Catalytically Inactive Cyclooxygenase 2 and Absence of Prostaglandin E2 Biosynthesis in Murine Peritoneal Macrophages following In Vivo Phagocytosis of Heat-Killed Mycobacterium bovis Bacillus Calmette-Guérin

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Catalytically Inactive Cyclooxygenase 2 and Absence of Prostaglandin E\textsubscript{2} Biosynthesis in Murine Peritoneal Macrophages following In Vivo Phagocytosis of Heat-Killed *Mycobacterium bovis* Bacillus Calmette-Guérin\textsuperscript{1}

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Over 25 years ago, it was observed that peritoneal macrophages (Mφ) isolated from mice given heat-killed *Mycobacterium bovis* bacillus Calmette-Guérin (HK-BCG) i.p. did not release PGE\textsubscript{2}. However, when peritoneal Mφ from untreated mice are treated with HK-BCG in vitro, cyclooxygenase 2 (COX-2), a rate-limiting enzyme for PGE\textsubscript{2} biosynthesis, is expressed and the release of PGE\textsubscript{2} is increased. The present study of peritoneal Mφ obtained from C57BL/6 mice and treated either in vitro or in vivo with HK-BCG was undertaken to further characterize the cellular responses that result in suppression of PGE\textsubscript{2} release. The results indicate that Mφ treated with HK-BCG in vivo express constitutive COX-1 and inducible COX-2 that are catalytically inactive, are localized subcellularly in the cytoplasm, and are not associated with the nuclear envelope (NE). In contrast, Mφ treated in vitro express catalytically active COX-1 and COX-2 that are localized in the NE and diffusely in the cytoplasm. Thus, for local Mφ activated in vivo by HK-BCG, the results indicate that COX-1 and COX-2 dissociated from the NE are catalytically inactive, which accounts for the lack of PGE\textsubscript{2} production by local Mφ activated in vivo with HK-BCG. Our studies further indicate that the formation of catalytically inactive COX-2 is associated with in vivo phagocytosis of HK-BCG, and is not dependent on extracellular mediators produced by in vivo HK-BCG treatment. This attenuation of PGE\textsubscript{2} production may enhance Mφ-mediated innate and Th1-acquired immune responses against intracellular infections which are suppressed by PGE\textsubscript{2}. *The Journal of Immunology*, 2007, 179: 7072–7078.

Phagocytosis of intracellular bacteria by macrophages (Mφ)\textsuperscript{1} results in cellular activation with expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for PGE\textsubscript{2} biosynthesis. PGE\textsubscript{2} down-regulates innate and Th1-mediated immune responses induced by bacteria in autocrine and paracrine fashions. For example, PGE\textsubscript{2} inhibits inducible NO synthase/NO synthesis, NAPDH oxidase/superoxide anion release, and IL-12/IL-18/TNF-\textalpha synthesis (1–3). In contrast, PGE\textsubscript{2} promotes IL-10 production by Mφ (4, 5), a Th1-to-Th2 shift of acquired immune responses (4, 5), dendritic cell Ag presentation (6), regulatory T cell differentiation and function (7), bone marrow progenitor cell migration via the CXCR4/stromal cell-derived factor-1 (CXCL12) system (8), IL-23 production (9), and Mφ production of matrix metalloproteinase 9 (10). Regulation of these events, therefore, may depend on the regulation of PGE\textsubscript{2} release by COX-2\textsuperscript{+} Mφ (11). The formation of these PGE\textsubscript{2}-releasing, activated Mφ (PGE\textsubscript{2}-Mφ) appears to be regulated by multiple bacterial and host factors, including the tissue origin of the Mφ (12–14).

Humes et al. (15) reported for the first time in 1980 that PGE\textsubscript{2} release by peritoneal Mφ isolated from mice that are given heat-killed *Mycobacterium bovis* bacillus Calmette-Guérin (HK-BCG) or HK-*Corynebacterium parvum* (*Propionibacterium acnes*) i.p. is significantly reduced compared with that released by untreated peritoneal Mφ. Studies by other groups including our own confirmed this phenomenon (13, 16, 17). Because peritoneal Mφ elicited by i.p. HK-*C. parvum* in both monocyteic and control mice showed diminished PGE\textsubscript{2} biosynthesis, the phenomenon is not dependent on monocyte-derived Mφ migration but rather is dependent on the direct interaction between local Mφ and bacteria (13). A precise explanation for the suppression of PGE\textsubscript{2} release has not been established. In sharp contrast to long-held views, recent studies indicate that in vitro these bacteria induce COX-2 expression and PGE\textsubscript{2} biosynthesis by various Mφ preparations including normal peritoneal Mφ, blood monocytes, and Mφ cell lines (18–26). Although the regulation of COX-1 and COX-2 expression has been extensively studied (27), exact mechanisms for regulation of PGE\textsubscript{2} biosynthesis by Mφ activated with HK-BCG in vitro or in vivo are still unclear (12).

PGE\textsubscript{2} biosynthesis is initiated by activation of phospholipase A\textsubscript{2} (PLA\textsubscript{2}) to release arachidonic acid (AA), which is metabolized by constitutive COX-1 and inducible COX-2 yielding PGH\textsubscript{2}, which is converted to PGE\textsubscript{2} by cytosolic PGE synthase or microsomal

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\textsuperscript{3}Abbreviations used in this paper: Mφ, macrophage; COX, cyclooxygenase; HK, heat killed; BCG, bacillus Calmette-Guérin; NE, nuclear envelope; ER, endoplasmic reticulum; PGES, PGE synthase; cPLA\textsubscript{2}, cytosolic phospholipase A\textsubscript{2}; PI, propidium iodide; AA, arachidonic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; CFDA, carboxyfluorescein diacetate.

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PGES (28, 29). Catalytically active COX-1 and COX-2 are localized in the nuclear envelop (NE) and endoplasmic reticulum (ER) of PGE₂-releasing cells (30–32). More recent studies have suggested that for functional coupling and PGE₂ biosynthesis, cytosolic PLA₂, COXs, and PGESs appear to be localized in the perinuclear region (28, 33). Previously, we demonstrated that i.p. administration of HK-BCG results in at least two different forms of COX-2+ splenic Mφ: Mφ obtained beginning at 1 day following HK-BCG treatment, which have catalytically inactive COX-2 dissociated from the NE and do not release PGE₂, and Mφ obtained 7 days following treatment which have catalytically active COX-2 localized at the NE and release PGE₂. The dissociation of COX-2 from the NE is associated with in vivo phagocytosis of HK-BCG (34). Although capable of phagocytosis, the splenic Mφ expressing catalytically active COX-2 do not contain intracellular HK-BCG (14, 34). Neither of these COX-2+ Mφ subsets can be induced in vitro, where only catalytically active COX-2+ Mφ are seen, and only in the presence of phagocytosed HK-BCG (34). Because formation of splenic and peritoneal PGE₂-Mφ have distinct features (13), we sought to determine whether catalytically inactive COX-2 is induced in peritoneal Mφ that phagocytose HK-BCG in vivo. We found that endogenous factors involved in phagocytosis of HK-BCG in vivo, but not extracellular signaling molecules produced locally by HK-BCG, are responsible for regulating the subcellular localization COX-2.

Materials and Methods

Animals

Nonpregnant female C57BL/6 mice, 8–14 wk old, were obtained from Harlan Breeders. Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water ad libitum. Experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee at Florida Atlantic University.

Intraperitoneal administration of HK-BCG, HK-C. parvum, or LPS

As described previously (35), cultured M. bovis BCG Tokyo 172 strain were washed, autoclaved, and lyophilized. This HK-BCG powder was suspended in pyrogen-free saline and dispersed by brief (10 s) sonication immediately before use. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 endotoxin units/ml), as determined by the Limulus amebocyte lysate assay (Sigma-Aldrich) (33). Groups of mice (three per group) received 1, 0.1, or 0.01 mg of HK-BCG (5 × 10⁷ bacilli/mg) i.p. Controls received 0.1 ml of saline. Peritoneal lavage was performed at 6 or 24 h. As comparison controls, peritoneal Mφ were also obtained from mice given 1 mg of HK-C. parvum (13) or 100 µg of LPS (Escherichia coli:0111:B4, phenol; Sigma-Aldrich) i.p. The schedules were identical with those used for HK-BCG.

Peritoneal Mφ preparation and treatment with HK-BCG, HK-C. parvum, or LPS in vitro

Peritoneal lavage was performed as previously described (13). Nucleated peritoneal cells were counted in a Coulter counter (model Z1; Beckman Coulter). Differential cell counts were performed on cytopsin preparations (Shandon Southern Instruments) stained with Diff-Quik. To enrich plastic-adherent Mφ, peritoneal cells at 1 × 10⁷ cells/ml suspended in RPMI 1640 plus 5% FBS were incubated in culture dishes (Falcon) for 2 h. Nonadherent cells (lymphocytes) were removed by washing with warmed medium. Adherent cells were cultured with 100 µg/ml HK-BCG, HK-C. parvum, or 1 µg/ml LPS for an additional 6 or 24 h. In some experiments, plasma and cell-free peritoneal lavage fluid isolated from the HK-BCG-treated mice were added to peritoneal Mφ cultures with HK-BCG.

Cocultures of peritoneal Mφ treated with HK-BCG in vitro and in vivo

Peritoneal Mφ were labeled with 1 µM carboxyfluorescein diacetate (CFDA; Molecular Probes) at 37°C for 15 min and washed with RPMI 1640 plus 5% FBS. CFDA-labeled Mφ (10⁶ cells/ml) were cultured with 100 µg/ml HK-BCG for 6 h and mixed with peritoneal cells at 10⁶ cells/ml isolated from mice in which 1 mg of HK-BCG was given i.p. 6 h before harvest. The mixed cells were cultured for an additional 18 h.

PGE₂ assay

For assay of PGE₂ release, plastic adherent peritoneal Mφ (1 × 10⁶ cells/ml) were cultured in serum-free RPMI 1640 with 1 µM calcium ionophore A23187 (Sigma-Aldrich) for 2 h. PGE₂ levels in the culture supernatants were measured by competitive ELISA (Cayman Chemical).

Subcellular localization of COX-1 and COX-2 by confocal microscopy

Peritoneal Mφ prepared as described above were fixed with 4% paraformaldehyde in PBS for 30 min. The fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated in blocking buffer consisting of PBS with 10% FBS for 3 h at 22°C before incubation with anti-COX-1 or anti-COX-2 Ab (Cayman Chemical), 1:500 in blocking buffer, overnight at 4°C. Subsequently, cells were washed with PBS three times and incubated with FITC-conjugated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories) for 1 h at 22°C. For detection of nuclei and HK-BCG, propidium iodide (PI) was mixed at 10 µg/ml with the secondary Ab solution. After washing three times, cells were examined with a laser scanning confocal microscope (Bio-Rad Radiance 2100). The images were processed with Adobe Photoshop software.

Subcellular fractionation

The method for subcellular fractionation was modified from that published previously (36). Peritoneal Mφ prepared above were resuspended in 0.1 M Tris-HCl (pH 7.5), disrupted with a Dounce homogenizer, and forced through 26-gauge needles on ice. Disruption of cellular membranes was verified by microscopic examination. Cellular debris was removed by low-speed centrifugation (700 × g for 10 min), and the supernatants were further centrifuged at 10,000 × g for 10 min to collect nuclei. The resulting supernatants were subjected to ultracentrifugation at 100,000 × g for 90 min to isolate microsomal membrane and cytosolic fractions. Nuclear and membrane fractions were resuspended in 0.1 M Tris-HCl (pH 7.5). Protein concentrations were measured with a bicinchoninic acid assay (Pierce) and BSA as standard.

COX activity assay

The peroxidase component of COX in isolated cellular fractions was measured with a COX assay kit (Cayman Chemical) briefly as follows. The activity was determined with AA as a substrate and N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD) as cosubstrate. Equal amounts of protein (20 µg) were incubated at 25°C in a reaction mixture consisting of AA, TMPD, and hemin in 0.1 M Tris-HCl (pH 7.5). The absorbance change, due to oxidation of TMPD during the initial 5 min, was measured at 590 nm. The specific enzyme activities were calculated and indicated as nanomoles per minute per milligram.

Western blot analysis

Peritoneal Mφ prepared as described above were washed three times with cold saline. Washed cells were resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 4 mM EDTA, 0.1% SDS, 1:500 protease inhibitor mixture (P8340; Sigma-Aldrich)), 1% Nonidet P-40, and 1% sodium deoxycholate. Debris was eliminated by centrifugation (10 min, 10,000 × g). Protein concentration in the lysate was measured with a bicinchoninic acid assay (Pierce) and BSA as standard. Equal amounts of protein from each sample were separated by SDS-PAGE. Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 10% nonfat dry milk and incubated with Abs (anti-COX-1, 1:2,000; anti-COX-2, 1:4,000; Cayman Chemical; anti-GAPDH, 1:4,000 (Novus Biologicals)) for the detection of GAPDH as constitutively expressed protein control) in 5% nonfat dry milk, overnight at 4°C. Following incubation with peroxidase-conjugated donkey anti-rabbit IgG (1:20,000; Jackson ImmunoResearch Laboratories), proteins were detected by chemiluminescence (ECL plus; Amersham) following the manufacturer’s instructions.

Statistics

Data for PGE₂ release were analyzed by one-way ANOVA. For cell culture studies, tissues isolated from at least three mice were pooled unless indicated; these cells were cultured in at least triplicate in each group. Differences between mean values for the COX activity assays were analyzed by Student’s t test with Statcel software. A value of p < 0.05 is considered statistically significant.
Results

Intraperitoneal administration of 1 mg of HK-BCG was chosen to achieve an inflammatory response in mice like that associated with mycobacterial infection. This dose was used previously to induce splenic PGE_2-Mφ resulting in a Th1-to-Th2 shift of immune response (5). Previous in vitro studies showed that Mφ phagocytose mycobacteria through TLR2 and that expression of COX-2 and PGE_2 biosynthesis are dependent on MAPK and NF-κB activation (19). Results shown in Fig. 1 indicate a difference in PGE_2 production by peritoneal Mφ dependent on exposure to HK-BCG in vitro or in vivo. Calcium ionophore A23187-elicited production of PGE_2 by resident peritoneal Mφ treated in vitro with HK-BCG is increased at 6 and 24 h, but is unchanged following in vivo treatment (Fig. 1, ■). At 6 h, the constitutive production of PGE_2 by peritoneal Mφ treated in vitro with HK-BCG is also increased, but is suppressed for cells treated in vivo (Fig. 1, □).

Distinct subcellular localization of COX in Mφ activated with HK-BCG in vivo and in vitro

We determined the COX levels in peritoneal Mφ activated with HK-BCG in vivo and in vitro. As shown in Fig. 2, in vitro treatment of peritoneal Mφ resulted in increased COX-2 levels, without a change in COX-1. In contrast, for Mφ from mice treated with 1 mg of HK-BCG in vivo, an increase in COX-2 was accompanied by a decrease in COX-1 (Fig. 2). The results were similar at 6 and 24 h.

We further determined the subcellular localization of COX-2 by confocal microscopy. As shown in Fig. 3, Mφ activated in vitro expressed COX-2 that was localized in the NE. In contrast, Mφ activated in vivo had a dense form of COX-2 distributed in the cytoplasm, but not localized in the NE (Fig. 3). COX-1 in untreated Mφ was consistently expressed in the NE and ER, which was not changed for Mφ treated in vitro. However, when Mφ were activated in vivo, COX-1 appeared as the dense form dissociated from the NE with a pattern similar to that seen for COX-2 (Fig. 4).

Differential COX distribution in Mφ subcellular fractions

To further confirm that COX-2 dissociated from the NE is catalytically inactive, peritoneal Mφ treated in vivo with 1 mg of HK-BCG were homogenized and subjected to differential centrifugation. For Mφ activated in vitro, relatively more COX protein and activity were detected in the nuclear and membrane fractions than in the cytosolic fraction (Fig. 5). The profiles of COX-1 and COX-2 distribution are similar to previous reports using various PGE_2-releasing cells (36, 37). In contrast, COX protein isolated from Mφ treated in vivo was predominantly detected in the membrane fraction (Fig. 5A), but the COX activity was not greater than the background level seen in the nuclear and cytosolic fractions (Fig. 5B).

Taken together, these results indicate that, following in vivo treatment with 1 mg of HK-BCG, COX-1 and COX-2 are dissociated from the NE and catalytically inactive. It appears that these

FIGURE 1. Differential PGE_2 biosynthesis by Mφ treated in vitro or in vivo with HK-BCG. For in vivo HK-BCG treatment, groups of C57BL/6 mice received 1 mg of HK-BCG i.p. After 6 or 24 h, peritoneal lavage cells were harvested. For in vitro HK-BCG treatment, normal resident peritoneal Mφ were incubated with 100 μg/ml HK-BCG for 6 or 24 h, and cells were harvested. C indicates cells exposed to saline for 24 h. To determine PGE_2 release, the Mφ suspension (10^6/ml) was stimulated with 1 μM A23187 (■) or medium (□), for 2 h. PGE_2 was assayed by ELISA. Mean ± SD, n = 3, * p < 0.005 compared with C (saline) in the same group. #, p < 0.005 and ##, p < 0.0005 compared with the corresponding in vitro group, respectively.

FIGURE 2. The detection of COX-1 and COX-2 in Mφ treated in vitro or in vivo with HK-BCG. Peritoneal Mφ treated in vivo or in vitro with HK-BCG were prepared as indicated in Fig. 1. COX-1, COX-2, and GAPDH were determined by Western blotting as indicated in Materials and Methods. GAPDH bands show equivalent loading of samples.

FIGURE 3. Subcellular localization of COX-2 in peritoneal Mφ after HK-BCG treatment. Peritoneal Mφ treated in vivo or in vitro with HK-BCG for 24 h were prepared as indicated in Fig. 1. Cells were examined by confocal microscopy following staining with anti-COX-2 (green) and PI (red) for the nucleus. HK-BCG is also stained by PI.
inactive forms may be associated with aggregation of COX protein. In contrast, peritoneal Mφ exposed to HK-BCG in vitro expressed both COX protein and activity in the nuclear and membrane fractions.

**FIGURE 4.** Subcellular localization of COX-1 in peritoneal Mφ after HK-BCG treatment. Peritoneal Mφ treated in vivo or in vitro with HK-BCG for 24 h were prepared as indicated in Fig. 1. Cells were examined by confocal microscopy following staining with anti-COX-1 (green) and PI (red) for the nucleus. HK-BCG is also stained by PI.

**FIGURE 5.** Distribution and activity of COX in subcellular fractions. Peritoneal Mφ treated with HK-BCG in vitro or in vivo for 24 h as indicated in Fig. 1 were homogenized and separated into nuclear (N), membrane (M), and cytosolic (C) fractions by differential centrifugation as detailed in Materials and Methods. A, Protein (20 μg) in each fraction was used for COX-1 and COX-2 detection by Western blotting. B, The COX activity in each fraction was measured by using a COX assay kit (Cayman Chemical) following the manufacturer’s instructions. The specific enzyme activities were calculated and indicated as nanomoles per minute per milligram. Mean ± SD, n = 3. *, p < 0.001 compared with the activity of the corresponding in vitro fraction.

**FIGURE 6.** Time and HK-BCG dose dependence of COX-1 localization. Groups of C57BL/6 female mice (three per group) received 0.01, 0.1, or 1 mg of HK-BCG i.p. on day 0. Peritoneal lavage cells were harvested at times indicated. Subcellular localization of COX-1 as well as identification of intracellular HK-BCG were performed by confocal microscopy as described under Materials and Methods. Mean ± SE, n = 3. Percentage of COX-1 Mφ (●), percentage of Mφ with NE-dissociated COX-1 (●), percentage of Mφ with NE-dissociated COX-1 and PI-stained BCG (●), percentage of Mφ with NE-associated COX-1 (●), and percentage of Mφ with NE-associated COX-1 and PI-stained BCG (●).

**Kinetics of COX localization and phagocytosis of HK-BCG**

To determine the kinetics of the subcellular translocation of COX-1 and expression of COX-2, peritoneal Mφ were harvested at 2, 6, and 24 h after i.p. administration of 1 mg of HK-BCG. The localization of COX-1 and COX-2 in each sample was analyzed by confocal microscopy. Fig. 6 shows that 97% of untreated peritoneal Mφ expressed COX-1, whereas COX-1 Mφ were reduced to 56, 53, and 47% at 2, 6, and 24 h after treatment with 1 mg of HK-BCG. This reduction corresponds to the reduced COX-1 protein levels determined by Western blot (Fig. 2). The percentage of COX-1 present in the NE-dissociated dense form increased from 52% (30% of total cells) at 2 h until at 24 h nearly all COX-1 (44% of total cells) was in this form. Interestingly, 90% of the NE-dissociated (dense form) COX-1 Mφ had phagocytosed HK-BCG that was stained by PI (Fig. 6). We previously demonstrated that HK-BCG stained with PI are almost totally costained with anti-BCG Abs (34).

**FIGURE 7.** Time and HK-BCG dose dependence of COX-2 localization. Groups of C57BL/6 female mice (three per group) received 0.01, 0.1, or 1 mg of HK-BCG i.p. on day 0. Peritoneal lavage cells were harvested at times indicated. Subcellular localization of COX-2 as well as identification of intracellular HK-BCG were performed by confocal microscopy as described under Materials and Methods. Mean ± SE, n = 3. Percentage of COX-2 Mφ (●), percentage of Mφ with NE-dissociated COX-2 (●), percentage of Mφ with NE-dissociated COX-2 and PI-stained BCG (●), percentage of Mφ with NE-associated COX-2 (●), and percentage of Mφ with NE-associated COX-2 and PI-stained BCG (●).
COX-2, which was present in <1% of untreated cells, was seen in 60% of Mφ at 2 h with the NE-dissociated dense form present in 41% of these cells (25% of total cells) (Fig. 7). These NE-dissociated (dense form) COX-2 cells increased to >90% of COX-2 cells (70% of total cells) at 6 h. As for COX-1, most (95, 95, and 93% at 2, 6, and 24 h, respectively) of Mφ expressing NE-dissociated COX-2 contained intracellular PI-stained HK-BCG. Figs. 6 and 7 also show that, at 24 h, COX-1 was lost from half of the cells originally containing COX-1, and at the same time 81% of cells now express COX-2. The results further suggest that some cells contain both isoforms of COX.

Dose dependence of COX localization

The subcellular translocation of COX-1 and localization of COX-2 were also investigated at lower doses of HK-BCG (0.1 or 0.01 mg). The results shown in Fig. 6 indicate that the fractions of Mφ-expressing COX-1 and NE-associated COX-1 are both reduced dose dependently at 24 h. Intracellular HK-BCG was detected in over 80% of NE-dissociated (dense form) COX-1 Mφ (Fig. 6). Our results clearly indicate that the NE-dissociated (dense form) COX-1 pattern is associated with phagocytosis of HK-BCG.

At 24 h, there were 44 or 35% COX-2 Mφ in response to 0.1 or 0.01 mg of HK-BCG, respectively, compared with 81% at 1 mg of HK-BCG (Fig. 7). Mφ with NE-dissociated (dense form) COX-2 increased dose-dependently to 98% of total COX-2 Mφ at 1 mg of HK-BCG. At all doses, nearly all of these NE-dissociated COX-2 cells contained PI-stained intracellular HK-BCG. Although this was the predominant COX-2 phenotype in response to 1 mg of HK-BCG at 24 h, there were a few cells observed with NE-associated COX-2 containing HK-BCG (Fig. 7). Thus, in vivo treatment with 1 mg of HK-BCG resulted in a dramatic shift from NE-associated COX-1 to NE-dissociated and catalytically inactive COX-2 at 24 h.

Intraperitoneal administration of H. C. parvum and LPS induces NE-dissociated and -associated COX-2, respectively, in peritoneal Mφ

Additional studies following intraperitoneal administration of bacterial endotoxin (LPS) or H. C. parvum showed that soluble LPS induced catalytically active NE-associated COX-2, and H. C. parvum induced catalytically inactive, NE-dissociated COX-2 at 24 h (data not shown). These results further support the conclusion that NE-dissociated (dense form) COX-2 expression depends on phagocytosis.

### Table 1. The effects of peritoneal fluid isolated from HK-BCG-treated mice on the localization of COX-2 in vitro

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<thead>
<tr>
<th>Cocultures</th>
<th>COX-2 Localization&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td>Responders with HK-BCG in vitro&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Additive from HK-BCG-treated mice&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Unlabeled Mφ</td>
<td>RPMI 1640</td>
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<td>Unlabeled Mφ</td>
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<td>Unlabeled Mφ</td>
<td>Sera</td>
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<td>CFDA Mφ</td>
<td>Peritoneal cells</td>
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<sup>a</sup> Cells were stained with Ab to COX-2 and examined by confocal microscopy. Percentage of COX-2<sup>+</sup> Mφ with NE-associated COX-2 or with NE-dissociated COX-2 is indicated.

<sup>b</sup> Peritoneal Mφ at 10⁶ cells/ml were labeled in vitro with CFDA or media (unlabeled), and stimulated with 100 μg/ml HK-BCG for 6 h before transferring additive.

<sup>c</sup> Mice received 1 mg of HK-BCG i.p. After 6 h, peritoneal lavage was performed with 1 ml of cold serum-free RPMI 1640 and the lavage fluid was centrifuged to isolate cells from the peritoneal fluid. Sera were also harvested. As stimulators, 10⁶ peritoneal cells/ml, 20% peritoneal fluid or 20% sera were added to the in vitro responder cells. These mixtures were cultured for an additional 18 h.

### The effects of peritoneal fluid isolated from HK-BCG-treated mice on localization of COX-2 in vitro

Our results suggest that extracellular factors produced by in vivo HK-BCG treatment regulates the localization of COX-2 in peritoneal Mφ. To test this hypothesis, CFDA-labeled or unlabeled normal peritoneal Mφ were challenged with HK-BCG and cocultured with peritoneal cells, cell-free peritoneal fluid, or sera isolated from HK-BCG-treated mice. All CFDA-labeled Mφ expressed NE-associated COX-2 in response to HK-BCG in vitro and the localization was unchanged by coculture with peritoneal cells, peritoneal fluid, or sera from HK-BCG-treated mice (Table I) or from normal mice (data not shown). Thus, it appears that extracellular mediators produced by HK-BCG treatment in vivo do not induce the NE-dissociated form of COX-2 in Mφ treated in vitro with HK-BCG.

### Discussion

#### NE-dissociated COX

It is well-established that catalytically active COX-2 is localized in the NE/ER of activated human monocytes and umbilical vein endothelial cells, as well as murine NIH 3T3 cells, and splenic Mφ, where it mediates PGE<sub>2</sub> biosynthesis (30–32, 34). However, our present and previous studies (34) demonstrate that most splenic and peritoneal Mφ isolated from mice 24 h after receiving 1 mg of HK-BCG i.p. express catalytically inactive COX-2. This inactive COX-2 appears as a densely stained structure and is completely dissociated from the NE. Constitutively expressed COX-1 is also detected in a dense form dissociated from the NE in these Mφ. Thus, catalytically inactive forms of COX-1 and COX-2 are present, without PGE<sub>2</sub> biosynthesis, in activated splenic (34) and peritoneal Mφ. However, it is of particular note that the stimulation of normal Mφ with HK-BCG in vitro results in expression of catalytically active COX-2, which is localized in the NE/ER. Therefore, factors induced in vivo by HK-BCG administration are obligatory for establishing the catalytically inactive form of COX and consequently the regulation of PG production.

### Role of phagocytosis

Another provocative finding is that intracellular HK-BCG is observed in nearly all NE-dissociated COX-2<sup>+</sup> peritoneal Mφ isolated from mice 24 h after i.p. administration of HK-BCG (Fig. 7). Thus, phagocytosis of HK-BCG in the local tissues is significantly associated with expression of the NE-dissociated COX-2, although there are a few COX-2<sup>+</sup> Mφ without intracellular HK-BCG and a
few COX-2− Mφ with intracellular HK-BCG in the same samples. These and our earlier studies indicate that the in vivo phagocytosis of HK-BCG by Mφ in various tissues results in the formation of catalytically inactive COX-2. In preliminary studies in which HK-BCG was administered intranasally, this phenomenon was also demonstrated in alveolar Mφ (data not shown). However, for mice receiving lower doses of HK-BCG, Mφ subsets with NE-associated (active) COX-2 and intracellular HK-BCG are present in relatively greater numbers in peritoneal Mφ (Fig. 7). In contrast, peritoneal Mφ activated in vitro with HK-BCG show only NE/ER-associated, catalytically active COX-2 with intracellular HK-BCG.

Furthermore, our studies with peritoneal Mφ in culture (Table I) do not support the hypothesis that extracellular factors produced in response to HK-BCG in the peritoneal cavity or present in sera regulate the subcellular localization of COX isozymes. Thus, it appears that other endogenous factors associated with phagocytosis in vivo but not in vitro are important for the formation of catalytically inactive COX-2. Because the coculture studies presented in Table I were performed for only one set of time intervals, it is possible that, for instance, the early transient expression of a particular cellular mediator is critical for regulating the subcellular localization of COX and its activity.

As presented in Fig. 5, the membrane (M) fraction from the Mφ treated in vivo has less COX activity than the same fraction taken from cells treated in vitro, despite almost equivalent COX protein levels. This suggests that either 1) the COX isozymes in the membrane fractions of the cells treated in vivo have been structurally modified, rendering them less active, or 2) there is a nonstructural (biochemical) basis for this difference. One possibility is that the reduction/oxidation (redox) states of the membrane fractions differ between Mφ exposed to HK-BCG in vivo and those exposed in vitro. The activities of both COX-1 and COX-2 are modulated by ambient hydroperoxide availability (38). It may be speculated that the sequestered NE-dissociated COX found in peritoneal Mφ from HK-BCG-treated mice is, at least in part, a consequence of a low “peroxide tone” which limits COX activity.

COX localization in other studies

Other recent studies indicate that COX-2 localizes not only to the NE but also other subcellular sites. D’Avila et al. (39) have reported that intraperitoneal administration of live BCG induces lipid-laden pleural Mφ in a TLR2-dependent but phagocytosis-independent manner. In these Mφ, COX-2 is expressed and localized at lipid bodies within 24 h, and mediates a large amount of PGE2 production. For phorbol ester (PMA)-stimulated bovine aortic endothelial cells, Liou et al. (40) found that COX-2 is present in cytosolic vesicle-like structures, and that PGI2 synthesis by these cells is not enhanced. In PMA- and IL-1β-treated fibroblasts, catalytically active COX-2 is found in the plasma membrane localized with caveolin (41). Girotti et al. (42) indicated that catalytically active COX-2 is localized in the phagosomes of peritoneal Mφ after in vitro phagocytosis of zymosan particles. The localization of cPLA2α followed by COX-2 to the phagosome correlated with the time course of PGE2 production, suggesting that the phagosome membrane may serve as a site for release of AA and prostanoid production. However, the magnitudes of PGE2 biosynthesis at the phagosome and NE were not reported (42). It is predicted that without colocalization of COX-2, cPLA2α, and PGES, PGE2 synthesis does not occur and COX-2 is apparently inactive. Thus, regulation of COX-2 activity associated with its subcellular localization appears to be complex, dependent on cell types and specific activating agents.

COX-2 is not associated with phagosome

In our study using Mφ activated in vivo, the dense form of COX-2 does not appear to be directly associated with intracellular HK-BCG (Fig. 3) or lysosome-associated membrane protein 1-positive late phagosomes (data not shown). Furthermore, activation of Mφ in vitro also indicated that there is no direct association of COX-2 with intracellular HK-BCG (Fig. 3). Spencer et al. (36) demonstrated in their mutation analysis of COXs that the mutant proteins, which lack membrane binding domains and enzyme activity, are distributed in the microsomal fraction. They suggested that these mutant proteins are mostly present as unfolded aggregates. Although the membrane-binding domains of COX-1 and COX-2 appear to be important for maintaining their catalytic activity, the mechanisms underlying NE dissociation and enzyme inactivation are still unknown.

Role of COX-2 localization

In response to bacterial components, Mφ become bactericidal with increases in NADPH oxidase/superoxide anion release, inducible NO synthase/NO production, and IL-12/IFN-α synthesis. PGE2 down-regulates Th1 responses and bactericidal activity toward intracellular organisms. It is therefore reasonable to speculate that catalytically inactive COX-2− Mφ enhance the development of bactericidal activities more effectively than Mφ with catalytically active COX-2. In our studies, at 24 h following treatment with 1 mg of HK-BCG, both COX-1 and COX-2 are NE dissociated and inactive. Compartmentalization of COX might aid the development of bactericidal activity by placing this enzyme in a location where catalysis cannot occur. Whether additional posttranslational modification is involved in this regulation of COX activity is not known.

Conclusion

Our present and previous findings (12, 34) indicate that normal peritoneal and splenic Mφ treated with HK-BCG in vitro express catalytically active COX-2 and release increased amounts of PGE2 within 24 h. However, administration of HK-BCG activates various tissue Mφ locally and systemically to express either catalytically active or inactive COX-2, dependent on route of administration, dose, timing, in vivo phagocytosis and the presence of bone marrow-derived PGE2−Mφ progenitors, which localize and mature at inflammatory sites. Although more studies are needed to elucidate regulatory mechanisms for the diversity of COX-2− Mφ formation in vivo, it appears that the distinct COX-2− Mφ subsets may play pro- and anti-inflammatory roles.

Disclosures

The authors have no financial conflict of interest.

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