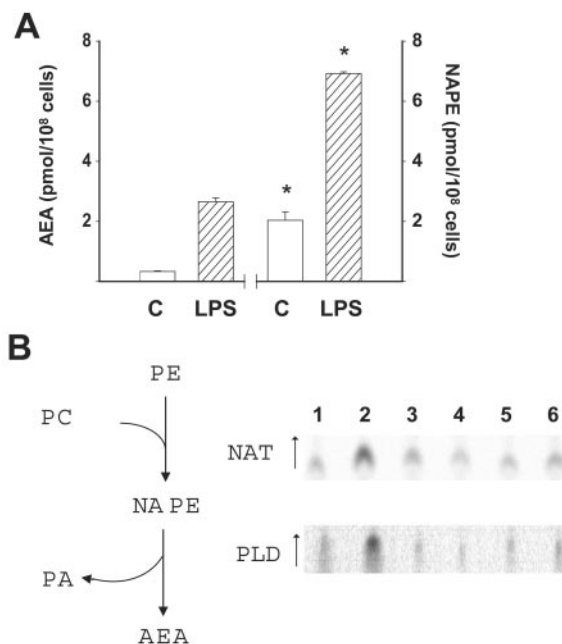


FIG. 5. The effects of LPS on AEA and NAPE levels (A) and on NAT and PLD activities (B) in RAW264.7 cells. In A, values represent means \pm S.E. from four to six experiments, and * indicates significant difference ($p < 0.05$) from AEA levels in the same treatment group (C, control). In B, radiochromatograms from representative experiments are shown. Mean values from replicate experiments are provided under "Results." Pretreatments were vehicle (lane 1), 10 ng/ml LPS (lane 2), $6 \mu\text{g/ml}$ actinomycin D (lane 3), actinomycin D + LPS (lane 4), $10 \mu\text{g/ml}$ cycloheximide (lane 5), and cycloheximide + LPS (lane 6). PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

LPS Increases Both *N*-Acyltransferase and NAPE-PLD Activities—As illustrated by a representative experiment, LPS treatment markedly increased NAT activity (Fig. 5B, lanes 1

and 2). In three replicate experiments, NAT activity was increased 3.6-fold, from 1.58 ± 0.60 to 4.02 ± 0.24 pmol⁻¹ min⁻¹ mg of protein⁻¹ ($p < 0.05$). LPS also increased PLD activity (Fig. 5B, lanes 1 and 2) by a mean of 2.8-fold, from 13.3 ± 2.7 to 36.6 ± 7.6 pmol min⁻¹ mg of protein⁻¹ in vehicle- versus LPS-treated cells, respectively ($n = 4$, $p < 0.05$). The LPS-induced increases in both NAT and PLD activity were completely blocked in the presence of either actinomycin D (lanes 3 and 4) or cycloheximide (lanes 5 and 6). These experiments were also replicated two more times with similar results.

LPS-treated Macrophages Elicit Hypotension in Rats—We tested the ability of vehicle- and LPS-treated macrophages to induce hypotension in urethane-anesthetized rats. Intravenous injection of 10^7 RAW264.7 cells preincubated with 10 ng/ml LPS for 90 min caused long lasting (>2 h) hypotension, which could be prevented by pretreatment of the recipient rat with the CB₁ receptor antagonist SR141716 (3 mg/kg). No hypotension was observed when rats were injected with cells preincubated with vehicle only or with LPS in the presence of actinomycin D (Fig. 6A).

In similar experiments, the hypotensive response to the injection of mouse peritoneal macrophages correlated with their AEA content (Fig. 6B). Vehicle-treated FAAH^{+/+} cells elicited no significant change in blood pressure, whereas FAAH^{+/+} cells treated with 5 μg/ml LPS and vehicle-treated FAAH^{-/-} cells caused a small but significant hypotensive response. A much greater decrease in blood pressure was elicited by LPS-treated FAAH^{-/-} cells, which had the highest AEA content. 2-AG levels were similar in vehicle-treated FAAH^{+/+} and FAAH^{-/-} cells (126 ± 48 versus 142 ± 38 pmol/10⁸ cells, $n = 7$) and were unaffected by LPS in both groups (197 ± 84 in FAAH^{+/+} versus 198 ± 65 pmol/10⁸ cells in FAAH^{-/-} cells, $n = 5$).

PAF Is Not Involved in the LPS Induction of AEA in RAW264.7 Cells—LPS can increase both the number of functional PAF receptors (31) and the production of PAF in macrophages (18), and PAF has been implicated as an autocrine mediator of the effects of LPS on macrophages (19, 20). We therefore tested the effects of PAF and a PAF receptor antagonist on endocannabinoid production in RAW264.7 cells in the absence or presence of LPS treatment. Incubation of cells with 100 nM of SR27417A, a potent and selective PAF receptor antagonist (32), did not affect the LPS-induced increase in AEA (Fig. 2). Incubation of cells with 30 nM PAF itself did not influence AEA levels but caused a small, statistically significant increase in 2-AG within 2 min (Fig. 7A), which was unaffected by actinomycin D (not shown) but was blocked by pretreatment with 100 nM SR27417A. The PAF-induced increase in 2-AG is consistent with the observation in anesthetized rats that the acute hypotensive response to an intravenous injection of 5 ng/kg PAF is inhibited by pretreatment with the CB₁ receptor antagonist SR141716 (3 mg/kg, Fig. 7B).

DISCUSSION

We have demonstrated that murine macrophages are capable of generating both AEA and 2-AG and that the cellular levels of these two endocannabinoids are differentially regulated. The results indicate that LPS acts through the cell surface CD14 molecule to selectively stimulate AEA synthesis through a transcriptional mechanism independent of PAF that targets the AEA biosynthetic enzymes *N*-acyltransferase and NAPE-PLD. In contrast, PAF acts through PAF receptors to elicit a more rapid increase in 2-AG but not AEA levels. The results also indicate that macrophage-derived AEA, but not 2-AG, is likely to contribute to the hypotensive response to LPS, whereas 2-AG may contribute to the acute hypotensive response to PAF.

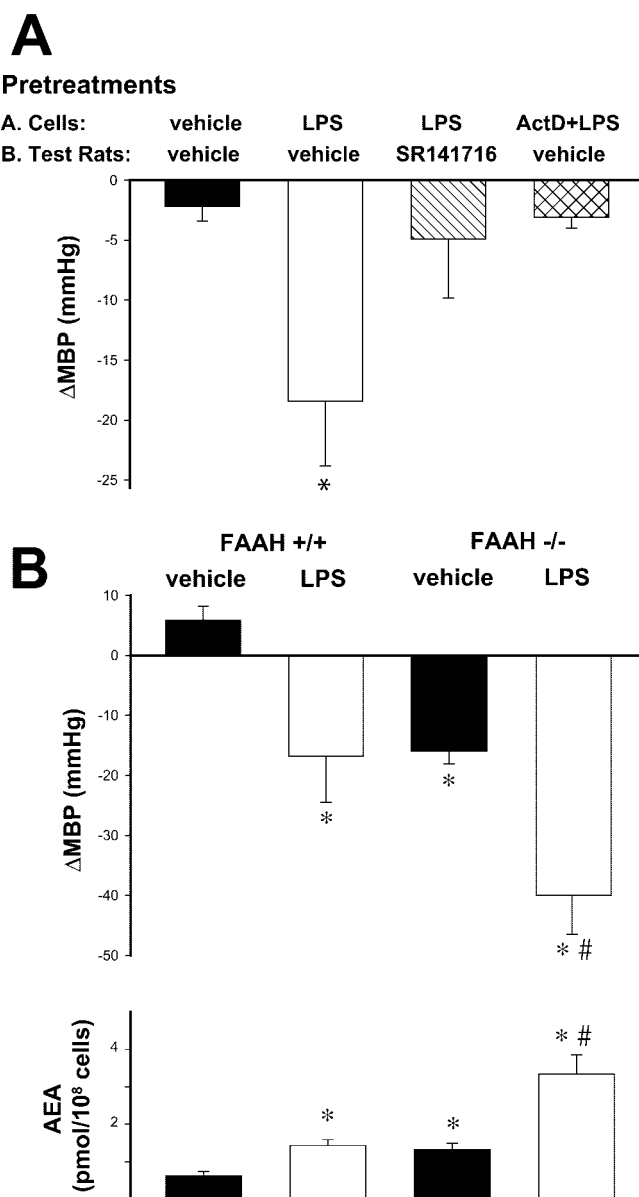


FIG. 6. Hypotensive response of anesthetized rats to intravenous injection of RAW264.7 cells (A) or mouse peritoneal macrophages (B) correlates with cellular AEA levels. A, RAW264.7 cells and test rats were pretreated as indicated. Basal mean blood pressure (MBP) was 89 ± 2 , 99 ± 2 , 95 ± 1 , and 92 ± 1 mm Hg in the control ($n = 4$), LPS ($n = 6$), LPS + Sr141716 ($n = 4$), and ActD + LPS groups ($n = 5$), respectively. Vertical bars represent means \pm S.E. * indicates significant difference from baseline blood pressure ($p < 0.05$). ActD, actinomycin D. B, blood pressure response (top) and AEA content (bottom) of peritoneal macrophages from FAAH^{+/+} and FAAH^{-/-} mice preincubated with vehicle or LPS. Significant difference ($p < 0.05$) from corresponding values in vehicle-treated (*) or LPS-treated (#) FAAH^{+/+} cells.

The calcium and PLD-dependent synthesis of AEA by macrophages, first reported in the rat JM771 macrophage cell line (3) and later also in RAW264.7 cells (33), is similar to the biosynthetic pathway of AEA in neurons (2). Subsequent studies have implicated macrophage- and platelet-derived endocannabinoids as mediators of the hypotension associated with various forms of shock (4, 5, 11) as well as the hypotension and vasodilated state in advanced liver cirrhosis (34, 35). Liver cirrhosis is often associated with endotoxemia (36), and bacterial endotoxin may be the common factor responsible for the increase in endocannabinoid production in these conditions. Indeed, *in vitro* incubation with LPS was shown to increase both AEA and

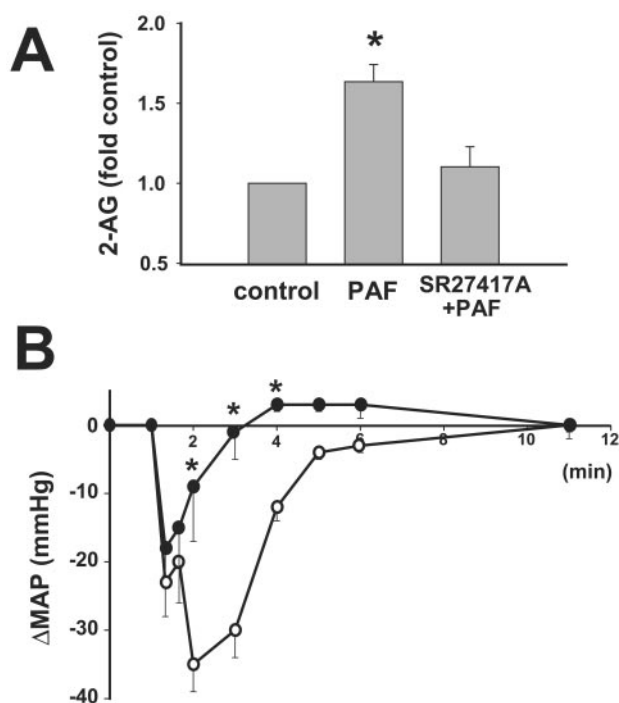


FIG. 7. PAF stimulates the production of 2-AG in RAW264.7 cells (A) and elicits cannabinoid receptor-mediated hypotension in anesthetized rats (B). (A) Cells were preincubated as indicated before measurement of their AEA and 2-AG contents by LC/MS. AEA levels were similar in the 3 groups (not shown). Columns and bars represent means \pm S.E. from three to five experiments, and * indicates significant difference from control value ($p < 0.05$). (B) PAF was injected at 1 min to control rats (\circ , $n = 4$) or rats pretreated with SR141716 (\bullet , $n = 4$). Vertical bars represent S.E. * indicates significant difference from corresponding value in controls ($p < 0.05$).

2-AG levels in circulating macrophages isolated from normal rat blood (5, 37).

PAF is an autocrine mediator of LPS-induced effects in macrophages (19, 20) and can increase 2-AG in P388D1 macrophages (21). Therefore, we tested whether PAF may be an obligatory intermediate of the LPS-induced production of endocannabinoids in RAW264.7 cells. LPS caused a 10-fold increase in AEA without affecting 2-AG levels, and the LPS-induced increase in AEA was unaffected by a PAF receptor antagonist (Fig. 2). This clearly indicates that PAF is not an intermediary in LPS-induced AEA synthesis. At the same time, we confirmed that PAF rapidly stimulates 2-AG production, but not AEA production, via PAF receptors (Fig. 7A). The effect of PAF was smaller than that reported in P388D1 cells (21), the difference most likely being related to the uniquely high expression of PAF receptors in P388D1 cells as compared with other macrophage cell lines including RAW264.7 (38). Therefore, different mechanisms are involved in the regulation of the two endocannabinoids, which could suggest that they have distinct physiological functions.

Monocytes isolated from LPS-treated rats elicit cannabinoid receptor-mediated hypotension when injected into normal rats (5). This implicated monocyte-derived endocannabinoids, but the respective roles of AEA and 2-AG could not be established. LPS-treated RAW264.7 cells also elicited SR141716-sensitive hypotension (Fig. 6A), and coinubation of the cells with actinomycin D prevented both the LPS-induced increase in AEA synthesis (Fig. 2) and the ability of the cells to elicit hypotension in recipient rats (Fig. 6A). This strongly suggests that this latter response is mediated by AEA and not 2-AG. Further support of this is provided by the finding that LPS-treated FAAH^{-/-} peritoneal macrophages elicited much greater hypo-

tension than similarly treated FAAH^{+/+} cells or vehicle-treated FAAH^{-/-} cells (Fig. 6B), which correlated with their AEA but not their 2-AG content.

LPS is known to promote macrophage adherence to endothelial cells by actions on both types of cells (39) and, although the lipophilic AEA may remain largely cell-associated (2, 3), this mechanism would deliver it in a "juxtacrine" manner to its site of action on the endothelium. Vascular endothelial cells express CB₁ receptors (28, 34, 40) as well as SR141716-sensitive receptors distinct from CB₁ or CB₂ (10, 41, 42). The involvement of these latter receptors is suggested by our observation² that the SR141716-sensitive hypotensive response to LPS remains unchanged in CB₁ knockout or CB₁/CB₂ double knockout mice.

The potentiation of the LPS-induced increase of AEA in FAAH^{-/-} macrophages is in good agreement with the LPS induction of FAAH in RAW264.7 cells, as both findings indicate that FAAH activity limits the ability of LPS to increase AEA levels. This is different from the situation in human lymphocytes, in which much higher concentrations of LPS (10–100 μ g/ml) have been reported to decrease rather than increase FAAH expression (43).

In contrast to the effects of LPS, exposure of RAW264.7 cells to PAF caused a PAF receptor-mediated, rapid (<2 min) increase in 2-AG without affecting AEA levels, and *in vivo* treatment of rats with PAF elicited an acute hypotensive response that was partially inhibited by the cannabinoid CB₁ receptor antagonist SR141716. This suggests that 2-AG may contribute to the acute hypotensive response to PAF. AEA and 2-AG may therefore have complementary biological roles as mediators of gradually developing, prolonged responses *versus* more rapid, acute physiological effects, respectively, at least within the cardiovascular system.

The effect of LPS on AEA levels peaked at 90 min and was prevented by inhibitors of RNA transcription (actinomycin D, α -amanitin) or protein synthesis (cycloheximide), suggesting the transcriptional activation of a factor or factors involved in AEA biosynthesis. The finding that the levels of NAPE, the immediate precursor of AEA, increased less than AEA itself suggests that LPS increases both the generation of NAPE via an *N*-acyltransferase and its further metabolism into AEA via a NAPE-specific PLD. Indeed, we could show that LPS increases both *N*-acyltransferase and PLD activity in RAW264.7 cells. These effects of LPS were similarly blocked by actinomycin D or cycloheximide (Fig. 5B), suggesting that LPS induces the expression of the genes for these enzymes, or of factors involved in their activation, such as protein kinases. Although the identity of the specific protein targets of LPS in the AEA biosynthetic pathway is not yet known, their transcription appears to be regulated by NF- κ B and p44/42 MAP kinase, but not by protein kinase C, Jun terminal kinases, or p38 kinase (Fig. 3). Neither the *N*-acyltransferase nor the NAPE-PLD have yet been identified by cloning, and the ability of LPS to induce AEA synthesis in RAW264.7 cells may be exploited to determine the molecular identity of these enzymes or the proteins that regulate their activity.

The transcription factor NF- κ B is a key signaling intermediate for LPS-induced responses in macrophages (15). Activation of NF- κ B can occur through activation of the I κ B kinase-2 (IKK β), which promotes I κ B degradation (44), and also through phosphoinositide-3 kinase-dependent phosphorylation of the transactivation domain of p65 (45). We found that either wortmannin, a phosphoinositide-3 kinase inhibitor, or MG132, a proteasome inhibitor that blocks I κ B degradation (46), is able

² Z. J arai, S. B atkai, P. Pacher, J. A. Wagner, and G. Kunos, submitted for publication.

to inhibit LPS-induced AEA synthesis, which implicates NF- κ B activation in this process. A recent report that AEA inhibits NF- κ B activation in lung adenocarcinoma cells (47) could suggest the existence of a negative feedback loop between NF- κ B and AEA, which could affect other NF- κ B-dependent mediators, such as TNF α (48). The relationship between AEA and TNF α is also interesting in terms of their relative role in the early (<6 h) phase of LPS-induced hypotension. Both TNF α and interleukin-1 β elicit hypotension in rats, which could be prevented by pretreatment with antibodies against these cytokines (49). The finding that similar pretreatment failed to significantly influence LPS-induced hypotension (49) suggests that these cytokines, although their production is increased by LPS (12), do not significantly contribute to the hemodynamic response to LPS.

AEA synthesis by macrophages is also induced in hemorrhagic shock (4), where the role of LPS is less clear. Oxidative stress was found to cause NF- κ B activation in hemorrhagic shock (50); thus, NF- κ B may be a common link among hypotensive conditions in which a paracrine vasodilator mechanism mediated by macrophage-derived endocannabinoids is activated. The present results indicate that AEA is likely to be this mediator.

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