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Pivotal Role of Reactive Oxygen Species in Differential Regulation of Lipopolysaccharide-Induced Prostaglandins Production in Macrophages

Guiqing Zhao, Rui Yu, Jing Deng, Qiong Zhao, Yongchao Li, Myungsoo Joo, Richard B. van Breemen, John W. Christman, and Lei Xiao

Department of Medicine, Sections of Pulmonary, Critical Care, Sleep and Allergy (G.Z., J.D., M.J., J.W.C., L.X.) & Cardiology (G.Z., L.X.), Center for Cardiovascular Research (G.Z., Q.Z., L.X.), and Department of Medicinal Chemistry and Pharmacognosy (R.Y., Y. L., R.B.v.B.), University of Illinois at Chicago, Chicago, IL; Department of Medicine (Q.Z.), Northwestern University, Chicago, IL; Department of Applied Medicine (M.J.), Pusan National University, Yangsan, Republic of Korea; The Jesse Brown Veterans Affairs Medical Center, Chicago, IL (J.W.C.).

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Running Title: Pivotal role of ROS in LPS-induced PGD_2 production in BMDM

Address correspondence to: Lei Xiao, M.D., Ph.D.
Department of Medicine, M/C: 719
University of Illinois at Chicago
840 S. Wood Street
Chicago, IL 60612
Telephone: 312-996-9078
Fax: 312-996-4665
E-mail: leixiao@uic.edu

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Abbreviation: LPS, lipopolysaccharide; ROS, reactive oxygen species; PG, prostaglandin; BMDM, bone marrow-derived macrophages; NOX, NADPH oxidase; METC, mitochondrial electron transport chain; zymosan, zymosan A; MnTMPyP, Mn(III)-terakis-(1-methyl-4-pyridyl)-porphyrin pentachloride; EUK-134, Chloro[[2,2'-[1, 2-ethanediylbis[(nitrilo-κN)methylidyne]]bis[6-methoxyphenolato-κO]]]-manganese; H-PGDS, hematopoietic PGD synthase; HQL-79, 4-(diphenylmethoxy)-1-[3-(1H-tetrazol-5-yl)propyl-piperidine; L-PGDS, lipocalin PGD synthase; AT-56, 4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-1-[4-(2H-tetrazol-5-yl)butyl]-piperidine; COX, cyclooxygenase; cPGES, cytosolic PGE synthase; mPGES-1, microsomal PGE synthase-1; NOS, nitric oxide synthase; L-NAME, L-N^G-nitroarginine methyl ester; SOD, superoxide dismutase; DPI, diphenylene iodonium; PAO, phenylarsine oxide; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PVDF, polyvinylidene difuoride; BHT, butylated hydroxytoluene; LC-MS-MS, liquid chromatography-tandem mass spectrometry; HPLC, high performance liquid chromatography; SRM, selected reaction monitoring; TLR4, Toll-like receptor 4.

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Abstract

Gram-negative bacterial endotoxin lipopolysaccharide (LPS) triggers the production of inflammatory cytokines, reactive oxygen species (ROS), and prostaglandins (PGs) by pulmonary macrophages. Here, we investigated if ROS influenced PGs production in response to LPS treatment in mouse bone marrow-derived macrophages (BMDM). We observed that pretreatment of BMDM with two structurally-unrelated ROS scavengers, MnTMPyP and EUK-134, not only prevented LPS-induced ROS accumulation, but also attenuated the LPS-induced PGD_2, but not PGE_2 production. Conversely LPS-induced PGD_2, but not PGE_2 production, was potentiated with the co-treatment of BMDM with H_2O_2. These data suggest that ROS differentially regulates PGD_2 and PGE_2 production in BMDM. In addition, selective inhibition of the ROS generator NADPH oxidase (NOX) using either pharmacologic inhibitors or its $p47^{phox}$ subunit deficient mouse BMDM also attenuated LPS-induced PGD_2, but not PGE_2 production, suggesting the critical role of NOX-generated ROS in LPS-induced PGD_2 production in BMDM. We further found that both hematopoietic PGD synthase (H-PGDS) siRNA and its inhibitor HQL-79, but not lipocalin PGDS (L-PGDS) siRNA and its inhibitor AT-56, significantly attenuated LPS-induced PGD_2 production, suggesting that H-PGDS, but not L-PGDS, mediates LPS-induced PGD_2 production in BMDM. Furthermore, data from our *in vitro* cell-free enzymatic studies showed that co-incubation of the recombinant H-PGDS with either MnTMPyP, EUK-134 or catalase significantly decreased PGD_2 production; whereas co-incubation with H_2O_2 significantly increased PGD_2 production. Taken together, our results show that LPS-induced NOX-generated ROS production differentially and specifically regulates the H-PGDS-mediated production of PGD_2, but not PGE_2, in mouse BMDM.

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Introduction

Cyclooxygenase (COX) converts arachidonic acid to PGH_2, which is the precursor of distinct PGs. PGs are a group of lipid compounds derived from fatty acids in nearly every cell type (Funk, 2001; Cao et al., 2008) and mediate a variety of important physiologic functions *in vivo* (McAdam et al., 2000; Burleigh et al., 2002). For example, PGE_2 promotes tumor initiation, progression and metastasis (Samuelsson et al., 2007); while PGD_2 triggers asthmatic responses (Matsuoka et al., 2000). Both PGE_2 and PGD_2 are converted from PGH_2 by various isoforms of PGE synthase (PGES) and PGDS (Samuelsson et al., 2007; Yu et al., 2011), respectively. We recently showed that BMDM express all 3 types of PGES including cytosolic PGES (cPGES), microsomal PGES-1 (mPGES-1), and mPGES-2; as well as H-PGDS (Xiao et al., 2010). H-PGDS is a cytosolic, GSH-dependent enzyme that catalyzes the isomerization of PGH_2 to PGD_2, whereas L-PGDS is an *N*-glycosylated, GSH-independent protein (Urade and Eguchi, 2002). HQL-79 and AT-56 are reported isoform-selective PGDS inhibitors interdicting H-PGDS- and L-PGDS-mediated PGD_2 production both *in vivo* and *in vitro*, respectively (Matsushita et al., 1998a, b; Irikura et al., 2009).

ROS are chemical reactive molecules containing oxygen generated during normal and disease-related metabolic processes (Bedard and Krause, 2007; Xiao et al., 2002), including three major species superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2). Several major ROS-generating systems in different tissues have been identified, including NOX (Xiao et al, 2002; Cross and Segal, 2004), mitochondrial electron transport chain (METC) (Balaban et al., 2005), and nitric oxide synthase (NOS) (Xia et al., 1998). METC inhibitors, rotenone and antimycin A, have been shown to induce mitochondrial ROS generation from mitochondria Complex I and Complex III, respectively (Li et al., 2003). The NOS-selective inhibitor L-NAME

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prevented NOS-produced NO or ROS generation (Kim et al., 2007). ROS have been reported to play an important role in carcinogenesis (Benhar et al., 2002), cardiac myocyte hypertrophy (Amin et al., 2001), and augmented airway obstruction in asthma (Henricks and Nijkamp, 2001). We have shown that in BMDM, LPS induces both generation of ROS and PGs, which are important mediators in host defense (Park and Christman, 2006; Xiao et al., 2010). MnTMPyP is a membrane-permeable and non-toxic superoxide dismutase (SOD)/catalase mimetic that efficiently scavenges ROS (i.e. $O_2^{\bullet-}$ and H_2O_2) both *in vivo* and *in vitro* (Amin et al., 2001; Xiao et al., 2002; Zhao et al., 2011). EUK-134 is another structurally-unrelated and membrane-permeable synthetic SOD/catalase mimetic that has been commonly used as a scavenger for intracellular ROS and peroxynitrite (Rong et al., 1999). Catalase catalyzes the decomposition of H_2O_2 to water and oxygen (Yu et al., 2006).

NOX is a membrane-bound multi-subunit enzyme complex (Bedard and Krause, 2007; Lambeth, 2004; Geiszt and Leto, 2004) that contains 2 transmembrane subunits $gp91^{phox}$ and $p22^{phox}$, and at least 4 cytosolic subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac. Currently, 6 additional isoforms (Nox1, 3, 4 & 5, and Duox1 & 2) of the NOX catalytic subunit $gp91^{phox}$ (Nox2) have been reported (Lambeth, 2004; Geiszt and Leto, 2004; Shiose et al., 2000). The primary enzymatic activity of NOX is to generate superoxide ($O_2^{\bullet-}$) by transferring a single electron from NADPH/NADH to O_2 (Leto, 1999). The NOX-generated $O_2^{\bullet-}$ radicals are rapidly converted to H_2O_2 by intracellular SOD, which is further decomposed by catalase to water and oxygen. Diphenylene iodonium (DPI) is a commonly used NOX-selective inhibitor although it also inhibits other flavin protein-containing enzymes including NOS or METC (Bedard and Krause, 2007; Jaquet et al., 2009). The membrane-permeable protein tyrosine phosphatase

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inhibitor phenylarsine oxide (PAO) is also reported as a potent NOX2-selective inhibitor (Xiao et al., 2002; Jaquet et al., 2009).

We and others have previously shown that LPS induced production of both PGD_2 and PGE_2 in macrophages via the expression of COX-2 (Park and Christman, 2006). We also recently showed that LPS stimulated the activation and expression of the NOX enzyme as well as PGs production in BMDM (Zhao et al., 2010). ROS are known to play important roles in carcinogenesis (Benhar et al., 2002) and asthma (Henricks and Nijkamp, 2001) that are mediated by PGE_2 (Samuelsson et al., 2007) and PGD_2 (Matsuoka et al. 2000), respectively. However, it is unclear if LPS-induced production of PGs is functionally connected with NOX-dependent ROS generation in BMDM. Here, we thus explored the potential regulatory roles and signaling mechanisms of ROS in LPS-induced PGs production in BMDM.

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Materials and Methods

Animals. The $p47^{phox}$-deficient mice (10- to 12-weeks old) were kindly provided from Dr. Steven M. Holland (National Institutes of Health, Bethesda, MD). Age- and gender-matched wild type (WT) C57BL/6 male mice from Harlan were used with the $p47^{phox}$-deficient mice in paired experiments. Mice were housed at the University of Illinois at Chicago (UIC) animal facility in a temperature-controlled room with a 12:12-h light-dark cycle and were given standard chow and bottle water. All procedures and protocols using mice were approved by the UIC animal care committee and complied with the Animal Welfare Act.

Materials. Hanks' Balanced Salt solution, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech (Manassas, VA). Lysis buffer was purchased from Cell Signaling Technology (Danvers, MA). Laemmli sample buffer was purchased from Bio-Rad Laboratories (Richmond, CA). Polyvinylidene difluoride (PVDF) membrane was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). SuperSignal chemiluminescent substrate solution and enhanced chemiluminescence solution were purchased from Thermo Fisher Scientific (Rockford, IL). Amaxa Mouse Macrophage Nuclefector Kit (catalog # VPA-1009) was purchased from Lonza (Walkersville, MD), and siRNA's were purchased from Thermo Dharmacon (Lafayette, CO). The primary antibody for L-PGDS (M-17), Actin and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibodies for COX-2, H-PGDS and L-PGDS (catalog # 10004344), human recombinant H-PGDS, human recombinant L-PGDS, human recombinant mPGES-1, GSH, EUK-134, HQL-79, AT-56, PGH_2, PGD_2, PGE_2, d_4-PGD_2 and d_4-PGE_2 were purchased from Cayman Chemicals (Ann Arbor, MI). Isoluminol, NADPH,

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zymosan A (zymosan), LPS, rotenone, antimycin A, catalase, H_2O_2, Tris base, hydrogen peroxide, citric acid, and EDTA were purchased from Sigma-Aldrich (St. Louis, MO). MnTMPyP, DPI, PAO, L-NAME, and formic acid were purchased from EMD Chemicals (San Diego, CA). Hydrochloric acid and butylated hydroxytoluene (BHT) were purchased from Thermo Fisher Scientific (Rockford, IL). Purified water was prepared using a Millipore (Billerica, MA) Milli-Q purification system. All organic solvents were HPLC grade or better and were purchased from Thermo Fischer (Rockford, IL), and all other chemicals and solvents were ACS reagent grade, unless stated otherwise.

BMDM isolation and culture. BMDM were isolated from WT C57BL/6 or $p47^{phox}$-deficient mice as we previously described (Yu et al., 2011). Briefly, after mice were euthanized, bone marrow was flushed from the rear femurs. The cells were washed and resuspended in DMEM medium containing 10% endotoxin-free FBS and 10% (v/v) L929 cell-conditioned medium as a biologic source of macrophage colony-stimulating factor. The medium was then replenished at Day 4 in culture, and the non-adherent cells were removed. The adherent bone marrow cells were used for experiments after Day 7 in culture, corresponding to a mature macrophage phenotype.

Western blot assay. Western blot method was similar as we previously described (Cao et al., 2008; Xiao et al., 2002). Briefly, BMDM were harvested in protein lysis buffer and sonicated for 10 s to shear genomic DNA. Protein concentration was determined by the Bradford assay. Equal amounts of the denatured proteins in Laemmli sample buffer were subjected to SDS-PAGE, and transferred to a PVDF membrane, blocked with 5% nonfat dry milk, then incubated with primary antibody at 4°C overnight. Protein was detected with horseradish peroxidase-conjugated secondary antibody using SuperSignal enhanced chemiluminescent method.

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Supernatant sample preparation. Supernatant sample preparation was similar to what we previously described (Cao et al., 2008; Yu et al., 2011). Briefly, a 500-µl aliquot of supernatant was spiked together with d_4-PGE_2 and d_4-PGD_2 as surrogate standards. Next, citric acid and BHT were added to prevent free radical-catalyzed peroxidation. Prostaglandins were extracted by adding hexane/ethyl acetate (50:50, v/v). The upper organic phase was collected, and the extraction procedure was repeated, then the organic phases were combined and evaporated to dryness. Immediately before analysis using liquid chromatography-tandem mass spectrometry (LC-MS-MS), each extract was reconstituted in methanol/water (50:50, v/v). Standards for calibration curves and quality control measurements were prepared by spiking 500 µl aliquots of cell culture medium with measured amounts of PGD_2 and PGE_2. These standards were then processed as described above. The concentrations of PGD_2 and PGE_2 in these standards ranged from 0.1 to 1000 ng/ml.

siRNA transfection. Primary BMDM were transfected with either ON-TARGET *plus* control siRNA (25 nM) or siRNA's for H-PGDS or L-PGDS (25 nM) using the Amaxa Mouse Macrophage Nuclefector Kit. After 48-h-post-transfection, BMDM were stimulated by LPS (1 µg/ml) for 16 h.

ROS detection by chemiluminescence assay. The isoluminol-enhanced chemiluminescent assay for ROS detection in BMDM was similar to the previous reports (Dahlgren and Karlsson, 1999; Maeda et al., 2010). Briefly, primary cultured BMDM were seeded into 96-well culture plate (E&K Scientific) at 5 x 10^4/well in triplicate, and primed with 100 ng/ml LPS for 16 h prior to ROS measurement. The culture medium was then replaced with phenol red- and serum-free DMEM for subsequent ROS measurement. After pre-incubation with 50 M isoluminol, 40 U/ml HRP, and 100 µM NADPH at 37°C in dark for 5 min, the BMDM

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were stimulated with either LPS (1 μg/ml) or the known phagocyte ROS stimulus zymosan (200 μg/ml). Chemiluminescence signals of each well were continuously recorded for 2 h post-stimulation in a BioTek Synergy 2 multi-detection microplate reader (BioTek, Winooski, VT), and the averaged net increase of the plateau chemiluminescence signals was used to quantify the ROS production in each sample.

The *in vitro* cell-free enzyme assay. Equal amount of recombinant PG synthase, including mPGES-1 and H-PGDS from Cayman Chemicals, was prepared in Tris•HCl buffer (pH 8.0 at 37°C) on ice in the presence of enzyme cofactor GSH. When necessary, an aliquot of MnTMPyP, catalase, or H_2O_2 was added into the appropriate sample, and pre-incubated at 37°C for 10 min. The reaction was initiated by adding the enzyme substrate PGH_2, followed by incubation for 30 min at 37°C. Identical amounts of PGD_2 and PGE_2 were added in separate tubes as quantitative controls. Mixture of d_4-PGD_2 and d_4-PGE_2 was added as internal standards. The reaction was terminated by adding HCl, and each sample was extracted using hexane/ethyl acetate (50:50, v/v), and the organic phase was removed, evaporated to dryness, and reconstituted in methanol/water (50:50, v/v) immediately prior to quantitative analysis using LC-MS-MS (Yu et al., 2011).

Mass spectrometry. For the quantitative analysis of PGD_2 and PGE_2, HPLC separations were carried out using a Shimadzu (Columbia, MD) Prominence HPLC system with a Waters (Milford, MA) XTerra MS C_{18} (2.1 mm × 50 mm, 3.5 μm) analytical column and a 5-min isocratic mobile phase consisting of acetonitrile/aqueous 0.1% formic acid (37:63, v/v) at a flow rate of 200 μl/min. The HPLC system was interfaced to a Thermo-Finnigan (San Jose, CA) TSQuantum triple quadruple mass spectrometer that was operated using negative ion electrospray. Isomeric PGD_2 and PGE_2 were measured using a SRM transition of *m/z* 351 to *m/z*

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271, and the SRM transition of *m/z* 355 to *m/z* 275 was selected for the internal standards d_4-PGE_2 and d_4-PGD_2 (Yu et al., 2011; Cao et al., 2010; Cao et al., 2011).

Statistical analysis. Samples were run in triplicate (unless stated otherwise), and values were expressed as mean ± SEM. Statistical significance was assessed using either student *t* test or one-way analysis of variance (ANOVA), and p value < 0.05 were considered significantly different.

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Results

LPS and zymosan induced ROS generation in BMDM. The Gram-negative bacterial endotoxin LPS is known to selectively activate Toll-like receptor 4 (TLR4) in the mammalian immune system and triggers the secretion of inflammatory cytokines and PGs in macrophages (Azim et al., 2007). As shown in Figure 1A, LPS treatment concentration-dependently (0.01, 0.1, 1, 5 μg/ml, 16 h) stimulated both PGD_2 and PGE_2 production in BMDM. Since 1 and 5 μg/ml LPS treatment showed similar plateau levels of PGs production, we thus used 1 μg/ml LPS treatment in the following studies. Similarly, LPS treatment also concentration-dependently (0.01, 0.1, 1 μg/ml) induced ROS generation in BMDM (Fig. 1B).

Although LPS is known to stimulate ROS production in neutrophils, the reports of LPS-induced ROS generation in macrophages have been controversial in the literature (Deschacht et al., 2010; Pfeiffer et al., 2007; Maeda et al., 2010; Szefler et al., 1989). In contrast, zymosan consistently stimulates ROS generation in macrophages (Russwurm et al., 1994; Bramble and Anderson, 1998), and thus was used as a positive ROS stimulus in our studies. Like zymosan stimulation, LPS significantly induced ROS generation in BMDM to a level similar to that of zymosan (Fig. 1B and 1C), which was completely abolished by two structurally-unrelated ROS scavengers MnTMPyP (50 μM, Fig. 1C) or EUK-134 (5 μM Fig. 2C), suggesting that the ROS scavenger MnTMPyP (50 μM) and EUK-134 (5 μM) pretreatment could efficiently and completely prevent LPS-stimulated ROS generation in BMDM.

ROS regulated PGD_2 production in BMDM. We have previously reported that LPS induced both PGD_2 and PGE_2 production in macrophages via TLR4 pathway (Park and Christman, 2006; Xiao et al, 2010). In the current studies, we found that pretreatment of BMDM with the ROS scavenger MnTMPyP not only concentration-dependently attenuated the LPS-

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stimulated ROS generation (Fig. 2A), but also significantly and selectively decreased LPS-induced PGD_2 production by about 85% at 50 μM (Fig. 2B); whereas the LPS-induced PGE_2 production was not affected (Fig. 2B). Similarly, pretreatment of BMDM with another structurally-unrelated ROS scavenger EUK-134 also concentration-dependently (0.5, 2, 5 μM) attenuated the LPS-stimulated ROS generation (Fig. 2C) and production of PGD_2, but not PGE_2 (Fig. 2D). Conversely, when increasing the overall cellular ROS level by directly adding H_2O_2 (0.3 mM) in BMDM, the LPS-induced production of PGD_2, but not PGE_2, was selectively and significantly enhanced by over 65%. In contrast, H_2O_2 treatment alone could not induce any detectable PGs production in BMDM (Fig. 2E). When BMDM were pretreated with a COX-2-selective inhibitor NS-398 (0.5 h), LPS-induced both PGE_2 and PGD_2 production were concentration-dependently (0.001 to 0.1 μM), but non-selectively inhibited to the same extent at each tested concentration (Fig. 2F). This result is completely different from our above observation using ROS scavengers or H_2O_2 (only production of PGD_2, but not PGE_2, was affected), suggesting that the observed ROS effect on PG production is not likely acting on the COX-2 enzyme.

NOX regulated PGD_2 production in BMDM. To further investigate the potential ROS source in BMDM, we next used a series of selective inhibitors targeting various reported potential ROS generating systems in our studies, including NOX (DPI and PAO), METC (rotenone and antimycin A), and NOS (L-NAME). Among the above pharmacological inhibitors, both DPI (0.5, 1.5, 5 μM, Fig. 3A) and PAO (0.1, 0.3, 1 μM, Fig. 3B) pretreatment of BMDM concentration-dependently inhibited LPS-induced production of PGD_2, but not PGE_2, similar to that seen with the MnTMPyP or EUK-134 pretreatment. The LPS-induced PGD_2 production was reduced by about 87% with 5 μM DPI pretreatment (Fig. 3A), while 1 μM PAO inhibited LPS-

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induced PGD_2 production by around 80% (Fig. 3B). Since DPI may also inhibit other potential ROS generating enzymes containing flavin-protein (i.e., NOS or mitochondrial enzymes), we thus next tested the effects of selective inhibitors for NOS or mitochondria on LPS-induced PGD_2 production. In contrast, pretreatment of BMDM with mitochondrial inhibitor rotenone (1 μM) and antimycin A (1 μM), or the NOS inhibitor L-NAME (1 mM) had no effect on LPS-induced production of either PGD_2 or PGE_2 (Fig. 3C), thus excluding the possibility of mitochondria and NOS as the potential ROS generators in BMDM regulating LPS-stimulated PGD_2 production. Taken together, these data suggested that NOX, but not mitochondria or NOS, was the potential ROS generator in response to the LPS treatment in mouse BMDM.

In order to confirm our above finding using pharmacological inhibitors, we next used molecular approach to test our hypothesis in our studies. NOX is a multi-subunit enzyme that requires the presence of a critical cytosolic subunit $p47^{phox}$ for its enzyme activity of ROS generation. The $p47^{phox}$-deficient transgenic mice showed significantly attenuated ability of NOX-mediated ROS production in neutrophils (Bäumer et al., 2008; Leto et al., 2009). Similar to the above inhibitory effects observed with MnTMPyP, EUK-134, DPI and PAO, the LPS-induced production of PGD_2, but not PGE_2, was significantly lowered by about 60% in BMDM from the $p47^{phox}$-deficient mice compared to that from the WT mice; whereas DPI and PAO showed no further inhibitory effect on LPS-induced PGD_2 production in $p47^{phox}$-deficient BMDM (Fig. 3D). This result from the $p47^{phox}$-deficient transgenic mouse confirmed that NOX/$p47^{phox}$ was involved in regulating LPS-induced PGD_2 production.

Both DPI and PAO inhibited the LPS-induced ROS generation in WT BMDM (Fig. 3E), confirming their inhibitory effects on NOX-mediated ROS production in response to LPS stimulation. The LPS-induced ROS generation in $p47^{phox}$-deficient BMDM decreased by about

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70% (Fig. 3E), indicating that in response to LPS stimulation, the $p47^{phox}$-deficient BMDM still could generate a lower but yet significant amount of ROS, which was completely abolished by the ROS scavenger MnTMPyP (Fig. 3E). Similarly, the LPS-induced production of PGD_2, but not PGE_2, was attenuated by about 60% in $p47^{phox}$-deficient BMDM (Fig. 3D).

H-PGDS mediates LPS-induced PGD_2 production in BMDM. LPS stimulation is known to generate PGs in BMDM via the expression and activation of COX-2 enzyme (Park and Christman, 2006; Xiao et al., 2010). Two isoforms of PGD synthases have been reported, namely H-PGDS and L-PGDS (Matsushita et al., 1998a, b; Irikura et al., 2009). Our data showed that pretreatment of BMDM with MnTMPyP (50 μM, Fig. 4A), DPI (5 μM), or PAO (1 μM) (Fig. 4B) had no effect on either H-PGDS or LPS-induced COX-2 expression, suggesting that the inhibitory effect of MnTMPyP, DPI and PAO on PGD_2 production was not via the inhibition of COX-2 or H-PGDS enzyme expression. In addition, using two different isoform-selective antibodies targeting for mouse L-PGDS isomerase with the mouse brain tissue as a positive control, our Western blot results indicated no detectable L-PGDS protein expression in mouse BMDM (Fig. 4C), suggesting that L-PGDS was not likely to be the PGDS isoform that mediated LPS-induced PGD_2 production. To further identify the PGDS isomerases involved in this signaling pathway, siRNA's for both H-PGDS and L-PGDS, H-PGDS-selective inhibitor HQL-79, and L-PGDS-selective inhibitor AT-56 were used. Selective inhibition of H-PGDS protein expression using its siRNA (Fig. 4D) significantly attenuated LPS-induced PGD_2 production by about 55% in BMDM (Fig. 4E); whereas siRNA for L-PGDS did not show any inhibitory effect on LPS-induced PGD_2 production (Fig. 4E). Additionally, the H-PGDS-selective inhibitor HQL-79 concentration-dependently (5, 20, 100 μM) attenuated LPS-induced PGD_2 production in BMDM (Fig. 5A); whereas the L-PGDS-selective inhibitor AT-56 (5, 20, 100 μM) had no such

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effect (Fig. 5B). Furthermore, additive effects on the inhibition of LPS-induced PGD_2 production were observed after co-pretreatment of BMDM with HQL-79 (20 μM) and DPI (0.5 μM) or PAO (0.1 μM) (Fig. 5C), suggesting the roles of both NOX and H-PGDS in regulation of LPS-induced PGD_2 production. Taken together, our data suggested that NOX regulated the enzymatic function of H-PGDS that mediated LPS-induced PGD_2 production in mouse BMDM.

ROS directly regulate H-PGDS-mediated PGD_2 production *in vitro*. In order to test if ROS could directly regulate H-PGDS-mediated PGD_2 production *in vitro*, ROS scavenger MnTMPyP, EUK-134, catalase and H_2O_2 were used in our *in vitro* cell-free enzymatic assay. Co-incubation of recombinant enzyme H-PGDS and its substrate PGH_2 with either MnTMPyP (10, 50, 500 μM, Fig. 6A), EUK-134 (2, 5, 20 μM, Fig. 6B), or catalase (10, 100, 1000 units, Fig. 6D) showed concentration-dependent attenuation of H-PGDS-mediated PGD_2 production; whereas co-incubation with H_2O_2 (1, 5, 10 μM) significantly promoted H-PGDS-mediated PGD_2 production (Fig. 6E). In contrast, co-incubation of recombinant enzyme mPGES-1 and its substrate PGH_2 with MnTMPyP, EUK-134, catalase, or H_2O_2 showed no significant effect on PGE_2 production under the same experimental conditions *in vitro* (Fig. 6F). Since there is no other cytosolic factors or enzymes present in this simple cell-free reaction system except for the recombinant isomerase (i.e., either H-PGDS or mPGES-1) and its substrate PGH_2 in PBS buffer, these *in vitro* cell-free enzyme assay results confirmed that the ROS level or redox state surrounding the H-PGDS enzyme directly affect its enzymatic capability of PGD_2 production. Unlike mPGES-1, an adequate basal level of ROS is required for H-PGDS activity of PGD_2 production *in vitro*. To strengthen our conclusion, we further determined the basal oxidant level in the above enzyme reaction mixture, which showed significant decrease of ROS signals (i.e.,

superoxide) when MnTMPyP or EUK-134 was added into the reaction mixture (Fig. 6C), suggesting the changes of ROS level or redox state in the reaction mixture by ROS scavengers.

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Discussion

In our studies, we showed that inhibition of LPS-stimulated ROS production in BMDM also selectively inhibited the production of PGD_2, but not its isomer PGE_2. LPS-induced PGD_2 production in BMDM was mediated via H-PGDS isomerase, but not L-PGDS. LPS-induced H-PGDS-mediated PGD_2 production was sensitive to and dependent on the NOX-generated ROS in BMDM. In contrast, the LPS-induced PGE_2 production in BMDM was ROS-independent.

To our knowledge, this is the first report of the role of ROS in differential regulation of LPS-induced PGD_2 and PGE_2 production. The novel finding of our study is that the modulation of intracellular ROS levels in macrophages could selectively regulate LPS-induced production of PGD_2, but not PGE_2. Therefore, it is impossible that the ROS or any NOX/ROS inhibitors exert their selective effects on PGD_2 production via the modification of COX-2 enzyme in BMDM. Because if there is any modifications of the COX-2 protein expression or its enzyme activity by the above inhibitors or H_2O_2, the production of both PGE_2 and PGD_2 would accordingly change uniformly in the same direction as shown in Fig. 2F with the COX-2 selective inhibitor NS-398, but not unilaterally with only one product PGD_2, as COX-2 is the common upstream rate-limiting PGs synthase for both PGE_2 and PGD_2. Thus this ROS effect must occur further downstream from the COX-2 enzyme and is only specific for the PGD_2 signaling pathway. Although previous reports have not shown a consensus on whether ROS could regulate COX-2 expression in different cell types (Feng et al., 1995), in our hands, LPS-induced ROS production clearly had no inhibitory effect on either COX-2 protein expression or its enzyme activity in BMDM as shown by the unaffected downstream product PGE_2 generation.

Previous reports indicated that ROS generated from NOX played important roles in regulating the expression of several pro-inflammatory genes in macrophages (Hsu and Wen,

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2002; Sanlioglu et al., 2001). In our studies, both NOX inhibitors DPI and PAO significantly attenuated the LPS-induced production of PGD_2, but not PGE_2, suggesting the role of NOX as the ROS generator in response to LPS stimulation in WT BMDM. Although both DPI and PAO are known to have other NOX-unrelated potential side effects in cells (i.e., inhibition of flavin proteins by DPI or proteases by PAO), the inhibitory effect of DPI on ROS and PGD_2 productions in BMDM was not likely achieved via non-specific inhibition of other flavin-containing ROS generators as the METC inhibitors rotenone, antimycin A, and NOS inhibitor L-NAME showed absolutely no effect on LPS-induced PGs production, thus eliminating the possibility of mitochondria or NOS as potential ROS generators in this signaling pathway. Furthermore, the LPS-induced production of ROS and PGD_2, but not PGE_2, in $p47^{phox}$-deficient BMDM was also significantly attenuated compared to that in WT BMDM, confirming the role of NOX/$p47^{phox}$ in LPS-induced production of ROS and PGD_2. Taken together, our data from DPI, PAO, $p47^{phox}$-deficient BMDM, and the negative results from METC and NOS inhibitors, collectively confirmed the critical role of NOX as the main ROS source in BMDM in response to LPS stimulation.

Interestingly, although the LPS-induced production of PGD_2 and ROS was significantly attenuated in $p47^{phox}$-deficient BMDM, the production of neither PGD_2 nor ROS was completely prevented, and was further attenuated by MnTMPyP pretreatment. It was reported that the lack of $p47^{phox}$ subunit is sufficient to inactivate the $p47^{phox}$-dependent NOX2 isoform enzyme activity in neutrophils. However, we found that there was still a low level of LPS-induced ROS and PGD_2 production in $p47^{phox}$-deficient BMDM, suggesting that unlike neutrophils, other non-$p47^{phox}$-dependent and low-level expression NOX isoforms (i.e., NOX1 or NOX4, Xiao's lab unpublished data) or other potential ROS generating systems in macrophages might compensate

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for the loss of NOX2/$p47^{phox}$ system and contribute to the residual ROS production in $p47^{phox}$-deficient BMDM.

In order to understand the ROS-dependent signaling mechanism of PGD_2 generation, we first identified the PGDS isoform(s) that mediated LPS-induced PGD_2 production. Between the two cloned PGDS isoforms, L-PGDS was not detected in mouse BMDM with or without LPS stimulation, and inhibition of L-PGDS by either AT-56 or its siRNA failed to prevent LPS-induced PGD_2 production in BMDM. Therefore, the potential role of L-PGDS in LPS-induced PGD_2 production in BMDM is eliminated. In contrast, we found strong H-PGDS expression in BMDM, and H-PGDS-selective inhibitor HQL-79 or its siRNA significantly attenuated LPS-induced PGD_2 production. In addition, co-pretreatment of BMDM with HQL-79 and either DPI or PAO showed additive inhibitory effects on LPS-induced PGD_2 production, suggesting that NOX-regulated LPS-induced PGD_2 production via H-PGDS isoform. Taken together, these data confirmed that H-PGDS mediated LPS-induced PGD_2 production in BMDM.

In order to determine the mechanism of ROS regulation on H-PGDS, an *in vitro* cell-free enzyme assay was conducted. Scavenging ROS in the H-PGDS reaction system by either SOD/catalase mimetics MnTMPyP and EUK-134, or catalase concentration-dependently attenuated H-PGDS-mediated PGD_2 production, which was significantly enhanced by the addition of H_2O_2. In contrast, these reagents have not inhibitory effect on PGE_2 production mediated by mPGES-1. These results indicated that the ROS levels could directly affect the *in vitro* enzyme activity of H-PGDS, but not mPGES-1, and a certain amount of ROS is required to maintain H-PGDS enzyme activity. We showed that MnTMPyP or EUK-134 could further decrease the ROS level in the enzyme reaction mixture, confirming the presence of a basal level of oxidants (i.e. superoxide) in this enzyme reaction solution. These enzyme assay data strongly

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supported our findings that intracellular ROS regulate LPS-induced PGD_2 production of via H-PGDS, and a basal level of ROS is required to maintain H-PGDS enzyme activity.

Although our enzyme assay results confirmed that ROS could directly modify H-PGDS enzyme activity *in vitro*, the precise and complete molecular mechanism of ROS regulation on H-PGDS-mediated PGD_2 production in macrophages is still not entirely defined. Our data didn't completely exclude the possibility that ROS may also work through other intracellular signaling intermediates (e.g., kinases) to modify H-PGDS enzyme activity in macrophages in addition to the above confirmed mechanism of direct ROS interaction with H-PGDS. The potential mechanisms of ROS regulate H-PGDS-, but not L-PGDS-, mediated PGD_2 production in BMDM may result from the differences between H-PGDS and L-PGDS as follows: 1) The enzyme activity of H-PGDS is glutathione (GSH)-dependent, whereas L-PGDS enzyme activity doesn't require GSH (Urade and Eguchi, 2002). GSH is a well-known antioxidant protein with its thiol groups acting as reducing agents, preventing damage to important cellular enzymes or components caused by ROS. GSH reduces disulfide bonds formed within cytoplasmic proteins to cysteines, and is thus converted to its oxidized form glutathione disulfide (GSSG), which can be reduced back by glutathione reductase, using NADPH as an electron donor. It is possible that the NOX inhibitors or ROS scavengers changed the cytosolic ratio of GSH/GSSG, and thus affected the enzyme activity of H-PGDS. 2) H-PGDS and L-PGDS may have different sensitivities to redox environment. Since the L-PGDS is not detectable in our system, thus the different sensitivities of the two isomerases to redox environment are not likely to be the main mechanism of ROS regulated PGD_2 production in BMDM. 3) ROS may facilitate the formation of a hydrogen bond that is a required for H-PGDS activation (Uchida et al., 2010). Our *in vitro* enzyme assay results strongly suggested the critical role of ROS-(H-PGDS) interaction in

modulation of H-PGDS activity, suggesting ROS may serve as an indispensable co-factor for maintaining the normal enzyme configuration and activity of H-PGDS; whereas depletion of ROS would decrease or abolish its enzyme activity.

In our studies, MnTMPyP (50 μM) inhibited PGD_2 production more efficiently in BMDM (~85% inhibition) compared to that from the cell-free enzyme assay (~60% inhibition). This could be explained by the non-enzymatic conversion of PGH_2 in aqueous solution. We recently reported the spontaneous conversion of PGH_2 to PGE_2 (42.7%) and PGD_2 (24.2%) without the presence of PGES or PGDS enzymes in the cell-free enzyme assay (Yu et al., 2011). These findings suggested that unlike in macrophages, a large portion of PGD_2 was spontaneously converted from PGH_2 independent of H-PGDS enzyme activity in this cell-free enzyme assay, and thus would not be affected by the changes of its surrounding ROS levels (i.e., MnTMPyP concentrations). Currently, it is still unclear if the spontaneous conversion of PGH_2 to PGD_2 *in vitro* also occurs in live cells (i.e., BMDM). However, this observation could explain why MnTMPyP reduced the PGD_2 production by 60% in the cell-free enzyme assay, but more efficiently blocked PGD_2 production in BMDM.

In conclusion, our results, for the first time, showed that LPS-induced PGD_2 production in BMDM was mediated by H-PGDS that required NOX-derived ROS to maintain its proper enzymatic function. ROS could directly modulate the H-PGDS enzyme activity of PGD_2 production *in vitro*, but not the mPGES-1 enzyme activity of PGE_2 production. Our findings not only illustrate the critical role of intracellular ROS in differential regulation of PGs production in macrophages, but also implicate a potential new therapeutic strategy in selectively regulating PGD_2 production in the treatment of PGs-involved diseases by regulating the intracellular ROS levels.

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Authorship Contributions

Participated in research design: Zhao G, Yu, Zhao Q, and Xiao.

Conducted experiments: Zhao G, Yu, Deng, Li, and Xiao.

Contributed new reagents or analytic tools: Christman, Zhao Q, Joo, van Breemen, and Xiao.

Performed data analysis: Zhao G, Yu, and Xiao.

Wrote or contributed to the writing of the manuscript: Yu, Zhao G, van Breemen, Zhao Q, Christman, and Xiao.

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Footnotes

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Figure Legends

Figure 1. LPS concentration-dependently stimulated production of both PGs and ROS in BMDM. *A*. Primary cultured WT BMDM were treated with LPS for 16 h, and the concentrations of PGD_2 and PGE_2 in culture medium were determined using LC-MS-MS. LPS concentration-dependently (0.01, 0.1, 1, and 5 μg/ml) stimulated both PGD_2 and PGE_2 production in BMDM (n=3). $p < 0.05$ represents significant differences in productions of both PGE_2 and PGD_2 vs. their individual counterparts between the compared LPS concentrations. *B*. The primed WT BMDM was treated with LPS for ROS measurement using isoluminol-enhanced chemiluminescence assay. LPS concentration-dependently (0.01, 0.1, and 1 μg/ml, n=3) stimulated ROS generation in BMDM as measured by increased chemiluminescent signals. *C*. Both LPS- and the phagocyte ROS stimulus zymosan-induced ROS generation in BMDM was completely prevented by pretreatment of BMDM with the ROS scavenger MnTMPyP (50 μM, 0.5 h prior to stimulation, n=6).

Figure 2. ROS selectively regulated LPS-induced production of PGD_2, but not PGE_2, in BMDM. Primary cultured WT BMDM were stimulated with 1 μg/ml LPS for detection the production of PGs (16 h) and ROS using LC-MS-MS or chemiluminescence assay, respectively. The pretreatment of BMDM with the ROS scavenger MnTMPyP not only concentration-dependently (1, 5, and 50 μM, 0.5 h) prevented the LPS-induced ROS generation (n=8) *(A)*, but also the LPS-induced (n=9) production of PGD_2, but not PGE_2 (*B).* Similarly, another structurally-unrelated ROS scavenger EUK-134 also concentration-dependently (0.5, 2, and 5 μM, 0.5 h, n=3) prevented LPS-induced ROS generation *(C)* and the production of PGD_2, but not PGE_2, in

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BMDM (*D).* Conversely, co-incubation of BMDM with (0.3 mM, n=4) enhanced LPS-induced production of PGD_2, but not PGE_2, in BMDM; whereas H_2O_2 treatment alone (16 h) doesn't increase PGs production in BMDM *(E).* In contrast, COX-2-selective inhibitor NS-398 pretreatment (0.5 h) concentration-dependently (0.001 to 0.1 μM), but non-selectively inhibited both LPS-induced PGE_2 and PGD_2 production in BMDM to the same extent at each tested concentration (*F*).

Figure 3. NOX-generated ROS selectively regulated LPS-induced production of PGD_2, but not PGE_2, in BMDM. Primary cultured BMDM from either WT or $p47^{phox}$-deficient mice were treated with 1 μg/ml LPS for 16 h, and the concentrations of PGD_2 and PGE_2 in culture medium were determined using LC-MS-MS. *A-C.* LPS-induced production of PGD_2, but not PGE_2, in WT BMDM was concentration-dependently inhibited by NOX inhibitors DPI (0.5, 1.5, and 5 μM, n=10, *A*) or PAO (0.1, 0.3, and 1 μM, n=12, *B*), but not affected by METC inhibitors rotenone (1 μM, n=3, *C*) and antimycin A (1 μM, n=3, *C*), or NOS inhibitor L-NAME (1 mM, n=3, *C*); *D.* LPS-induced production of PGD_2, but not PGE_2, in $p47^{phox}$-deficient BMDM was significantly decreased compared to that in WT BMDM (n=4). The LPS-induced production of PGD_2, but not PGE_2, was selectively further inhibited by MnTMPyP (50 μM, n=5) in $p47^{phox}$-deficient BMDM; whereas DPI (0.5 μM) or PAO (0.1 μM) didn't affect the production of either PGD_2 or PGE_2 in $p47^{phox}$-deficient BMDM. *E.* Inhibition of NOX by pretreatment of WT BMDM with DPI (0.5 μM, 0.5 h, n=6), PAO (0.1 μM, 0.5 h, n=6), or using $p47^{phox}$-deficient BMDM (n=5) attenuated LPS-induced ROS generation as measured by chemiluminescence assay. LPS-induced low-level ROS generation in $p47^{phox}$-deficient BMDM was further prevented by MnTMPyP pretreatment (50 μM, 0.5 h, n=5).

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Figure 4. H-PGDS, but not L-PGDS, mediated LPS-induced PGD_2 production in BMDM. *A-B*. Primary cultured WT BMDM were treated with LPS (1 μg/ml) with or without ROS/NOX inhibitors pretreatment (0.5 h) including 50 μM MnTMPyP (*A*), 1.5 μM DPI (*B*), and 1 μM PAO (*B*). The protein expressions of COX-2 and H-PGDS in BMDM were determined by Western blot using actin as protein loading control. LPS treatment did not change the protein expression of H-PGDS, but significantly induced COX-2 expression (n=3). LPS-induced expressions of COX-2 and H-PGDS were not affected by any of the above inhibitors. *C*. Mouse brain tissue lysate was used as a positive control for L-PGDS protein expression that was detected using two different antibodies (Cayman & Santa Cruz) specific for mouse L-PGDS. In contrast, the expression of L-PGDS protein was not detectable in mouse BMDM. *D-E*. Primary cultured WT BMDM were transfected with either control siRNA, or siRNA for H- or L-PGDS for 48 h prior to LPS treatment. The concentrations of PGD_2 and PGE_2 were determined using LC-MS-MS. The protein expression of H-PGDS by Western blot (*D*) and LPS-induced PGD_2 production (*E*) were selectively attenuated by H-PGDS siRNA (n=3), but not by L-PGDS siRNA (n=2); whereas siRNA for either H- or L-PGDS had no effect on LPS-induced PGE_2 production. Densitometric quantification of the relative protein expression of COX-2, H-PGDS, or L-PGDS (all normalized to its actin expression) determined by Western blots were also shown in *A-D*.

Figure 5. The H-PGDS-selective inhibitor HQL-79, but not the L-PGDS-selective inhibitor AT-56, attenuated LPS-induced PGD_2 production in BMDM. Primary cultured WT BMDM were treated with 1 μg/ml LPS for 16 h in the presence of various concentrations of HQL-79 (*A*) or AT-56 (*B*). The concentrations of PGD_2 and PGE_2 were determined using LC-MS-MS. *A*. HQL-

79 concentration-dependently (5, 20, and 100 μM) inhibited LPS-induced production of PGD_2, but not PGE_2, in BMDM (n=6); *B.* AT-56 did not affect LPS-induced either PGD_2 or PGE_2 production in BMDM (n=3); *C.* Co-pretreatment (0.5 h) of WT BMDM with HQL-79 (20 μM) and either DPI (0.5 μM) or PAO (0.1 μM) showed additive and selective inhibitory effects on LPS-induced production of PGD_2, but not PGE_2, in BMDM (n=3).

Figure 6. ROS directly regulated H-PGDS-mediated PGD_2 production in cell-free enzymatic assay *in vitro*. Recombinant enzymes H-PGDS (0.1 unit) or mPGES-1 (3 units) was pre-incubated with MnTMPyP, EUK-134, catalase, H_2O_2, or equal volume of vehicle for 10 min at 37°C in test tubes *in vitro* prior to the addition of the enzyme substrate PGH_2 (2 μM). The concentrations of PGD_2 and PGE_2 in each sample buffer were determined after the reaction using LC-MS-MS. Co-incubation of H-PGDS with either MnTMPyP (n=8, *A*), EUK-134 (n=3, *B*), or catalase (n=7, *D*) attenuated H-PGDS-mediated PGD_2 production in a concentration-dependent manner; whereas co-incubation with H_2O_2 (n=4, *E*) concentration-dependently enhanced H-PGDS-mediated PGD_2 production. In contrast, mPGES-1-mediated PGE_2 production was not affected by co-incubation with MnTMPyP (50 μM), EUK-134 (5 μM), catalase (100 units), or H_2O_2 (10 μM) (n=3, *F*). The basal oxidant level in the reaction mixture was also determined using isoluminol-enhanced chemiluminescence assay. Either MnTMPyP (50 μM) or EUK-134 (5 μM) significantly decreased the basal oxidant levels in the reaction mixture (n=3, *C*).

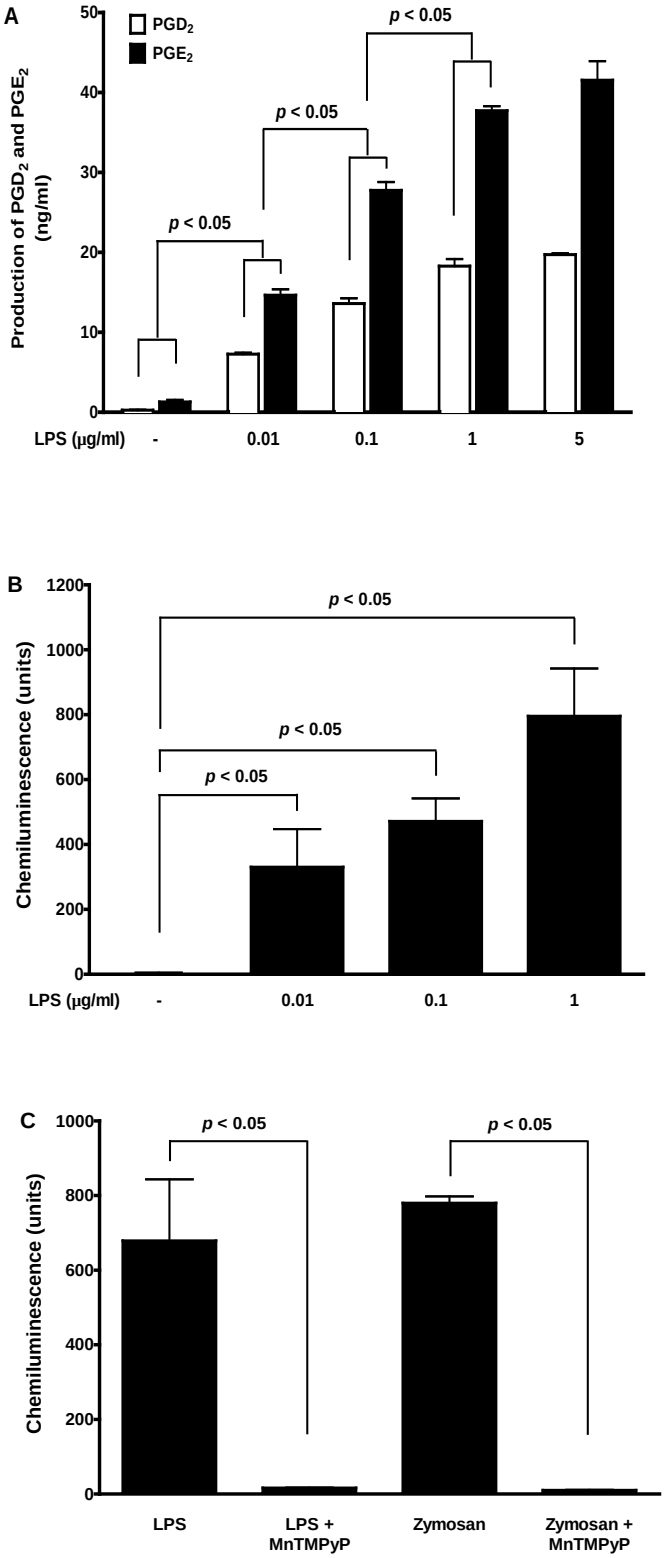
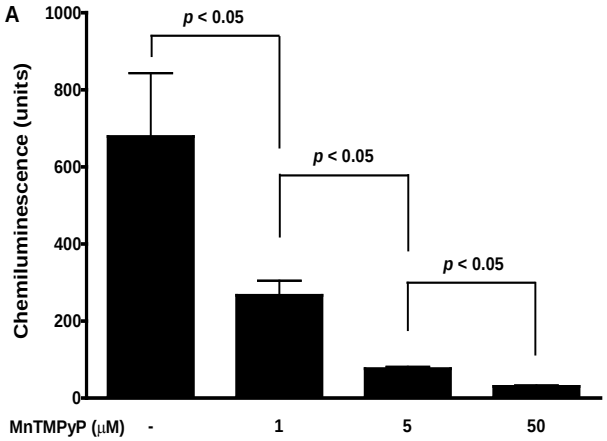
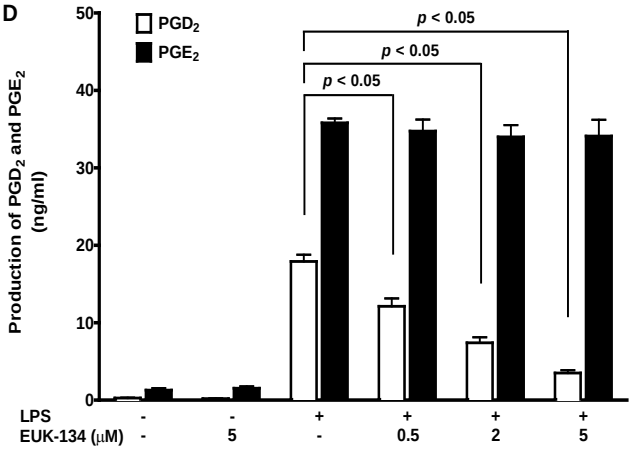
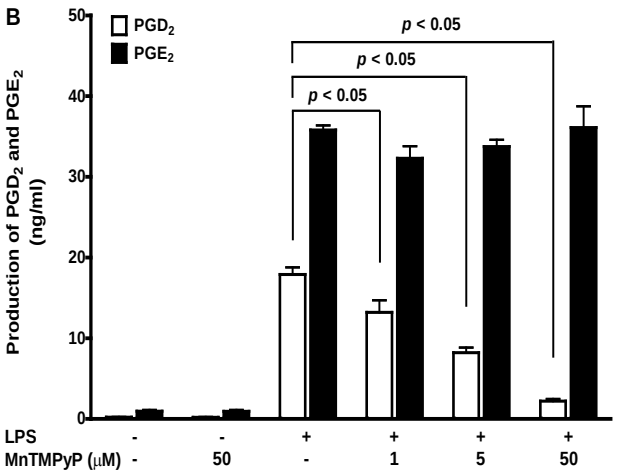
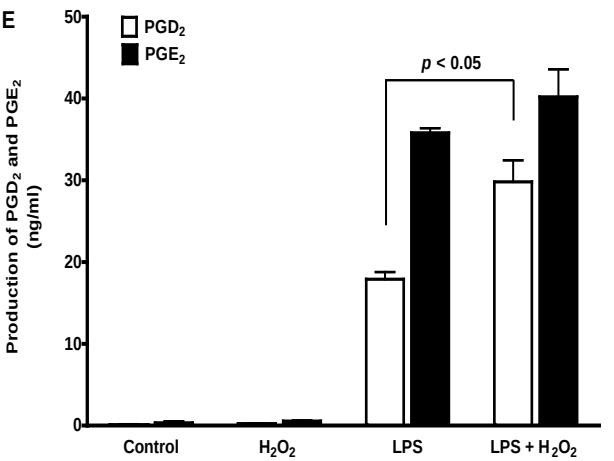

A
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
p < 0.05
p < 0.05
p < 0.05
LPS (µg/ml)
-
0.01
0.1
1
5
B
Chemiluminescence (units)
p < 0.05
p < 0.05
p < 0.05
LPS (µg/ml)
-
0.01
0.1
1
C
Chemiluminescence (units)
p < 0.05
p < 0.05
LPS
LPS + MnTMPyP
Zymosan
Zymosan + MnTMPyP

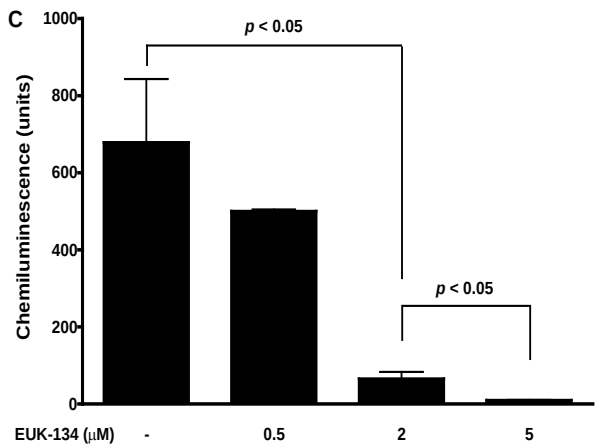
Figure 1


A
Chemiluminescence (units)
1000
800
600
400
200
0
p < 0.05
p < 0.05
p < 0.05
MnTMPyP (μM) - 1 5 50


D
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
50
40
30
20
10
0
p < 0.05
p < 0.05
p < 0.05
LPS - - + + + +
EUK-134 (μM) - 5 - 0.5 2 5


B
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
50
40
30
20
10
0
p < 0.05
p < 0.05
p < 0.05
LPS - - + + + +
MnTMPyP (μM) - 50 - 1 5 50


E
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
50
40
30
20
10
0
p < 0.05
Control
H2O2
LPS
LPS + H2O2


C
Chemiluminescence (units)
1000
800
600
400
200
0
p < 0.05
p < 0.05
EUK-134 (μM) - 0.5 2 5

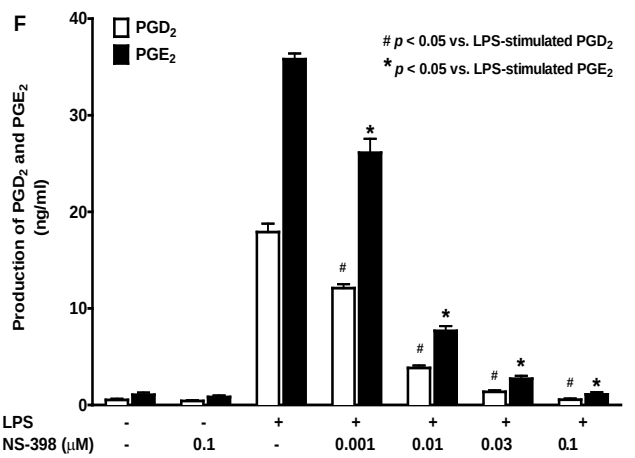
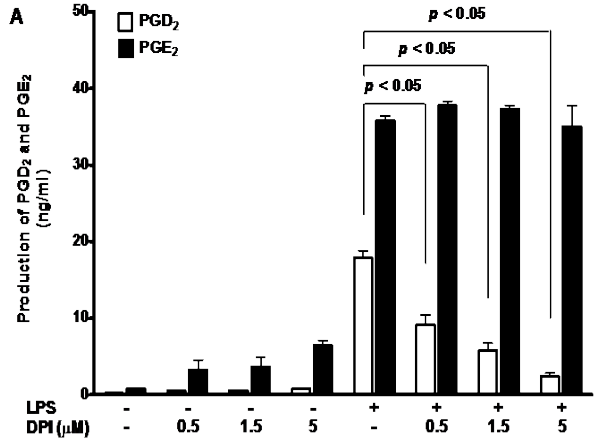
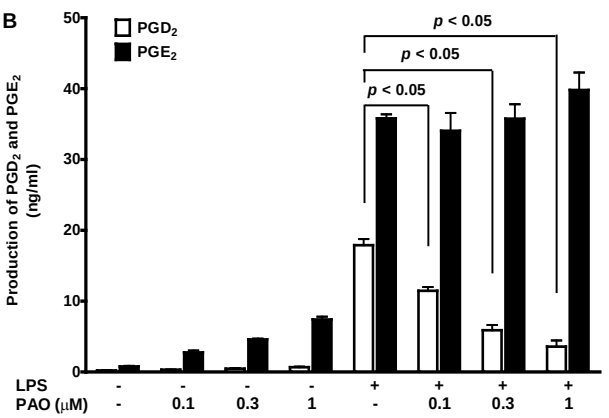
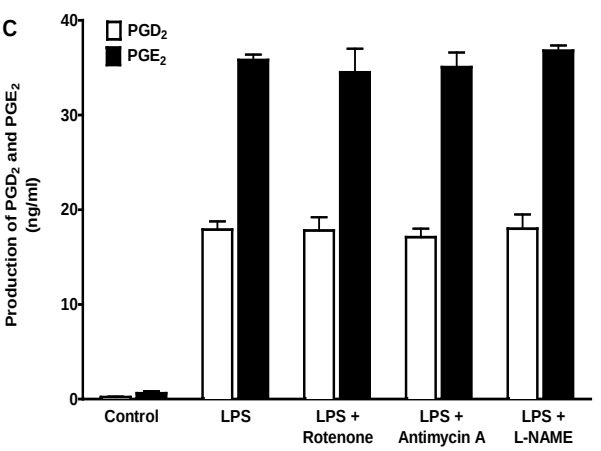
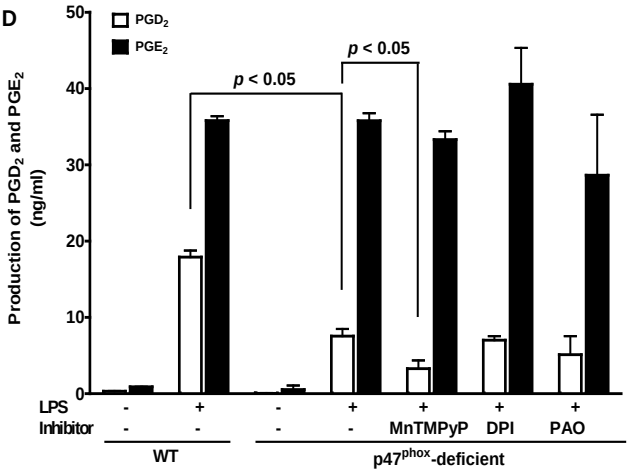

F
PGD2
PGE2
# p < 0.05 vs. LPS-stimulated PGD2
* p < 0.05 vs. LPS-stimulated PGE2
Production of PGD2 and PGE2 (ng/ml)
40
30
20
10
0
LPS - - + + + + +
NS-398 (μM) - 0.1 - 0.001 0.01 0.03 0.1

Figure 2


A
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
p < 0.05
p < 0.05
p < 0.05
LPS
DPI (μM)
- - - - + + + +
- 0.5 1.5 5 - 0.5 1.5 5


B
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
p < 0.05
p < 0.05
p < 0.05
LPS
PAO (μM)
- - - - + + + +
- 0.1 0.3 1 - 0.1 0.3 1


C
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
Control
LPS
LPS + Rotenone
LPS + Antimycin A
LPS + L-NAME


D
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
p < 0.05
p < 0.05
LPS
Inhibitor
- + - + + + +
- - - - MnTMPyP DPI PAO
WT
p47phox-deficient

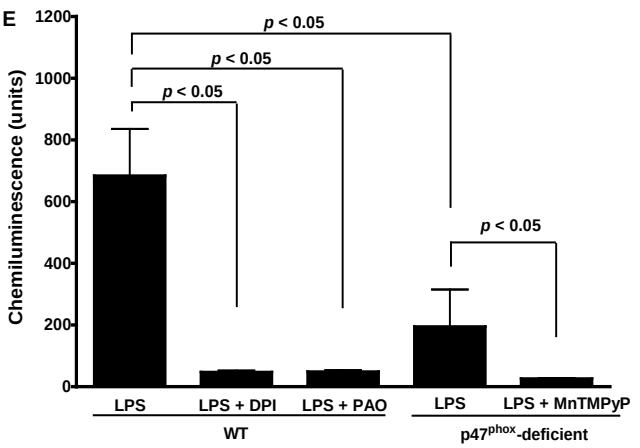

E
Chemiluminescence (units)
p < 0.05
p < 0.05
p < 0.05
p < 0.05
LPS
LPS + DPI
LPS + PAO
LPS
LPS + MnTMPyP
WT
p47phox-deficient

Figure 3

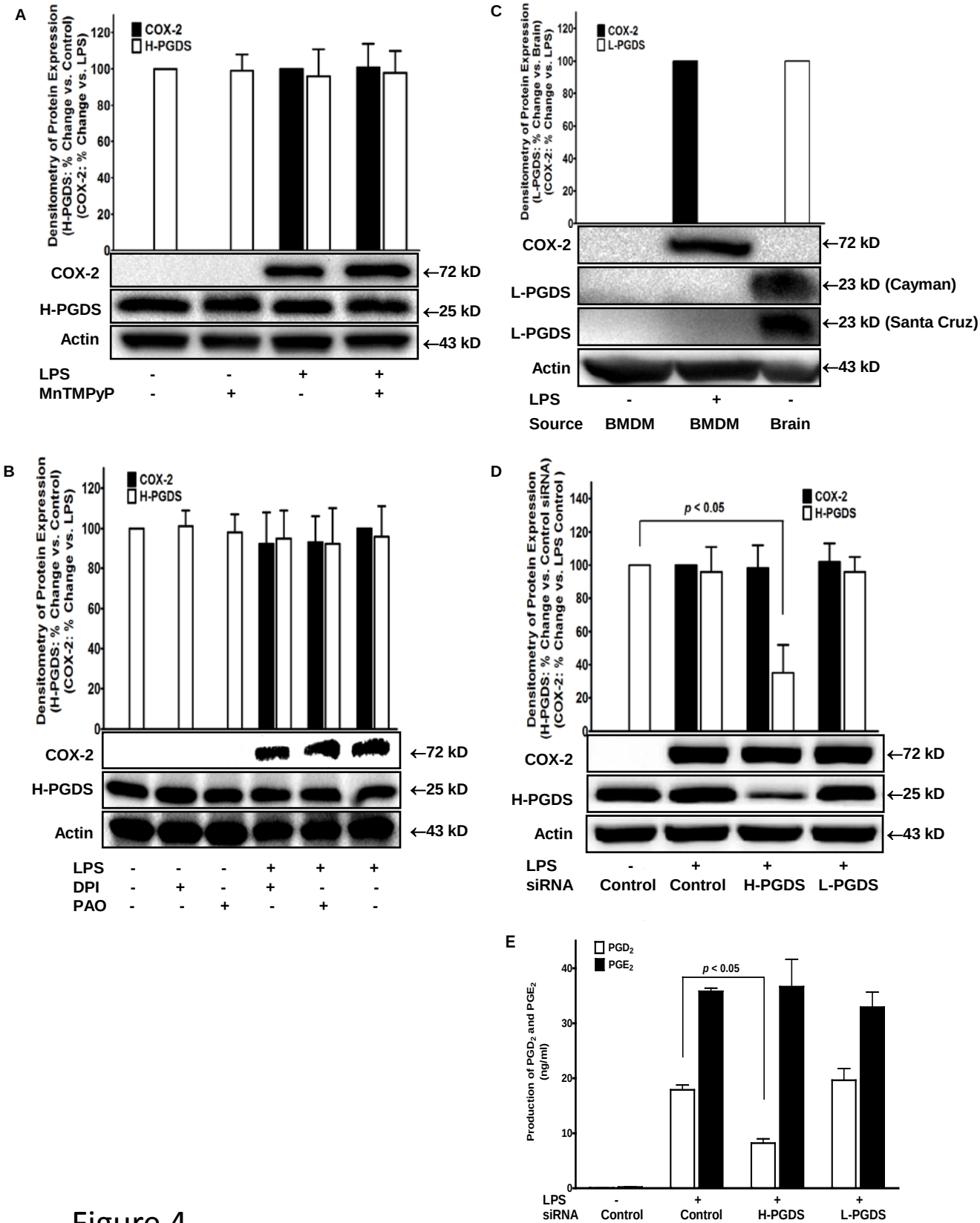
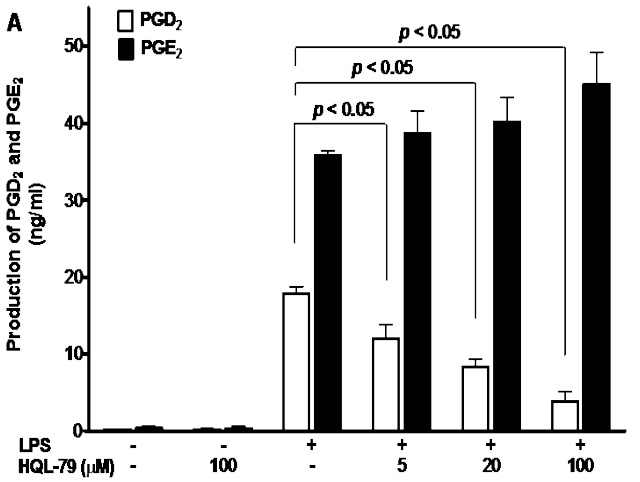
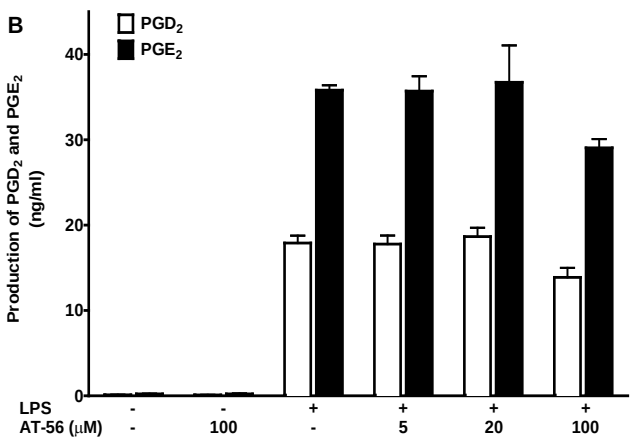

A
Densitometry of Protein Expression
(H-PGDS: % Change vs. Control)
(COX-2: % Change vs. LPS)
COX-2
H-PGDS
COX-2
H-PGDS
Actin
←72 kD
←25 kD
←43 kD
LPS
MnTMPyP
B
Densitometry of Protein Expression
(H-PGDS: % Change vs. Control)
(COX-2: % Change vs. LPS)
COX-2
H-PGDS
COX-2
H-PGDS
Actin
←72 kD
←25 kD
←43 kD
LPS
DPI
PAO
C
Densitometry of Protein Expression
(L-PGDS: % Change vs. Brain)
(COX-2: % Change vs. LPS)
COX-2
L-PGDS
COX-2
L-PGDS
L-PGDS
Actin
←72 kD
←23 kD (Cayman)
←23 kD (Santa Cruz)
←43 kD
LPS
Source
BMDM
BMDM
Brain
D
Densitometry of Protein Expression
(H-PGDS: % Change vs. Control siRNA)
(COX-2: % Change vs. LPS Control)
p < 0.05
COX-2
H-PGDS
COX-2
H-PGDS
Actin
←72 kD
←25 kD
←43 kD
LPS
siRNA
Control
Control
H-PGDS
L-PGDS
E
PGD2
PGE2
p < 0.05
Production of PGD2 and PGE2
(ng/ml)
LPS
siRNA
Control
Control
H-PGDS
L-PGDS

Figure 4


A
PGD2
PGE2
p < 0.05
p < 0.05
p < 0.05
Production of PGD2 and PGE2 (ng/ml)
0
10
20
30
40
50
LPS - - + + + +
HQL-79 (μM) - 100 - 5 20 100


B
PGD2
PGE2
Production of PGD2 and PGE2 (ng/ml)
0
10
20
30
40
LPS - - + + + +
AT-56 (μM) - 100 - 5 20 100

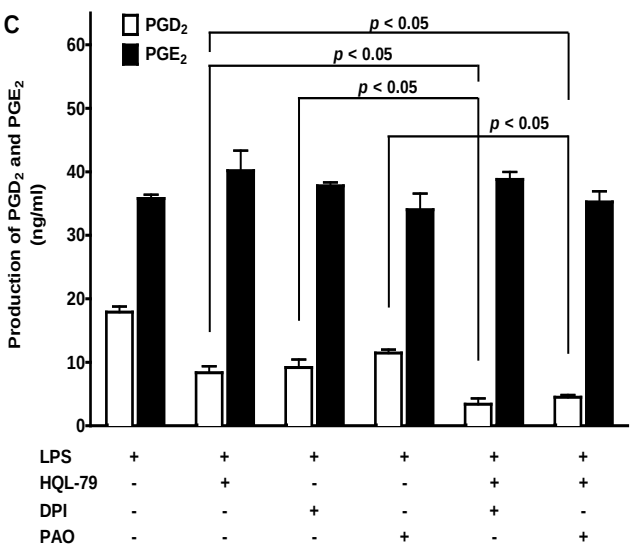

C
PGD2
PGE2
p < 0.05
p < 0.05
p < 0.05
p < 0.05
Production of PGD2 and PGE2 (ng/ml)
0
10
20
30
40
50
60
LPS + + + + + +
HQL-79 - + - - + +
DPI - - + - + -
PAO - - - + - +

Figure 5

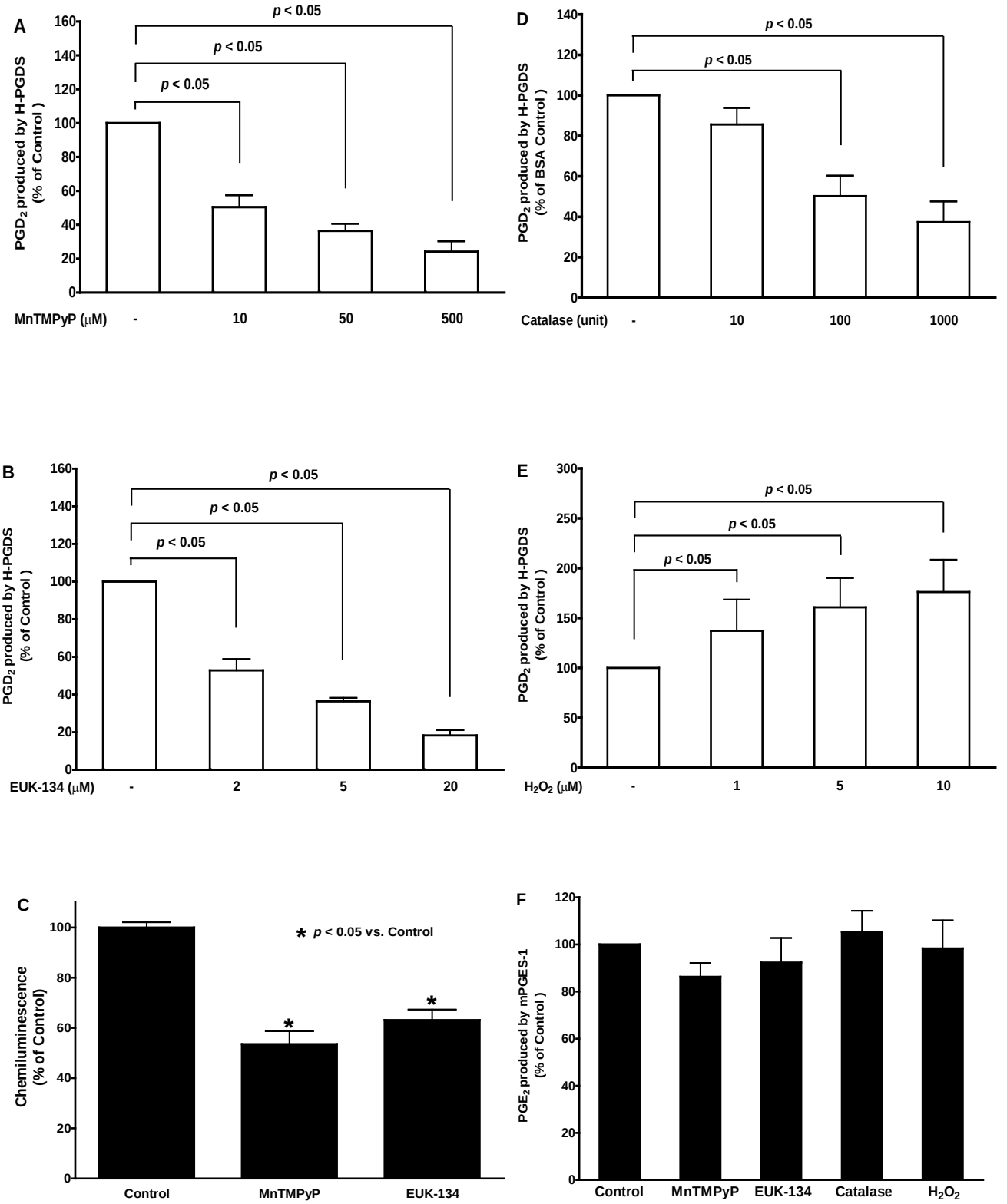

A
PGD2 produced by H-PGDS (% of Control)
p < 0.05
p < 0.05
p < 0.05
MnTMPyP (μM) - 10 50 500
B
PGD2 produced by H-PGDS (% of Control)
p < 0.05
p < 0.05
p < 0.05
EUK-134 (μM) - 2 5 20
C
Chemiluminescence (% of Control)
* p < 0.05 vs. Control
Control MnTMPyP EUK-134
D
PGD2 produced by H-PGDS (% of BSA Control)
p < 0.05
p < 0.05
Catalase (unit) - 10 100 1000
E
PGD2 produced by H-PGDS (% of Control)
p < 0.05
p < 0.05
p < 0.05
H2O2 (μM) - 1 5 10
F
PGE2 produced by mPGES-1 (% of Control)
Control MnTMPyP EUK-134 Catalase H2O2

Figure 6