Cyclooxygenase-1-Dependent Prostaglandin Synthesis
Modulates Tumor Necrosis Factor Alpha Secretion in
Lipopolysachharide-Challenged Murine Resident Peritoneal
Macrophages

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Running title: COX-1-Mediated TNF-α Suppression
Abstract: Comprehensive studies of prostaglandin (PG) synthesis in murine resident peritoneal macrophages (RPM) responding to bacterial lipopolysaccharide (LPS) revealed that the primary PGs produced by RPM were prostacyclin and PGE$_2$. Detectable increases in net PG formation occurred within the first hour, and maximal PG formation had occurred by 6 to 10 hr after LPS addition. Free arachidonic acid levels rose and peaked at 1-2 hr after LPS addition and then returned to baseline. Cyclooxygenase-2 (COX-2) and microsomal PGE synthase levels markedly increased upon exposure of RPM to LPS, with the most rapid increases in protein expression occurring 2 to 6 hr after addition of the stimulus. RPM constitutively expressed high levels of COX-1. Studies using isoform selective inhibitors and RPM from mice bearing targeted deletions of ptgs-1 and ptgs-2 demonstrated that COX-1 contributes significantly to PG synthesis in RPM, especially during the initial 1-2 hr after LPS addition. Selective inhibition of either COX isoform resulted in increased secretion of tumor necrosis factor-alpha (TNF-$\alpha$), however this effect was much greater with the COX-1 than with the COX-2 inhibitor. These results demonstrate autocrine regulation of TNF-$\alpha$ secretion by endogenous PGs synthesized primarily by COX-1 in RPM, and suggest that COX-1 may play a significant role in the regulation of the early response to endotoxemia.

**Key Words:** prostaglandin ⋅ macrophage ⋅ lipopolysaccharide ⋅ cyclooxygenase-1 ⋅ cyclooxygenase-2 ⋅ arachidonic acid ⋅ cytosolic phospholipase A$_2$ ⋅ tumor necrosis factor-alpha
Cyclooxygenase (prostaglandin G/H synthase, COX) catalyzes the first two steps in the conversion of arachidonic acid (20:4) to prostaglandins (PG), prostacyclin, and thromboxane (1-4). Two isoforms of COX have been identified and characterized (5). They share a 60% sequence identity, with nearly superimposable three-dimensional structures, and highly similar active sites (6-9). Kinetically, the two isoforms are virtually indistinguishable when 20:4 is used as substrate (10). However, subtle differences in the active sites allow the COX-2 isoform to use neutral ester and amide derivatives of 20:4 that are not utilized as effectively by COX-1 (11,12). These structural differences in the active sites have allowed the development of selective inhibitors for each isoform (13-19).

Although the structures and kinetics of the two COX isoforms are very similar, the enzymes differ considerably with regard to transcriptional regulation. Cells that contain COX-1 usually express the enzyme constitutively, whereas COX-2 expression is induced in response to a variety of inflammatory and proliferative stimuli, including cytokines, growth factors, and tumor promoters. As a consequence of these varied expression patterns, COX-1 is believed to be primarily responsible for "housekeeping" functions, such as gastric cytoprotection and regulation of platelet aggregation. In contrast, COX-2 is thought to be involved in the inflammatory response, pyresis, and in carcinogenesis (20-25). Consistent with these varying roles, two patterns of PG synthesis have been recognized. "Immediate" PG production occurs in response to stimuli that cause a rapid release of 20:4 from cellular phospholipid through strong activation of cytosolic phospholipase A₂ (cPLA₂). The release of 20:4 occurs quickly, and PG synthesis utilizes whichever of the two enzymes (COX-1 or COX-2) is already present in the cells. In most cases, PG formation is complete within one to two hours. Alternatively, "delayed" PG production occurs in response to stimuli that result in transcription of the COX-2 gene. Under
these circumstances, PG formation is usually not detectable until two or more hours after introduction of the stimulus, and may continue for prolonged periods (up to 24 hours or more). REF (26-28). Previous studies have demonstrated that delayed PG synthesis is solely COX-2-dependent, even when COX-1 is present in the cells (27-35). This has led to the suggestion that delayed PG synthesis results from the release of a restricted pool of 20:4 that is available only to COX-2. This may occur, in part, through selective coupling of COX isoforms to specific secretory PLA₂s or through distinct subcellular localization (28,36). Others have suggested that the release of 20:4 during delayed PG synthesis is too slow to provide adequate oxidant to activate COX-1. Both enzyme isoforms require activation through a reaction with hydroperoxide. However, COX-1 has been shown to require higher concentrations of peroxide than COX-2 for activation in vitro (4,28,35,37-48).

Bacterial lipopolysaccharide (LPS) is a stimulus of delayed PG synthesis in a variety of cells including macrophages. Macrophages challenged with LPS also secrete a number of cytokines, among them tumor necrosis factor-alpha (TNF-α). Prior studies have demonstrated potential interrelationships between PG synthesis and TNF-α secretion in LPS-treated macrophages. In particular, PGE₂ and prostacyclin have been shown to suppress TNF-α secretion in macrophages and/or peripheral blood monocytes responding to LPS. (49-56). Most of these studies have focused on the effects of exogenous PGs on LPS-stimulated TNF-α secretion. However, if adequate quantities of PGE₂ and/or prostacyclin are produced with appropriate timing, the potential exists for autocrine regulation of TNF-α secretion by endogenous PGs in macrophage populations.

Here we report detailed studies of the LPS-mediated release of 20:4 and the synthesis of PGs by murine primary resident peritoneal macrophages (RPM). Our studies reveal that RPM
release PGs much more rapidly and in greater quantities than can be explained entirely by COX-2-dependent synthesis. These cells constitutively express high quantities of the COX-1 isoform, and studies with selective isoform inhibitors and cells from mice harboring a targeted deletion of the ptgs-1 or ptgs-2 gene indicate that COX-1 contributes significantly to PG synthesis by RPM in response to LPS. Furthermore, we demonstrate that TNF-α secretion is suppressed in LPS-treated RPM by endogenous PGs primarily synthesized by COX-1.

EXPERIMENTAL PROCEDURES

RPM Cultures - All studies involving animals were done with the approval of the Institutional Animal Care and Use Committee of Vanderbilt University. Female wild-type (WT) C3H/HeN and CD-1 mice (25-30g) were obtained from Charles River Laboratories. Female mice (11-12 weeks of age) bearing a targeted deletion of the gene for COX-1 (COX-1−/−) on a C57BL/6 background (57) and WT littermate controls (COX-1+/+) were from Taconic (B6;129P2-Ptgs1tm1 Unc homozygous and WT). Female mice (11-12 weeks of age) bearing a targeted deletion of the gene for COX-2 (COX-2−/−) on a C57BL/6 background (58) and WT littermate controls (COX-2+/+) were also from Taconic (B6;129P2-Ptgs2tm1 Smi homozygous and WT). Female COX-1−/− and COX-2−/− mice on a CD-1 background (25-30 g) were bred in the Vanderbilt University animal care facility by the S.K. Dey laboratory as described (59). Mice were euthanized with carbon dioxide, and the peritoneal cavities were lavaged with a total of 3 mL of ice cold calcium- and magnesium-free phosphate-buffered saline (PBS). Peritoneal cells were collected by centrifugation of lavage fluid and resuspended at a concentration of 2 to 3 x 10⁶ cells/mL in Minimal Essential Alpha Medium supplemented with Gluta-Max (Invitrogen), containing 10% heat inactivated serum (Summit Biotechnologies) plus 100 Units/mL penicillin.
and 0.10 mg/mL streptomycin (Sigma) (a-MEM/FCS). The cell suspension was plated onto 35 mm tissue culture dishes at 2 mL per dish and incubated for 2 hours at 37°C in a humidified 5% CO₂ atmosphere. Non-adherent cells were removed by washing the plates four times with PBS, and the cultures were then incubated overnight in 2 mL of fresh a-MEM/FCS. The mean protein content of RPM cultures was 100 ± 10 µg/dish (8.2 ± 0.8 x 10⁵ cells/dish).

LPS Treatment of Macrophage Cultures - Cultures of RPM were washed twice with PBS at 37°C, and then overlaid with 1 mL of fresh, serum-free a-MEM. Following a 1 hr incubation, an additional 1 mL of medium with or without LPS (200 ng/mL) (E. coli 011:B4, Calbiochem) was added. Cells were incubated for the desired time periods. For the determination of COX-1, COX-2, cPLA₂, and microsomal PGE synthase-1 (mPGES-1) expression as well as PG synthesis and TNF-α secretion, the medium was collected from each culture, and the cells were then washed twice with ice cold PBS. Cell monolayers were scraped into 200 µL of lysis buffer (50 mM Tris-HCl, pH 7.5, plus 150 mM NaCl, 4 mM EDTA, 50 mM NaF, 0.1 mM Na₃VO₄, 0.2% Triton X-100, 0.1% NP40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 1 mg/mL AEBSF, and 5 µg/mL each of antipain, leupeptin, chymostatin, and pepstatin (all components from Sigma)). Cell lysates were allowed to stand for 30 min on ice with occasional vortex mixing, and particulate material was then removed by centrifugation for 10 min at 16,000 x g. Samples of culture medium and cell lysates were stored at –80°C until analyses could be completed. For the measurement of free 20:4, the medium was removed from the cell cultures and combined with 1 mL of ice cold acetonitrile containing 100 ng of 20:4-d₈ (Cayman). The cells were scraped twice into a total volume of 1 mL of ice-cold methanol, and the resulting cell lysate was added to the medium solution. In some experiments, the medium was collected and placed in one tube containing 1 mL of the internal standard solution, and then the cell lysate in 1 mL of methanol
was placed into a separate tube containing 1 mL of the internal standard solution. This allowed separate determination of lipid levels in the medium versus cells.

For the evaluation of the effects of selective COX inhibitors, cells were incubated in serum-free medium containing 100 nM of the COX-2 inhibitor, SC-236, and/or the COX-1 inhibitor, SC-560 (both from Calbiochem) for 1 hr prior to LPS addition. Incubation with LPS was then carried out in the ongoing presence of the inhibitors. The inhibitors were added as 100 μM stock solutions in DMSO, and the DMSO concentration in all cultures was maintained at 0.1% for these experiments. In some experiments in which cells had been pretreated with SC560, PGE₂ and or carbaprostacyclin (cPGI₂) (both from Cayman) were added to the culture medium at the same time as LPS. These compounds were prepared as stock solutions in DMSO prior to addition to cell cultures. The final DMSO concentration in all cultures was maintained at 0.2% for these experiments.

**Immunoblotting for Protein Expression** - The protein concentrations of 20 μL aliquots of macrophage cell lysates were determined using a BCA Protein Assay kit (Pierce) according to manufacturer’s directions. Macrophage lysate samples containing 15 μg of protein were then subjected to SDS polyacrylamide gel electrophoresis using an 8% gel (12% for mPGES-1) overlaid with a 3% stacking gel. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore), which was then incubated with 5% milk protein (Santa Cruz) in 1 mM Tris-HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20 (TTBS). COX-2 protein was detected following incubation with a rabbit polyclonal antibody directed against amino acids 570-598 of the murine enzyme (Cayman) diluted 1:30,000 in TTBS plus 0.1% milk protein. COX-1 protein was detected following incubation with a rabbit polyclonal antibody directed against amino acids 274-299 of the murine enzyme (Cayman) diluted 1:1,000. cPLA₂
protein was detected following incubation with a rabbit polyclonal antibody directed against amino acids 1-216 from the human enzyme (Santa Cruz) diluted 1:500. mPGES-1 protein was detected following incubation with a rabbit polyclonal antibody directed against amino acids 59-75 from the human enzyme (Cayman) diluted 1:1000. The secondary antibody for detection of all proteins was horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) used at a 1:2000 dilution in TTBS plus 0.1% milk protein. After the membranes were overlaid with ECL detection reagent (Amersham Pharmacia), the chemiluminescence signal intensity was measured using a Fluor-S Max Multi-Imager (BioRad). The membranes were also exposed to Hyperfilm ECL film (Amersham Pharmacia) to obtain photographic images.

Assay of PGs - PG levels in culture medium were determined by negative ion gas chromatography/mass spectrometry (GC/MS) of pentafluorobenzyl ester derivatives as previously described (60), or by selected reaction monitoring of the ammoniated ions by positive ion electrospray ionization liquid chromatography/mass spectrometry (LC/MS/MS) as described2.

Determination of Total 20:4 Levels - Samples were extracted with 12 mL of a solution of ethylacetate:hexane (90:10), and the organic extracts were evaporated to dryness under a stream of argon. The dried organic extracts were reconstituted in 1 mL of hexane and each was applied to a dry silica solid phase extraction column (Sep Pak Vac, 100 mg, Waters). The columns were washed with 3 mL of hexane. The columns were then eluted with 4 mL of ethylacetate:methanol:acetic acid (94.9:5:0.1, v:v:v) the first mL of which was used to rinse the sample tubes. The eluates were evaporated to dryness, reconstituted in 100 μL of acetonitrile, and analyzed for 20:4 by silver ion coordination LC/MS/MS as described (61).
Assay for TNF-α - The concentration of TNF-α in cell culture medium was determined by using an OptEIA assay kit (PharMingen) according to manufacturer’s instructions.

RESULTS

Synthesis of PGs by RPM in Response to LPS - Murine RPM from C3H/HeN mice were incubated in the presence or absence of 100 ng/mL of LPS for periods of up to 24 hr, and the culture medium was analyzed for PGs by GC/MS. RPM secreted primarily prostacyclin (detected as its hydrolysis product, 6-keto-PGF₁₀⁻⁻) and PGE₂ in response to LPS (along with minor amounts of TXA₂, which was detected as its hydrolysis product, TXB₂) (Fig. 1A). Increased levels of PGs were detectable at the earliest time point measured (1 hr), but the most rapid rate of PG synthesis occurred from 1 to 6 hr (Fig. 1B). It is notable that PGE₂ was not the major product secreted by LPS-challenged RPM, but as shown in Fig. 1C, the relative proportion of PGE₂ increased in RPM cultures during the course of the incubation. By 24 h, 6-keto-PGF₁₀⁻⁻ reached levels of 3,300 ± 300 pmol/10⁷ cells, and PGE₂ reached levels of 2,100 ± 300 pmol/10⁷ cells (Fig. 1A, and Table I). In the absence of LPS, 6-keto-PGF₁₀⁻⁻ and PGE₂ levels remained constant at 70-120 pmol/10⁷ cells and 18-27 pmol/10⁷ cells, respectively.

Changes in 20:4 Levels in RPM During the LPS Response - In order to assess the availability of substrate for PG synthesis by LPS-challenged RPM, we measured total 20:4 levels (medium plus cells) in RPM cultures at varying times after addition of the stimulus. An elevation of free 20:4 was observed by 1 to 2 hr after LPS addition with maximal values reaching 250-400 pmol/10⁷ cells, (Fig. 1D and Table II). Levels returned to control values by 6 hr. In the absence of LPS, free 20:4 remained constant at 90-140 pmol/10⁷ cells in RPM cultures.
The data in Fig.1D reflect total free 20:4 present in the combined medium and macrophage cell lysates. In separate experiments, RPM cultures were incubated for 0, 2, or 6 hr in the presence of LPS, and cells plus the medium were analyzed separately for free 20:4 content. The results showed that approximately 73-77% of the free 20:4 was localized to the cells regardless of the period of incubation with LPS. These results indicate that the maximum intracellular 20:4 content in RPM during LPS incubation reached values of 180-300 pmol/10^7 cells.

**Changes in PG Synthetic Enzyme Levels in LPS-Challenged RPM** - Prior evidence has indicated that PG synthesis by macrophages in response to LPS is due exclusively to COX-2, implying that it cannot occur prior to COX-2 induction. As noted in Fig. 1A & B, however, a significant increase in net PG synthesis occurs in RPM within the first hour of incubation with LPS, before one would expect *de novo* COX-2 protein expression to have occurred. We therefore performed immunoblot analysis of RPM cell lysates to determine whether these cells constitutively express COX-2, and to ascertain the rate of appearance of new COX-2 protein. As shown in Fig. 2A & B, COX-2 levels were undetectable in RPM prior to LPS treatment and only a trace amount of the protein was observed after 1 hr of incubation. Protein levels markedly increased between 2 and 6 hr, and maximal levels were reached at 6 to 10 hr. Thus the PG synthesis occurring in RPM cultures during the first hour of LPS incubation could not easily be attributable to constitutive COX-2 expression.

PGE_2_ synthesis is dependent on three isoforms of PGE synthase, one that is cytosolic (cPGES), and two that are microsomal (mPGES-1 and mPGES-2) (62,63). LPS-dependent induction of mPGES-1 has been described in a number of cell systems (64-66), and such induction should explain the change in relative PGE_2_ synthesis observed in RPM during the LPS
response in these cells. As shown in Fig. 2C & D mPGES-1 protein levels markedly increased in RPM cultures in the presence of LPS.

The primary enzyme responsible for the release of free 20:4 in response to LPS is cPLA$_2$, and induction of cPLA$_2$ in macrophages by LPS treatment has been reported (67,68). As shown in Fig. 2E & F, cPLA$_2$ was constitutively expressed in RPM. A gradual increase in cPLA$_2$ expression was observed after LPS treatment, although this increase was small compared to those observed for COX-2 and mPGES.

**Role for COX-1 in the Macrophage LPS Response** - Although PG synthesis in response to LPS is believed to be exclusively attributable to COX-2 in most systems, the relatively rapid PG secretion by RPM during the first hour of LPS incubation led us to consider the possibility that this early response could be attributable to COX-1. As seen in Fig. 2G & H, a strong chemiluminescence signal corresponding to COX-1 protein suggested high levels of constitutive expression of this enzyme in RPM. This observation is not surprising, considering the ability of these cells to rapidly produce very large quantities of PGs in response to stimuli such as zymosan, immune complexes, phorbol ester, and calcium ionophores without prior treatment with agents that induce COX-2 expression (69-75).

In order to test the hypothesis that COX-1 contributes to LPS-mediated PG synthesis, we incubated RPM cultures with LPS in the presence and absence of 100 nM concentrations of SC560 and/or SC236. SC560 is reported to be a selective COX-1 inhibitor (IC$_{50}$ COX-1 = 9 nM, IC$_{50}$ COX-2 = 6.3 μM (76)), whereas SC236 is considered selective for COX-2 inhibition (IC$_{50}$ COX-2 = 5-10 nM, IC$_{50}$ COX-1 = 17 μM (77,78)). RPM were incubated for 1 hr in the presence or absence of these inhibitors, alone or in combination, followed by the addition of LPS. Cultures were incubated for 2 or 6 hr after LPS addition. As seen in Fig. 3A, after 2 hr of
incubation with LPS, SC236 caused an approximately 35% reduction in the synthesis of 6-ketoPGF\(_{1\alpha}\), and an approximately 30% reduction in the synthesis of PGE\(_2\). In contrast, SC560 caused a 90% inhibition of the synthesis of both PGs. The presence of both inhibitors resulted in a slightly greater inhibition than that seen with SC560 alone. In contrast, after 6 hr of incubation, each of the inhibitors resulted in an 85 - 90% inhibition of both PGE\(_2\) and 6-ketoPGF\(_{1\alpha}\) synthesis, and the presence of both inhibitors resulted in a nearly 100% inhibition of PG formation (Fig. 3B). The inhibitors had no effect on free 20:4 content in the cell cultures after 2 or 6 hr of incubation with LPS, indicating that they did not cause a marked alteration in the availability of substrate (Fig 3C). A small (approximately 20%) decrease in COX-2 protein expression was observed in the presence of either or both inhibitors as judged by chemiluminescence signal on immunoblot analysis (Fig. 3D).

The greater degree of inhibition of PG synthesis by SC560 than SC236 after 2 hr of incubation with LPS supports the hypothesis that COX-1 plays a role in this process. Since COX-2 levels are still relatively low at the 2 hr time point in RPM, it is reasonable that the contribution of COX-1 should be relatively greater at this time point. However, the observation that both SC236 and SC560 caused a >85% inhibition of PG synthesis at the 6 hr time point is inconsistent with the assumption that each inhibitor affects only the activity of the COX isoform for which it is selective. This apparent lack of specificity, which will be discussed in greater detail below, makes it difficult to draw conclusions concerning the role of COX-1 in LPS-mediated PG synthesis with complete confidence based solely on inhibitor data.

In order to further explore the possible role of COX-1 in LPS-mediated PG synthesis, we isolated RPM from WT mice and mice bearing a targeted deletion of the gene encoding either COX-1 or COX-2 on the CD-1 background. RPM cultures were incubated for 0, 2, or 6 hr in the
presence of 100 ng/mL LPS. Cell lysates were then analyzed by immunoblot for COX-1, COX-2, and cPLA₂ protein expression. Constitutive COX-1 levels were similar in COX-2⁻/⁻ and WT macrophages, whereas this protein was undetectable in COX-1⁻/⁻ macrophages (Fig. 4A & B). The induced expression of COX-2 was slightly lower in COX-1⁻/⁻ than in WT macrophages, and undetectable in COX-2⁻/⁻ cells (Fig. 4C & D). The expression of cPLA₂ was similar in macrophages of all three genotypes, with a tendency toward slightly increased expression in COX-2⁻/⁻ cells (Fig. 4E & F).

RPM from CD-1 COX-1⁻/⁻ mice produced very low levels of PGs after 2 hr of incubation with LPS, and by 6 hr, the levels achieved were only approximately 20% as high as those produced by CD-1 WT cells (Table III). These results support the hypothesis that COX-1 contributes significantly to PG synthesis in RPM, and is relatively more important at the early time points. This conclusion was supported by the pattern of PG synthesis observed in CD-1 COX-2⁻/⁻ RPM. These cells produced large quantities of PGs after only 2 hr of incubation, reaching levels approximately 14-fold greater than those produced by WT cells in the same time period. Between 2 hr and 6 hr, COX-2⁻/⁻ RPM synthesized additional PGs, reaching levels approximately 83% as great as those produced by WT cells. Thus, COX-1 is clearly able to respond to the LPS stimulus with PG synthesis in CD-1 COX-2⁻/⁻ RPM. However, the time course of PG formation is markedly different from that seen in WT cells, suggesting that compensatory mechanisms actually promote COX-1 activity in this RPM population.

These experiments were repeated using COX-1⁻/⁻ and COX-2⁻/⁻ mice on a C57BL/6 background. Age and sex-matched WT littermates were used as controls. As observed in CD-1 mice, immunoblot analysis showed no difference in COX-1 expression between COX-2⁻/⁻ and WT mice or in COX-2 expression between COX-1⁻/⁻ and WT mice. The expression of cPLA₂
was similar irrespective of the genotypes. The immunoblots also confirmed the absence of the protein for which the gene had been deleted (data not shown).

RPM from C57BL/6 COX-1\textsuperscript{−/−} mice demonstrated a very similar pattern of PG synthesis as had been observed in the case of RPM from CD-1 COX-1\textsuperscript{−/−} mice. PG synthesis was very low 2 hr after LPS addition, and remained reduced as compared to littermate WT RPM (COX-1\textsuperscript{+/+}) after 6 hr of incubation (Table III). Notably, however, the COX-1\textsuperscript{−/−} RPM produced 45% as much PG as their WT controls, as compared to only 20% for the corresponding experiment using CD-1 COX-1\textsuperscript{−/−} RPM. In the case of RPM from C57BL/6 COX-2\textsuperscript{−/−} mice, the pattern of PG synthesis was very different from that obtained from CD-1 COX-2\textsuperscript{−/−} RPM. Rather than rapid, high levels of PG production, C57BL/6 COX-2\textsuperscript{−/−} produced very low levels of PGs throughout the 6 hr incubation period, reaching a maximum of only about 3% of the WT control RPM (COX-2\textsuperscript{+/+}). These results suggest that in RPM from C57BL/6 mice COX-1 contributes to LPS-dependent PG production, since RPM from COX-1\textsuperscript{−/−} mice synthesize lower quantities of PGs than their WT counterparts. However, the results also appear to indicate that COX-1 cannot function in the absence of COX-2 in C57BL/6 RPM, since COX-2\textsuperscript{−/−} RPM produce almost no PGs even though their expression of COX-1 is comparable to that in WT mice.

The marked difference observed in PG synthesis between C57BL/6 and CD-1 COX-2\textsuperscript{−/−} RPM suggests a fundamental difference between the two strains of mice in their adaptation to the COX-2 gene deletion. In this context, it is interesting to note that RPM from WT CD-1 mice produce higher levels of PGs in response to LPS than do RPM from C3H/HeN mice, which in turn, produce higher levels of PGs than do RPM from C57BL/6 mice (Table I). Furthermore, the increase in free 20:4 levels after 2 hr of incubation with LPS is similar in WT CD-1 and C3H/HeN RPM, but lower in WT C57BL/6 RPM (Table II). It is notable that this difference in
20:4 release was not due to a marked difference in cPLA<sub>2</sub> expression between the strains as demonstrated by comparative immunoblot analysis (data not shown). A direct comparison of free 20:4 in RPM used for the gene deletion experiments indicated that levels were similar in LPS-stimulated CD-1 COX-1<sup>+/+</sup> and WT RPM, but levels in COX-2<sup>−/−</sup> RPM were significantly higher (p < 0.05, Table IV). In contrast, there was little to no increase in free 20:4 levels after 2 hr of LPS treatment in any of the C57BL/6 RPM cultures (Table IV). The apparent lower release of free 20:4 may correlate with the lower quantities of PGs synthesized in C57BL/6 RPM as compared to RPM from the other strains that were studied, and may contribute to the differences in PG synthesis observed between CD-1 and C57BL/6 COX-2<sup>−/−</sup> RPM.

**COX-1-Dependent Autocrine Suppression of TNF-α Secretion in LPS-Challenged RPM**

The suppression of TNF-α secretion by PGE<sub>2</sub> and prostacyclin has been widely reported. This suppression is based on an inhibition of TNF-α mRNA synthesis, which occurs during the first 1 - 3 hr of LPS treatment(79-86). Therefore it was reasonable to hypothesize that the ability of RPM to produce significant quantities of PGs during the first two hours after LPS addition might lead to autocrine suppression of the secretion of this cytokine. As shown in Fig. 5A, TNF-α was undetectable in the medium of RPM cultured in the absence of LPS, and in LPS-treated cells, increases in the level of the cytokine were detectable only after 2 hr of incubation. TNF-α concentrations increased rapidly between 2 hr and 4 hr after LPS addition, reached a maximum (51 ± 20 ng/10<sup>7</sup> cells) at 6 hr of incubation and then decreased thereafter. The fact that TNF-α levels drop after 6 hr in RPM culture medium indicates that the cytokine must be unstable under these conditions and that RPM no longer produce it at a rate sufficient to compensate for the rate of disappearance. Whether the decrease in TNF-α is due to enzymatic degradation by RPM or to nonenzymatic decomposition is not known.
To test the hypothesis that early, COX-1-dependent PG synthesis suppresses TNF-α secretion, we examined the effects of SC236 and SC560, on the levels of this cytokine in the medium of LPS-treated RPM. Cells were preincubated for 1 hr with 100 nM of SC236 and then incubated for an additional 6 hr with LPS. This treatment effected a small (1.8-fold) but significant increase in LPS-dependent TNF-α secretion. In contrast, when SC560 was used at the same concentration, a 5.9-fold increase in TNF-α secretion occurred. Incubation with both inhibitors produced results similar to those with SC560 alone (Fig. 5B).

The finding that COX inhibitors cause increased TNF-α secretion in LPS-treated RPM suggests that endogenous PGs suppress TNF-α secretion in those cells. Furthermore, the fact that this effect is much more striking with SC560 than SC236 implies that COX-1 is the primary source of the suppressive PGs. The latter conclusion is confounded by the fact that both SC236 and SC560 cause a similar (85-90%) inhibition of PG synthesis in RPM after a 6 hr incubation with LPS (Fig. 3B). In should be remembered, however, that after only 2 hr of LPS exposure, the differential inhibition of PG synthesis between the two inhibitors in RPM is striking, as SC236 produces a much lower suppression of PG synthesis (30-35%) than that observed with SC560 (90%, Fig. 3A). This observation is attributable to the inability of SC236 to suppress the relatively high proportion of COX-1-dependent PG synthesis that occurs early in the RPM LPS response when TNF-α mRNA is being synthesized.

The above findings suggest that SC560 has a greater effect on RPM TNF-α secretion than does SC236 as a result of its better ability to inhibit the formation of PGs resulting primarily from COX-1 activity early in the LPS response. However, it is also possible that the effects of SC560 on the response of RPM to LPS are unrelated to its suppression of PG synthesis. In order to investigate this possibility, we treated RPM with LPS in the presence of SC560 plus varying
concentrations of PGE$_2$ and/or cPGI$_2$, a stable prostacyclin analog. The concentrations of PGs ranged from 3.5 to 57 nM, corresponding roughly to the concentrations of the PGE$_2$ and prostacyclin appearing in the medium of RPM during 1 through 6 hr of incubation with LPS in the absence of SC560 (Table I). The results (Fig. 5C) demonstrate that the increased TNF-α formation observed in SC560-treated RPM incubated with LPS is reversed efficiently by the combination of PGE$_2$ and cPGI$_2$. PGE$_2$ and cPGI$_2$ alone were not as effective as the combination of the two PGs. These results support the hypothesis that the effect of SC560 on TNF-α secretion in LPS-treated RPM is due to suppression of PG synthesis in these cells.

Our results using selective COX inhibitors suggest that COX-1-dependent PG synthesis inhibits TNF-α secretion in LPS-treated RPM. If this were the case, then one would expect that genetic mutation of the gene for COX-1 should lead to increased TNF-α secretion in RPM responding to LPS. We tested this hypothesis by comparing the LPS response in RPM from CD-1 WT mice with RPM from CD-1 COX-1$^{-/-}$ and COX-2$^{-/-}$ mice. CD-1 WT mice produced lower quantities of TNF-α (3.3 ± 0.4 ng/10$^7$ cells) than did RPM from C3H/HeN WT mice after a 6 hr incubation with LPS. However, as predicted, RPM from CD-1 COX-1$^{-/-}$ mice produced 2.4-fold higher levels of TNF-α than did RPM from CD-1 WT mice, a statistically significant increase (p < 0.05, Fig. 5D). COX-2$^{-/-}$ RPM produced slightly more TNF-α than did WT RPM, but this difference was not statistically significant.

DISCUSSION

*LPS-Stimulated PG Synthesis in RPM Cultures* - For nearly 30 years, macrophages have been recognized as a major cellular source of 20:4 metabolites. These cells contain an unusually
high percentage (20-25%) of 20:4 in membrane phospholipid (71, 87, 88). They respond to a variety of stimuli with the release of large amounts of free 20:4, and efficiently metabolize the free acid to an array of COX and LOX products (69-72, 89-92) (73-75, 93-95). In this study, we have investigated LPS-mediated 20:4 metabolism in RPM using mass spectrometric assays to evaluate quantities of all major PGs and both intracellular and extracellular 20:4. These methods offer a distinct advantage over commonly used radiolabel techniques in that they allow for absolute rather than relative quantitation of 20:4 and its metabolites. They also offer advantages over ELISA assays in that multiple metabolites may be monitored simultaneously. The results of our studies demonstrate that RPM produce predominantly prostacyclin and PGE\(_2\) in response to LPS, and that net increases in PG levels occur within the first hour after LPS addition. Marked induction of COX-2 and mPGES protein expression occurs in LPS-treated RPM, but detectable increases in protein levels are not observed until 2 hr of LPS treatment.

*Role for COX-1 in the Macrophage LPS Response -* Our finding that significant PG synthesis occurs in LPS-treated RPM prior to increased COX-2 expression led us to question whether COX-1 plays a significant role in the macrophage LPS response. We initially investigated this hypothesis using the selective COX inhibitors, SC560 and SC236. The concentration of inhibitor used in these studies (100 nM) was chosen to be well above the reported IC\(_{50}\) of each compound for the isoform for which it is selective, but well below the reported IC\(_{50}\) for the opposite isoform. Others have used this concentration in efforts to achieve selective inhibition in cultured macrophage populations under conditions similar to ours (96). We chose to study the effects of the inhibitors at both 2 hr and 6 hr after LPS administration. Based on the patterns of enzyme expression, COX-2 levels are still low after 2 hr of LPS exposure, so any contribution of COX-1 to PG synthesis would be relatively greater at that time.
Alternatively, by 6 hr COX-2 expression has reached near maximal levels so that its relative contribution to PG synthesis would probably have matched or exceeded that of COX-1. Thus, if COX-1 does contribute to PG synthesis, we predict that SC560 would be the more efficient inhibitor of PG synthesis at 2 hr, whereas by 6 hr, SC236 would be the most effective.

The results obtained with SC236 generally followed the predicted pattern, in that it inhibited PG synthesis less efficiently at 2 hr than at 6 hr. In contrast, SC560 caused a >80% inhibition of PG synthesis at both time points. The latter finding is inconsistent with inhibition of only COX-1 by SC560, leading us to conclude that SC560 is also inhibiting COX-2 to some degree, or that it has suppressive effects on PG synthesis by a mechanism other than direct COX inhibition. Relevant to the latter possibility, we found no change in free 20:4 levels in the presence of the inhibitors at either time point, indicating that they did not cause a marked reduction in gross availability of substrate. Both inhibitors caused a slight reduction in COX-2 expression, but this effect was too small to readily account for the levels of inhibition observed after 6 hr. It therefore seems highly likely that SC560 inhibited COX-2 directly. It should be noted that SC236 also might have exerted nonspecific effects, including COX-1 inhibition, in our experiments. However, the inhibitory effects of SC236 in RPM (30-35% inhibition at 2 hr and 85-90% inhibition at 6 hr) are consistent with expected contributions of COX-2 to LPS-dependent PG synthesis in these cells.

Our finding that SC236 only causes partial inhibition of LPS-mediated PG synthesis in RPM, especially at the earlier time points, was consistent with the hypothesis that COX-1 contributes to this process. We also applied a genetic approach using RPM from mice bearing targeted deletions of the gene for COX-1 or COX-2. Prior reports have shown that thioglycollate-elicited peritoneal macrophages from COX-1<sup>−/−</sup> C57BL/6 mice demonstrate
reduced PG synthesis in response to exogenous 20:4, and that RPM from COX-2<sup>−/−</sup> C57BL/6 mice show no LPS-dependent increase in PG synthesis in response to exogenous 20:4. These findings confirm that COX-1 expression is constitutive, and that LPS induces COX-2 expression in peritoneal macrophages. However since exogenous 20:4 was used in these experiments, they do not directly address the relative roles of the two isoforms in delayed PG synthesis from endogenous substrate (57,97). For our studies we used WT, COX-1<sup>−/−</sup>, and COX-2<sup>−/−</sup> mice on CD-1 and C57Bl/6 backgrounds. When compared to WT RPM, RPM from mice bearing a targeted deletion of either COX isoform showed no difference in the expression of the opposite isoform as measured by immunoblot analysis. This was true for mice from both sources. In addition, similar expression of cPLA<sub>2</sub> was detected in RPM from mutant mice as compared to WT controls except for a trend to slightly higher expression in CD-1 COX-2<sup>−/−</sup> cells. Thus, we detected no evidence that deletion of the gene for either COX-1 or COX-2 leads to notably increased compensatory expression of other relevant enzymes in RPM. This finding contrasts with studies reported previously by Kirtikara et al. who demonstrated that cultured lung fibroblasts from mice bearing targeted deletions of either COX enzyme exhibit compensatory increases in both cPLA<sub>2</sub> and the opposite COX isoform (98) and with a recent report by Wang et al. who found compensatory expression of COX-1 in the uteri of CD-1 COX-2<sup>−/−</sup> female mice (59).

When RPM from COX-1<sup>−/−</sup> mice of either strain were compared to WT controls, a consistent pattern was observed. In both sets of experiments, these RPM produced very low levels of PGs during the first 2 hr of incubation with LPS. By 6 hr, considerable PG formation had occurred, but levels were still much lower than those observed with WT cells. These results are totally consistent with the hypothesis that COX-1 contributes significantly to PG synthesis in
WT RPM, and that the contribution is proportionately larger at the earlier time points, before COX-2 expression has reached high levels.

Studies of COX-2\(^{-/-}\) RPM gave very different results for cells obtained from CD-1 versus C57BL/6 mice. CD-1 COX-2\(^{-/-}\) RPM produced very large amounts of PGs in response to LPS (>80% of PGs synthesized by WT RPM), and the majority of PG synthesis (>65%) had occurred after only 2 hr of LPS exposure. In stark contrast, C57BL/6 COX-2\(^{-/-}\) RPM produced very low levels of PGs (only 3% of PGs synthesized by WT RPM) throughout the entire 6 hr incubation period. The latter result, using C57BL/6 RPM, suggests that COX-1 cannot synthesize PGs in response to the LPS stimulus, a conclusion in direct conflict with the result from CD-1 COX-2\(^{-/-}\) RPM, which indicates robust COX-1-dependent PG synthesis. This conclusion is also inconsistent with the results from COX-1\(^{-/-}\) RPM that indicate that COX-1 contributes significantly to the total amount of PGs synthesized by WT RPM of either strain. In an attempt to reconcile these differences, we examined the peak levels of cellular free 20:4 present in each RPM culture during the LPS response. Results indicated that, in the absence of LPS, 20:4 levels were similar in C57BL/6 and CD-1 RPM regardless of genotype (Table III). However, C57BL/6 WT RPM showed only a small increase in free 20:4 after 2 hr of incubation with LPS, and C57BL/6 COX-1\(^{-/-}\) and COX-2\(^{-/-}\) RPM showed essentially no increase at all. In contrast, LPS induced a near doubling of free 20:4 in CD-1 WT RPM and COX-1\(^{-/-}\) RPM, while a four-fold increase was observed in COX-2\(^{-/-}\) RPM. Thus the ability of COX-1 to respond to LPS in COX-2\(^{-/-}\) RPM correlates with the presence of a high level of free 20:4 during LPS treatment.

Activation of COX requires oxidation of the heme prosthetic group. This is followed by an intramolecular electron transfer leading to the formation of the active site tyrosyl radical that serves as the direct oxidant for 20:4. The tyrosyl radical is regenerated during the course of the
cyclooxygenase reaction, so that many enzyme turnovers can occur following a single activation event. However, environmental reducing agents may return the enzyme to its native state, necessitating reactivation (4,43,44). Thus, maintenance of COX in the active state will depend on the relative concentration of oxidants and reductants within the cell (45,99). The source of the initial activating agent for COX in the intracellular environment is not known, although the product, PGG$_2$, can carry out this function (39,40). Therefore, the presence of high substrate concentrations would favor sustained activation through the rapid production of PGG$_2$, a condition particularly important for COX-1, because it requires higher concentrations of oxidant for activation than does COX-2 (47,48). These considerations may explain why it appears that COX-1 requires higher cellular concentrations of 20:4 for activity despite the fact that the two isoforms have similar $K_m$ values for 20:4 in vitro (10,45). They also explain why COX-1 remains inactive in C57BL/6 COX-2$^{-/-}$ RPM in which free 20:4 levels are low, but becomes rapidly and strongly activated in CD-1 COX-2$^{-/-}$ RPM in which 20:4 levels are high. It is important to note, however, that other factors such as LPS-mediated formation of lipoxygenase products or reactive oxygen species could also contribute to the oxidant tone in RPM, thereby promoting COX activation. If this is the case, then variations in these factors between CD-1 and C57BL/6 mice could also help to explain the different LPS responses of COX-2$^{-/-}$ RPM from the two strains. We did not directly measure these factors in the present study. However, we have found that inducible nitric oxide synthase does not appear in significant quantities in RPM until 4 hr after LPS addition (data not shown). Thus, nitric oxide-dependent generation of peroxynitrous acid, which has been shown to be an activator of COX (42), probably does not influence PG synthesis during the first 2 hr of the LPS response under our experimental conditions.
If it is true that COX-1 does not carry out LPS-dependent PG synthesis in COX-2\(^{-/-}\) C57BL/6 RPM due to low levels of 20:4 release, then it is reasonable to assume that COX-1 should also not contribute to PG synthesis in LPS-challenged C57BL/6 WT mice. However, results of experiments using C57BL/6 COX-1\(^{-/-}\) RPM indicate reduced PG synthesis as compared to WT cells, suggesting that COX-1 does contribute to this process in the WT RPM. A possible explanation for this apparent discrepancy is that the activation of COX-1 is dependent on newly expressed COX-2 in C57BL/6 WT RPM. COX-2 is readily activated at low concentrations of 20:4, and the PGG\(_2\) that it generates would then be available to activate COX-1. If correct, this hypothesis implies that COX-1 can contribute to PG synthesis in LPS-treated macrophages as long as the cells respond to the LPS with a robust release of 20:4, or as long as sufficient COX-2 expression has occurred to provide adequate levels of oxidant for COX-1 activation. It should be noted again however, that other oxidants that have not been directly explored in these studies may also be involved in COX activation.

Regardless of the mechanism, our data clearly indicate a marked difference between CD-1 and C57BL/6 RPM in their ability to compensate for the deletion of the gene for COX-2. Similar results have been reported recently by Wang et al. who observed that that female CD-1 COX-2\(^{-/-}\) mice have dramatically improved fertility in terms of ovulation, fertilization, and implantation, giving rise to live births, as compared to female C57Bl/6 COX-2\(^{-/-}\) mice, which are almost completely infertile. This improved fertility in CD-1 COX-2\(^{-/-}\) mice is due to a compensatory upregulation of COX-1 in the uterus that does not occur in C57BL/6 COX-2\(^{-/-}\) mice (59). Note, however, that a similar compensatory change in COX-1 expression was not observed in any of the COX-2\(^{-/-}\) RPM populations studied here. Thus compensatory mechanisms
used to overcome the effects of COX-2 gene deletion must be tissue specific as well as strain specific.

20:4 levels in LPS-Stimulated Macrophages - Others have suggested that COX-1 is inactive during delayed PG synthesis due to insufficient release of 20:4. This hypothesis is consistent with our conclusion that low levels of 20:4 release lead to COX-1 inactivity in COX-2⁻/⁻ C57BL/6 RPM. It should be noted, however, that few attempts have been made to actually determine the levels of 20:4 that are available to the COX enzymes during the delayed PG synthetic response. In these studies, we measured the absolute quantity of free, unmetabolized 20:4 in macrophage cultures, and determined its distribution between medium and cells. We demonstrated that 20:4 levels increase in response to LPS, reaching a peak at about 2 hr, and then returning to baseline values. This result is consistent with that of Brown, et al. (100) who used human alveolar macrophages preincubated with radiolabeled 20:4 to show that the appearance of unmetabolized radiolabeled 20:4 in the culture medium reached a maximum after 2 hr of LPS exposure. Based on this time course, it is tempting to speculate that 20:4 release is maximal at 2 hr of LPS exposure, because this period of time is required for full activation of cPLA₂, and that levels decrease thereafter because of increasing rates of consumption of the free acid as COX-2 expression and PG synthetic rates rise. This speculation is consistent with the fact that PG synthetic rates are highest as 20:4 levels drop back to baseline values in RPM cultures and suggests a logical inverse relationship between the rate of PG synthesis and intracellular free 20:4 levels. It is likely, however, that the above conclusions describe an overly simplistic relationship between free 20:4 levels and PG synthesis. Relevant to these considerations is our finding that total inhibition of PG synthesis by the combination of SC236 and SC560 caused no significant change in free 20:4 levels in RPM at 2 hr or 6 hr after LPS
addition. This rather unexpected result indicates that complete blockade of the rate of PG synthesis had no effect on free 20:4 pool size. This could only occur if compensatory changes occur in the rates of 20:4 release and/or re-esterification, or if the relative contribution of the PG synthetic rate to total 20:4 pool size is very small. Alternatively, this observation could be explained by inhibition of cPLA₂ by SC236 and SC560, however, the level of inhibition of each inhibitor would have to exactly match the effect of COX inhibition on 20:4 pool size, which seems unlikely.

The maximal levels of free 20:4 measured in CD-1 and C3H/HeN RPM were in the range of 200-300 pmol/10⁷ cells, approximately 75% of which is cell-associated. Assuming a cell volume of approximately 4 pL/cell (comparable to a spherical cell of 20 μm diameter), and assuming that the cell-associated 20:4 is uniformly distributed throughout the cell, an intracellular concentration of 150 - 220 pmol/10⁷ cells would correspond to 3 - 4 μM, a value well within the range of the reported in vitro K_Ms for COX enzymes (5 μM) (10). Local concentrations in the vicinity of cPLA₂ are likely to be much higher. Interestingly, baseline levels of 20:4 in the absence of LPS would correspond to approximately 1.5 μM, yet comparatively little PG synthesis occurs in the absence of a stimulus. This could be due to the unavailability of adequate oxidant activating factors for COX, or it could be due to sequestration of 20:4 in the cell so that it is not available to COX for metabolism.

**Suppression of LPS-Mediated TNF-α Secretion in LPS-Treated RPM** - Our data show that LPS-mediated TNF-α secretion is suppressed by endogenous PGE₂ and prostacyclin in RPM. Since PGs suppress TNF-α secretion by blocking the LPS-mediated increase in TNF-α mRNA, it is clear that they must be present at significant concentrations during the first 1-2 hr after LPS addition when the major increase in mRNA transcription takes place. Therefore, the
most likely source of endogenous PGs for suppression of TNF-α secretion is COX-1. Consistent with this hypothesis, the COX-2 selective inhibitor, SC236, had only a small effect (1.8-fold increase) on RPM TNF-α secretion over a 6 hr incubation with LPS, whereas the COX-1 selective inhibitor, SC560, had a much greater effect (5.6-fold increase). This finding correlates with the clear differential in PG synthesis inhibition, leading to much lower PG concentrations in the presence of SC560 than in the presence of SC236 during the critical initial 2 hr after LPS addition. Thus, during the period of maximal TNF-α mRNA transcription, SC560 has a much greater effect on PG synthesis than does SC236, and is therefore more effective than SC236 in attenuating the PG-mediated suppression of TNF-α secretion.

Since the discovery of COX-2, considerable effort has been made to define specific functions for each COX isoform. The definition of delayed PG synthesis as a solely COX-2-dependent process implied a unique role for this isoform in response to the inflammatory agents and cytokines that also invoke its expression. Our finding that COX-1 contributes to PG synthesis in LPS-stimulated macrophages suggests that the processes of immediate and delayed PG synthesis may not be as biochemically distinct as is presently believed. In fact, considering the significant rate of PG synthesis observed during the first hour of LPS stimulation in RPM, the term "delayed" in this case is, perhaps, inappropriate. It is interesting to note that RPM are unusual cells in terms of their remarkable ability to synthesize exceptional quantities of PGs in response to a wide variety of both immediate and delayed PG stimuli. Apparently, a significant factor contributing to this ability is their high level of constitutive COX-1 expression. Because resident macrophages will be among the first to encounter a bacterial insult, and because COX-1-mediated PG synthesis occurs early after LPS exposure, this phenomenon may play a critical role in modulating the subsequent consequences of the LPS response, as is shown here in the case of
TNF-α secretion. These findings have important implications for the full understanding of the inflammatory response to gram-negative infection and sepsis.

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**FOOTNOTES**

1The abbreviations used are: RPM, resident peritoneal macropohages; LPS, lipopolysaccharide; COX, cyclooxygenase (prostaglandin G/H synthase); PG, prostaglandin; 20:4, arachidonic acid; TNF-[], tumor necrosis factor-alpha; PLA2, phospholipase A2; LOX, lipoxygenase; WT, wild-type; COX-1, mice bearing a targeted deletion of *ptgs-1*; COX-2, mice bearing a targeted deletion of *ptgs-2*; PBS, calcium- and magnesium-free phosphate-buffered saline; [-]-MEM, minimum essential medium-alpha; DMEM, Dulbecco's modified eagle medium; FCS, fetal calf serum; mPGES, microsomal prostaglandin E synthase; TTBS, tris-buffered saline containing tween-20.

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FIGURE LEGENDS

Fig. 1. Time course of PG synthesis and 20:4 release by LPS-stimulated macrophages. (A) RPM were isolated from C3H/HeN mice as described in Experimental Procedures and incubated for the indicated times in the presence (LPS) or absence (CON) of 100 ng/mL LPS. The medium was collected from each culture and analyzed for the indicated PGs by GC/MS. (B) The data in (A) were used to calculate the average rate of total net PG synthesis above control (no LPS) levels over the indicated time periods after LPS addition. (C) The data in (A) were used to calculate the percent of total PG synthesis that is comprised by each individual PG. (D) RPM cultures were prepared and incubated in the presence or absence of LPS as described for (A). At the indicated times, the medium and cell lysates were combined and analyzed for 20:4 content by LC/MS/MS as described in Experimental Procedures. Each data point (A, C, & D) or bar (B) is the mean ± s.d. from the combined results of three separate experiments in which duplicate determinations were made.

Fig. 2. Expression of PG metabolizing enzymes during the LPS response. (A) RPM were isolated from C3H/HeN mice as described in Experimental Procedures and incubated for the indicated times in the presence of 100 ng/mL of LPS. Lysates (15 µg protein) prepared from the
RPM monolayers were subjected to immunoblot analysis to detect COX-2 protein content as described in Experimental Procedures. The results were visualized by exposure of Hyperfilm ECL film. (B) A Fluor-S Max Multi-imager was used to quantify the COX-2 chemiluminescence signal from immunoblots prepared as shown in (A) using cells incubated in both the presence (LPS) and absence (CON) of LPS. The quantitative results for each blot were normalized to the COX-2 signal obtained for cells at 6 hr of incubation. (C & D). Experimental conditions were the same as described for (A & B), but the blots were analyzed for the presence of mPGES-1. The quantitative data in (D) are normalized to the mPGES-1 chemiluminescence signal in the 24 hr samples. (E & F). Experimental conditions were the same as described for (A & B), but the blots were analyzed for the presence of cPLA₂. The quantitative data in (F) are normalized to the cPLA₂ chemiluminescence signal in the 0 hr samples. (G & H) Experimental conditions were the same as described for (A & B) but the blots were analyzed for the presence of COX-1. The quantitative data in (H) are normalized to the COX-1 chemiluminescence signal in the 0 hr samples. All quantitative results are the mean ± s.d. from three separate experiments in which duplicate samples were analyzed.

Fig. 3. Effect of SC236 and SC560 on the macrophage LPS response. (A) RPM were isolated from C3H/HeN mice as described in Experimental Procedures. Following a 1 hr preincubation in the presence of the indicated inhibitor(s) (100 nM) or vehicle (DMSO), LPS (100 ng/mL) was added and the cells were incubated for 2 hr. Levels of PGs in the culture medium were determined by GC/MS, and data are expressed as the percent of control PG synthesis (CON, no inhibitor) achieved in the presence of each inhibitor. (B) Same as in (A), except that cultures were incubated for 6 hr with LPS. (C) Cultures treated as described in (A & B) were prepared,
and the total amount of 20:4 in medium and cells was determined using LC/MS/MS as described in Experimental Procedures. (D) Cell lysates (15 µg of protein) from cultures prepared for (B) were subjected to immunoblot analysis for COX-2 expression. The chemiluminescence signal from the immunoblots was quantified using the Fluor-S Max, and results were normalized to the signal obtained from control cultures. All results are the mean ± s.d. from three separate experiments in which duplicate samples were analyzed.

Fig. 4. Expression of COX-1, COX-2, and cPLA₂ in RPM from mice bearing targeted deletions of the genes for COX-1 and COX-2. (A) RPM were isolated from CD-1 COX-1⁻/⁻, COX-2⁻/⁻, and WT mice as described in Experimental Procedures. The cells were incubated for the indicated times with LPS (100 ng/mL), and cell lysates (15 µg of protein) from the cultures were analyzed for COX-1 expression by immunoblot. (B) The COX-1 chemiluminescence signals from immunoblots as shown in A were quantitated using a Fluor-S Max Multi-imager. The results for each blot were normalized to the signal obtained for WT cells at 0 hr of incubation, and each bar represents the mean ± s.d. from the combined results of two separate experiments in which duplicate samples were analyzed. (B & C) Conditions were the same as in (A & B), respectively except that COX-2 protein expression was analyzed, and the quantitative results were normalized to the signal from WT cells at 6 hr of incubation. (E & F) Conditions were the same as in (A & B), respectively except that cPLA₂ protein expression was analyzed, and the quantitative results were normalized to the signal from WT cells at 6 hr of incubation.
Fig. 5. Autocrine regulation of TNF-α secretion in LPS-treated RPM. (A) RPM were isolated from C3H/HeN mice as described in Experimental Procedures and incubated for the indicated times in the presence (LPS) or absence (CON) of 100 ng/mL LPS. The medium was collected from each culture and analyzed for TNF-α concentration by ELISA. (B) RPM were isolated from C3H/HeN mice as described in Experimental Procedures. Following a 1 hr preincubation in the presence of the indicated inhibitor(s) (100 nM) or vehicle (DMSO), LPS (100 ng/mL) was added and the cells were incubated for 6 hr. Levels of TNF-α in the culture medium were determined by ELISA. The symbol (★★) designates that the values were statistically different from control (no inhibitor) at p < 0.001. (C) RPM from C3H/H3N mice were preincubated for 1 hr with or without SC236, and then LPS (100 ng/mL) was added along with the indicated concentrations of PGE₂, cPGI₂, or both. Cells were incubated for 6 hr and then the culture medium was harvested for determination of TNF-α by ELISA. Data are expressed as the percent of the TNF-α concentration in medium from control cultures (CON, no inhibitor and no exogenous PGs). The symbols (★) and (★★) designate that the values were statistically different (p < 0.05) from control RPM or RPM incubated with SC560 but without exogenous PGs, respectively. All data from (A through C) are the mean ± s.d. from the combined results of three separate experiments in which duplicate samples were analyzed. (D) RPM were isolated from CD-1 COX-1−/−, COX-2−/−, and WT mice and incubated overnight. Cultures were then incubated for 6 hr with LPS (100 ng/mL), and the culture medium was analyzed for TNF-α by ELISA. Each bar represents the mean ± s.d. from the combined results of two separate experiments in which duplicate samples were analyzed. The symbol (★) designates that the values were statistically different from WT (p < 0.05).
**TABLE I**

*Comparison of LPS-induced PG synthesis by RPM from mice of different genetic backgrounds.*

PRM cultures were prepared as described in Experimental Procedures and incubated for the indicated periods of time with LPS. PGs in culture medium were analyzed by GC/MS or LC/MS/MS. Total PGs include PGE$_2$, 6-ketoPGF$_{1 \alpha}$, and TXB$_2$ for C3H/HeN RPM, and PGE$_2$ plus 6-ketoPGF$_{1 \alpha}$ for CD-1 and C57BL/6 RPM. Percent PGE$_2$ represents the percent of indicated total PGs that was PGE$_2$. Results are the combined mean ± s.d. for data from three experiments in which duplicate determinations were made. ND indicates not determined.

| Cell Source | 0 Hr   | 2 Hr   | 6 Hr   | 24 Hr  |
|-------------|--------|--------|--------|--------|
|             | Total Prostaglandin (pmol/10$^7$ cells) | Concentration in Culture Medium (nM) | Percent PGE$_2$ |
| C3H/HeN     | 83 ± 14 | 780 ± 210 | 3,700 ± 500 | 5,600 ± 400 |
|             | {3.2 ± 0.6} | {31 ± 10} | {140 ± 30} | {220 ± 25} |
|             | [16 ± 2] | [18 ± 2] | [31 ± 1] | [38 ± 5] |
|             | 160 ± 60 | 320 ± 70 | 6,900 ± 1,400 | ND |
| CD-1        | {6.8 ± 3.1} | {13 ± 4} | {280 ±40} | ND |
|             | [41 ± 13] | [40 ± 9] | [53 ± 9] | |
|             | 140 ± 100 | 130 ± 120 | 2,900 ± 800 | |
| C57BL/6     | {5.7 ± 4.4} | {5.6 ±5.1} | {110 ±30} | ND |
|             | [35 ± 9] | [43 ± 8] | [55 ± 6] | |
**TABLE II**

*Comparison of 20:4 levels in LPS-treated RPM from mice of different strains.*

Experimental conditions were identical to those described in the legend to Table I, except that total 20:4 in culture medium plus cell lysates was analyzed by LC/MS/MS. Results are the combined mean ± s.d. for data from three experiments in which duplicate determinations were made.

|                | Arachidonic Acid (pmol/10⁷ cells) |
|----------------|-----------------------------------|
|                | 0 Hr                              | 2 Hr                              |
| C3H/HeN        | 140 ± 40                          | 320 ± 90                          |
| CD-1           | 110 ± 20                          | 210 ± 20                          |
| C57BL/6        | 80 ± 20                           | 110 ± 30                          |
**TABLE III**

*Comparison of LPS-induced PG synthesis of RPM from mice of different genetic backgrounds bearing targeted deletions of COX-1 and COX-2 genes.*

RPM cultures were prepared as described in Experimental Procedures and incubated for the indicated periods of time with LPS. PGs in culture medium were analyzed by LC/MS. CD-1 COX-1^{-/-} and CD-1 COX-2^{-/-} designate RPM from CD-1 mice bearing a targeted deletion for *ptgs-1* or *ptgs-2* respectively whereas CD-1 WT designates RPM from CD-1 wild-type mice. C57BL/6 COX-1^{-/-} and COX-1^{+/+} designate RPM from C57BL/6 mice bearing a targeted deletion of *ptgs-1* and matched littermate WT controls, whereas C57BL/6 COX-2^{-/-} and COX-2^{+/+} designate RPM from C57BL/6 mice bearing a targeted deletion of *ptgs-2* and matched littermate WT controls. Total PGs include PGE$_2$ plus 6-ketoPGF$_{1\alpha}$. Percent PGE$_2$ represents the percent of indicated total PGs that was PGE$_2$. Results are the combined mean ± s.d. for data from two experiments in which duplicate determinations were made (CD-1) or mean ± range from a single experiment in which duplicate determinations were made (C57BL/6).

| Cell Source | Total Prostaglandin (pmol/10$^7$ cells) | 0 Hr | 2 Hr | 6 Hr |
|-------------|----------------------------------------|------|------|------|
|             |                                        | [Percent PGE$_2$] |      |      |      |
| CD-1        |                                        |      |      |      |
| COX-2^{-/-} | 49 ± 34                                 | 4,100 ± 2,700 | 6,000 ± 1,800 |
|             | [24 ± 28]                               | [52 ± 9] | [56 ± 11] |
| CD-1        | 1.4 ± 2.8                               | 23 ± 19 | 1,500 ± 500 |
| COX-1^{-/-} | [21 ± 13]                               | [35 ± 20] | [67 ± 5] |
| CD-1        | 140 ± 50                                | 290 ± 19 | 7,200 ± 1,700 |
| WT          | [21 ± 13]                               | [44 ± 7] | [59 ± 4] |
| C57BL/6     | 64 ± 7                                  | 58 ± 16 | 110 ± 4 |
| COX-2^{-/-} | [57 ± 18]                               | [67 ± 16] | [67 ± 5] |
| C57BL/6     | 66 ± 17                                 | 64 ± 10 | 3,300 ± 200 |
| COX-2^{+/+} | [40 ± 2]                                | [46 ± 4] | [57 ± 3] |
| C57BL/6     | 0 ± 0                                   | 9.0 ± 4.0 | 900 ± 200 |
| COX-1^{-/-} | [-]                                     | [26 ± 53] | [52 ± 1] |
| C57BL/6     | 83 ± 83                                 | 56 ± 6 | 2,000 ± 190 |
| COX-1^{+/+} | [33 ± 22]                               | [40 ± 23] | [47 ± 2] |
TABLE IV

Comparison of 20:4 levels in LPS-treated RPM from mice of different genetic backgrounds bearing targeted deletions of the COX-1 and COX-2 genes.

Experimental conditions were identical to those described in the legend to Table III, except that total 20:4 in culture medium plus cell lysates was analyzed by LC/MS/MS. Results are the combined mean ± s.d. for data from two experiments in which duplicate determinations were made (CD-1) or mean ± range from a single experiment in which duplicate determinations were made (C57BL/6).

| Cell Source | Arachidonic Acid (pmol/10^7 cells) |
|-------------|-----------------------------------|
|             | 0 Hr                              | 2 Hr                              |
| CD-1        |                                    |                                   |
| COX-2-/-    | 120 ± 50                           | 400 ± 80                          |
| CD-1        |                                    |                                   |
| COX-1-/-    | 120 ± 10                           | 230 ± 19                          |
| CD-1        |                                    |                                   |
| WT          | 100 ± 10                           | 210 ± 23                          |
| C57BL/6     |                                    |                                   |
| COX-2-/-    | 110 ± 16                           | 120 ± 40                          |
| C57BL/6     |                                    |                                   |
| COX-2^+/+   | 100 ± 10                           | 120 ± 30                          |
| C57BL/6     |                                    |                                   |
| COX-1-/-    | 90 ± 50                            | 82 ± 5                            |
| C57BL/6     |                                    |                                   |
| COX-1^+/+   | 59 ± 10                            | 90 ± 45                           |
**Figure 2**

**A** COX-2 in RPM + LPS

**B** RPM COX-2 Signal Intensity (% of 8 hr)

**C** mPGES-1 in RPM + LPS

**D** RPM mPGES-1 Signal Intensity (% of 24 hr)

**E** cPLA₂ in RPM + LPS

**F** RPM cPLA₂ Signal Intensity (% of 0 hr)

**G** COX-1 in RPM + LPS

**H** RPM COX-1 Signal Intensity (% of 0 hr)
A  RPM PG Synthesis (2 hr)

B  RPM PG Synthesis (6 hr)

C  RPM 20:4 Levels

D  RPM COX-2 Levels (6 hr)
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Cyclooxygenase-1-dependent prostaglandin synthesis modulates tumor necrosis factor alpha secretion in lipopolysaccharide-challenged murine resident peritoneal macrophages

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