Lipopolysaccharide-induced murine embryonic resorption involves nitric oxide-mediated inhibition of the NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase

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Abstract

The initial inactivation of prostaglandins (PGs) is mediated by 15-hydroxyprostaglandin dehydrogenase (15-PGDH). PGs are potent mediators of several biological processes, including inflammation and reproduction. In uterus, PGs play a key role in infection-induced pregnancy loss, in which concentration of this mediator increased. This process is accompanied with the induction of nitric oxide synthase expression and a marked increase in uterine levels of nitric oxide. There is no information concerning nitric oxide contribution to potential changes in PG catabolism, but experimental evidence suggests that nitric oxide modulates PG pathways. The specific objectives of the study were to evaluate the protein expression of HPGD (15-PGDH) and to characterize the nitric oxide-dependent regulation of this enzyme in a model of lipopolysaccharide (LPS)-induced embryonic resorption. Results show that LPS decreased HPGD protein expression and augmented PGE synthase activity; therefore, PGE₂ levels increased in uterus in this inflammatory condition. Just as LPS, the treatment with a nitric oxide donor diminished HPGD protein expression in uterine tissue. In contrast, the inhibition of nitric oxide synthesis both in control and in LPS-treated mice increased 15-PGDH levels. Also, we have found that this enzyme and PGE₂ levels are not modulated by peroxynitrite, an oxidant agent derived from nitric oxide. This study suggests that LPS and nitric oxide promote a decrease in the ability of the uterus for PG catabolism during bacterially triggered pregnancy loss in mice.

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Introduction

Microbial infections are known to cause miscarriage and preterm labor (Romero *et al.* 1988, Lamont & Sawant 2005) but their mechanisms are still not fully elucidated. Lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria, induces activation of immune cells. Activated macrophages produce several inflammatory cytokines including tumor necrosis factor α , interleukin 6 and 12, which, when in excess, leads to the onset of reproductive disorders or diseases (Penta *et al.* 2003). We have previously developed a mouse model to study the mechanisms of LPS-induced pregnancy loss. The administration of 1 μ g/g of body weight (i.p.) of LPS in mice on day 7 of pregnancy produced complete embryonic resorption at 24 h. We have observed that the decidua, which envelops the embryo, was necrotized 24 h after LPS injection and this resorbed tissue was expelled the following day. In this model, nitric oxide and prostaglandins (PGs) are highly produced in implantation sites of mouse uterus during the infection/

inflammation-induced embryonic resorption process (Ogando *et al.* 2003, Aisemberg *et al.* 2007).

PG biosynthesis is catalyzed by cyclooxygenase 1 and 2 enzymes (Rouzer & Marnett 2009) and the first step of its inactivation is due to the NAD⁺-dependent 15-hydroxyprostaglandin dehydrogenase that catalyzes the conversion of primary PGs to their biologically inactive 15-keto derivatives (Tai *et al.* 2006). The products, 15-keto metabolites, exhibit greatly reduced biological activities rendering this enzyme being a key enzyme responsible for the biological inactivation of these eicosanoids. A tight control over PG metabolism is critical for the maintenance of pregnancy, in part because they act as potent uterotonins affecting myometrial contractility. During early gestation, HPGD (15-PGDH) activity has been shown to increase (Keirse *et al.* 1985), and for most of the duration of pregnancy, 15-PGDH minimizes myometrial contractility, keeping PGs synthesized by amnion or chorion (Sangha *et al.* 1994). Therefore, changes in HPGD protein levels or

Figure 1 Effect of LPS treatment on prostaglandin E synthase activity. BALB/c mice were injected on day 7 of gestation and they were killed after 6 h of LPS (1 µg/g) or vehicle (PBS) treatment. In uterus, enzyme activity was assessed by conversion of PGH₂ to PGE₂. PGE₂ levels were determined by RIA. Results were expressed as µg PGE₂/mg protein per h. Values are mean $\pm$ S.E.M., $n=5$ mice per group. Comparisons of means were made with Student's *t*-test. Dissimilar superscripts denote significantly different values ($P<0.05$).

activity play important roles in regulating the catabolism and availability of primary PGs that are implicated in physiological and pathological processes. Despite the physiological importance of 15-PGDH, little is understood about the mechanisms involved in regulating its expression. Expression and activity of 15-PGDH have been demonstrated in reproductive tissues of different species (Casey *et al.* 1980, Kankofer 1999, Gao *et al.* 2007). LPS treatment decreases activity of 15-PGDH in mouse and human fetal membranes at term (Brown *et al.* 1998, Wang & Hirsch 2003) and alters *Hpgd* mRNA and protein levels in mouse and rat lung during sepsis (Hahn *et al.* 1998, Ivanov & Romanovsky 2004).

As we mentioned earlier, in response to LPS treatment, implantation sites produce various bioactive molecules, including nitric oxide, PGs, reactive oxygen species, and pro-inflammatory cytokines. So the cellular toxic effects of inducible nitric oxide synthase derivative products contribute to the detrimental effects of the endotoxin on tissue functions. The peroxynitrite anion is a short-lived oxidant and nitrating molecule that is produced by the reaction of nitric oxide and superoxide radicals. Tyrosine nitration is becoming increasingly recognized as a functionally posttranslational modification that serves as an indicator of nitric oxide-mediated oxidative inflammatory reactions. We have previously found that after LPS administration, uterine cyclooxygenase enzyme was nitrated in tyrosine residues. Moreover, we found that cyclooxygenase-nitrated levels were higher than nonmodified cyclooxygenase expression (Aisemberg *et al.* 2007).

The increment in PG output induced by LPS could be caused by increased synthesis as well as by diminished PG degradation. The objectives of this work were to evaluate the participation of PG catabolism in modulating prostanoid levels in uterus as part of the mechanism by which LPS-induces embryonic resorption, and the possible regulation of 15-PGDH by nitric oxide and nitric oxide-derived reactive species.

Results

Effect of LPS on PGE synthase activity

We have previously found that injection of LPS causes total embryonic resorption after 24 h of treatment (Ogando *et al.* 2003). At the maternal–fetal interface, we have shown that there is an increase in PGE₂ levels due to regulation of cyclooxygenase 2, a key enzyme in eicosanoid biosynthesis (Aisemberg *et al.* 2007). Cyclooxygenase enzymes catalyze the conversion of arachidonic acid to PG endoperoxides (PGG₂ and subsequently to PGH₂), which acts as a substrate for multiple isomerases that are individually responsible for the generation of eicosanoid products, including PGE₂. PGE synthase isomerizes specifically to PGE₂. Our first objective was to evaluate whether treatment with LPS could alter PGE₂ production through the regulation of its specific synthase. The enzymatic activity through the conversion of PGH₂ to PGE₂ in uterine tissues was assessed. We observed that 6-h treatment with endotoxin significantly increases PGE synthase activity, consistent with high levels of PGE₂ observed (Fig. 1).

Effect of LPS on HPGD protein expression

There are several evidences about modulation of the PG pathways by LPS. Our main objective was to evaluate whether some type of regulation was possible in our model of early pregnancy loss, at level of PG catabolism. First, we evaluated whether treatment with endotoxin had an effect on HPGD protein expression of the uterus and decidua. Animals were injected with LPS (1 µg/g) on day 7 of gestation and were killed 6 h after treatment. Within the context of an infection, we identified the uterine expression of HPGD protein. On the contrary, this protein was barely detectable in decidua, both in control and in LPS-treated animals. As shown in Fig. 2, 15-PGDH expression is down-modulated in uterus under inflammatory conditions (at the time analyzed).

Figure 2 Effect of LPS treatment on HPGD protein expression. BALB/c mice were injected on day 7 of pregnancy with vehicle (PBS) or LPS (1 µg/g) and killed after 6 h of treatment. HPGD protein expression was determined in uterine tissue by western blot. Normalized data (OD 15-PGDH/OD actin) are expressed as mean $\pm$ S.E.M., $n=5$ mice per group. Comparisons of means were made with Student's *t*-test. Dissimilar superscripts denote significantly different values ($P<0.001$). OD, optical density.

Effect of LPS on PGE₂ catabolism

PG levels *in vivo* depend on the balance between the relative activities of synthesis and degradation, and metabolic enzymes play crucial roles in this process. In addition to modulation of protein levels of HPGD by LPS, we studied whether LPS was able to modify the production of inactive metabolites of PGE₂. Mice were treated for 6 h with LPS (1 µg/g) on day 7 of gestation and implantation sites (uterus and decidua) were obtained. Metabolite quantification was assessed by ELISA (as described in the Materials and Methods section) and PGE₂ levels were determined in the same sample by RIA. LPS administration to pregnant mice resulted in a significant increased PGE₂ levels both in uterus and in decidua (Fig. 3A and B respectively). A reduction of PGE metabolite levels was found only in uterine tissue after 6 h of treatment (Fig. 3A). These results suggest that under inflammatory conditions, LPS can regulate the levels of PGE₂ in uterus not only by increasing its synthesis but also by decreasing its catabolism.

Effect of nitric oxide and peroxynitrite on PGE₂ catabolism

It was reported that PGs may interact with nitric oxide biosynthetic pathway and *vice versa*. As mentioned repeatedly in this paper, there is an increase in the levels of PG and nitric oxide at implantation sites as a consequence of LPS injection. So there could be an interaction between these two pathways in our model of early pregnancy loss induced by infection.

We evaluated the effect of nitric oxide synthesis inhibition on HPGD protein expression. We treated mice with aminoguanidine, a selective inducible nitric oxide synthase inhibitor. As shown in Fig. 4A, this treatment increased 15-PGDH expression in control pregnant mice. In animals treated with LPS and aminoguanidine, we observed an increment on HPGD protein, which restored the levels of expression reduced by LPS injection (Fig. 4B). These data indicate that the expression of this catabolic enzyme is negatively modulated by endogenous nitric oxide levels both in physiological and inflammatory conditions.

The latter result suggests that nitric oxide is an LPS effector that contributes to the modulation of the expression of 15-PGDH. Given this, we administered a nitric oxide donor, *S*-nitroso-*N*-acetyl-penicillamine (SNAP), to evaluate the relevance of nitric oxide in modulating PG catabolism. We observed a significant reduction in HPGD protein levels after 6 h of SNAP treatment (Fig. 5).

Peroxyntirite, an oxidizing and nitrating agent, is produced by the reaction between nitric oxide and superoxide. It may react with other reactive species or free radicals or directly with proteins, lipids, and DNA causing oxidative stress (Szabó *et al.* 2007). Peroxyntirite

Figure 3 Effect of LPS treatment on PGE₂ and PGE metabolites levels. BALB/c mice were injected on day 7 of pregnancy with vehicle (PBS) or LPS (1 µg/g) and killed after 6 h of treatment. PGE₂ and PGE₂ metabolite (PGE₂-M) levels were determined in uterus (A) and decidua (B) by RIA and EIA respectively. Results were expressed as pg PGE₂ or PGE₂-M/mg tissue. Values are mean ± S.E.M., *n* = 4 per group. Comparisons of means were made with Student's *t*-test. Dissimilar superscripts denote significantly different values (*P* < 0.05).

is now being revealed as a key reactive species in pathological events associated with stimulation of tissue production of nitric oxide, as we previously observed in our model of pregnancy loss induced by LPS (Aisemberg *et al.* 2007). The formation of 3-nitrotyrosine in specific tyrosine residues can alter protein structure and function, implying that in certain pathological states, nitration is

Figure 4 Effect of a nitric oxide synthesis inhibitor (aminoguanidine) on HPGD protein expression. BALB/c mice were injected on days 6 and 7 of gestation (as indicated in Table 1) with vehicle (PBS) or aminoguanidine (6 mg/mouse) (A), LPS, and LPS + aminoguanidine (B) and killed after 6 h of LPS treatment. HPGD protein expression was determined in uterine tissue by western blot. Normalized data (OD 15-PGDH/OD actin) are expressed as mean $\pm$ S.E.M., $n=5$ mice per group. Comparisons of means for more than two groups were performed using ANOVA. Pairwise comparisons were made with Tukey's or Student's *t*-test. Dissimilar superscripts denote significantly different values ($P<0.01$). OD, optical density.

part of the disease process. To evaluate the possible regulatory role of endogenous peroxynitrite on PG catabolism, we investigated the effect of quercetin, a selective peroxynitrite scavenger on uterine PGE₂ levels, and HPGD protein expression. Mice were injected with LPS (1 μ g/g) and quercetin on day 7 of gestation and were killed 6 h after treatment. The coadministration did not prevent the increase in PGE₂ levels or decrease in HPGD protein expression induced by LPS in uterus (Fig. 6A and B respectively). These results indicate that alterations in the metabolism of uterine PGE₂ observed by LPS administration appear to be not associated with the production of this particularly detrimental molecule.

Discussion

In this study, the regulation of uterine PGE₂ catabolism under inflammatory conditions during early pregnancy was examined. As we mentioned earlier, we have previously developed a mouse model to study the

mechanisms of LPS-induced pregnancy loss. In BALB/c mice, the treatment with 1 μ g/g of body weight (i.p.) of LPS on day 7 of gestation produced complete embryonic resorption at 24 h. We have found that after LPS administration, HPGD protein is expressed predominantly in the uterus, while in the decidua, the expression is almost undetectable. This result agrees with Gao *et al.* (2007) who detected *Hpgd* mRNA and protein expression in the mouse uterus during embryo implantation. For this reason, we have focused on studying the modulation of 15-PGDH in the uterus.

We observed that administration of LPS decreased the HPGD protein expression after 6 h of treatment (Fig. 2). Our result is in agreement with Wang & Hirsch (2003) who have shown that after treatment with heat-killed *Escherichia coli*, the expression of *Hpgd* (mRNA) significantly decreased in the myometrium of *Tlr4* wild-type pregnant mice but not in *Tlr4*-mutant mice; toll-like receptor 4 (TLR4) is a critical component of the LPS signaling. Endotoxin administration also decreases the 15-PGDH expression in the lung of male *Bdf1* mice, showing the maximum reduction (mRNA and protein) after 6 h of treatment with LPS (Hahn *et al.* 1998). Moreover, Ivanov *et al.* (2003) showed a decrease in *Hpgd* expression (mRNA) of liver and lung in a model of systemic inflammation in rats (Ivanov & Romanovsky 2004). Contrary to what we have found, Menon *et al.* (2006) have documented an increment in *Hpgd* mRNA expression after LPS stimulation, but this study was performed with placental membranes in a tissue explant system.

Several evidences, including those provided by our laboratory, have shown that treatment with LPS increases PGE₂ levels in the uterus (Herath *et al.* 2006, Aisemberg *et al.* 2007). Figure 1 shows that this is due in part to increased uterine activity of the PGE synthase in our

Figure 5 Effect of a nitric oxide donor (SNAP) on HPGD protein expression. BALB/c mice were injected on day 7 of pregnancy with vehicle (PBS), LPS (1 μ g/g), or SNAP (3 mg/kg) and killed after 6 h of treatment. HPGD protein expression was determined in uterine tissue by western blot. Normalized data (OD 15-PGDH/OD actin) are expressed as mean $\pm$ S.E.M., $n=5$ mice per group. Comparisons of means were performed using ANOVA and Tukey's test. Dissimilar superscripts denote significantly different values ($P<0.001$). OD, optical density; SNAP, *S*-nitroso-*N*-acetyl-penicillamine.

Figure 6 Effect of a peroxynitrite scavenger (quercetin) on PGE₂ production and HPGD protein expression. BALB/c mice were injected on day 7 of pregnancy with vehicle, quercetin (10 mg/kg), LPS, and LPS+quercetin and killed after 6 h of LPS treatment. (A) PGE₂ levels ($n=5$ mice per group) and (B) HPGD protein expression ($n=7$ mice per group) were determined in uterus by RIA and western blot respectively. Mean $\pm$ S.E.M. was expressed as pg PGE₂/mg tissue (A) and the relative optical density of the 15-PGDH band to that of β -actin (B). Comparisons of means were performed using ANOVA and Tukey's test. Dissimilar superscripts denote significantly different values ($P<0.05$). OD, optical density.

model of pregnancy loss. In parallel was found that LPS treatment reduces levels of PGE metabolites (Fig. 3), which reinforces the reduced enzyme activity that we have previously observed. In accordance with this, Brown *et al.* (1998) were able to detect a significant decrease in the levels of PG metabolites following incubation with LPS in explants from human fetal membranes. Based on these results, we suggest that in our model, the mechanism by which LPS regulates the levels of PGE₂ is by increasing and decreasing PGE synthase activity and HPGD protein expression respectively.

The endometrium is a target tissue for the ovarian steroid hormones estradiol and progesterone. There is now compelling evidence that 15-PGDH (activity and expression) is induced by progesterone (Casey *et al.* 1980, Kelly *et al.* 1986, Greenland *et al.* 2000). In addition, administration of mifepristone, a progesterone receptor antagonist during the luteal phase, induces a decrease in HPGD protein levels in human and monkey

endometrium (Nayak *et al.* 1998, Hapangama *et al.* 2002). We found that LPS treatment (1 μ g/g, i.p., on day 7 of pregnancy) resulted in a significant decrease in serum progesterone levels after 6, 12, and 24 h of endotoxin administration (Aisemberg J, Vercelli CA, Bariani MV, Billi SC, Wolfson ML & Franchi AM 2012, submitted manuscript). This allows us to suggest that because LPS induces a decrease in progesterone levels, regulation of 15-PGDH may result from a direct effect of LPS or an indirect effect through progesterone or both.

To our knowledge, this is the first study reporting that 15-PGDH is modulated by nitric oxide in the uterus of pregnant mice. We have shown that nitric oxide contributes to down-modulation of HPGD protein levels under inflammatory conditions. First, we have found that treatment with aminoguanidine (the selective inducible nitric oxide synthase inhibitor) upregulates HPGD protein expression (Fig. 4A). In addition, the reduction in 15-PGDH expression due to LPS treatment leads to HPGD protein levels to control values by the coadministration of the inhibitor (Fig. 4B). These findings were reinforced by the data obtained from experiments in which the animals were injected with the nitric oxide donor, SNAP (Fig. 5).

During an inflammatory setting induced by endotoxins, PGs and nitric oxide are released simultaneously in large quantities (Ogando *et al.* 2003, Aisemberg *et al.* 2007). In our model of pregnancy loss, the administration of LPS increases PGs and nitric oxide production in the uterus. In this model, we have also observed that the administration of aminoguanidine, an inducible nitric oxide synthase inhibitor, is able to block the LPS-induced embryonic resorption. In this sense, inhibition of 15-PGDH expression by nitric oxide could partly explain the increase in PG levels. In addition, a model of preterm labor induced by infection was demonstrated that the co-administration of LPS and aminoguanidine prevents preterm birth and inhibits increased PGs and nitric oxide (Cella *et al.* 2010). Furthermore, no changes were found in the expression of 15-PGDH (Fig. 6B) and PGE₂ production (Fig. 6A) in the uterus from animals treated with LPS and quercetin, the scavenger of peroxynitrite. It is worth noting that previous work with this model showed that quercetin does not prevent embryonic resorption process (Aisemberg *et al.* 2007). These latter results indicate that peroxynitrite is not involved in the resorption process, at least in the modulation mechanism of PG catabolism, in this model of pregnancy loss induced by LPS.

In summary, this study demonstrated that LPS and nitric oxide modulate the expression of 15-PGDH enzyme and that the increase in uterine PGs levels induced by LPS treatment is also a product of their reduced catabolism. These results suggest that 15-PGDH may be a new target for regulating PGs levels in pathological situations of pregnancy such as abortion or premature delivery induced by endotoxins.

Materials and Methods

Reagents

(5, 6, 8, 11, 12, 14, 15-3H (N)) – PGE₂ (specific activity: 100–200 Ci/mmol) was provided by PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA, USA). LPS from *E. coli* 05:B55, aminoguanidine hydrochloride, Quercetin dihydrate, SNAP, anti- β -actin antibody, glutathione, standard, and antiserum for PGE₂ RIA were purchased from Sigma Chemical Co. PGH₂ and Prostaglandin E Metabolite EIA kit were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). TRIzol reagent, Moloney murine leukemia virus reverse transcriptase (M-MLVRT), RNase-free DNase, and random primers were purchased from Invitrogen Life Technologies Co. The anti-PGDH antibody was a gift from Dr Tai Hsin-Hsiung (Department of Pharmaceutical Sciences College of Pharmacy University of Kentucky Lexington, USA). HRP-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The western blotting reagents were obtained from Bio-Rad Laboratories. All other chemicals were analytical grade.

Animals and treatments

Virgin female mice of the BALB/c strain, 8- to 12-week-old and 25–30 g of weight, were paired with adult BALB/c males and the day of appearance of the *post-coitum* vaginal plug was taken as day 0 of gestation. Animals received food and water *ad libitum* and were housed under controlled conditions of light (12 h light/12 h darkness) and temperature (23–25 °C). Mice were killed by CO₂ inhalation. The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFYO–CONICET) and by The Institutional Committed for the Care and Use of Laboratory animals from the School of Medicine, University of Buenos Aires.

On days 6 and 7 of gestation, animals were divided randomly into different groups of treatment for the *in vivo* experiments. Table 1 shows the different protocols, doses, routes of administration, and vehicles used. We have previously shown (Ogando *et al.* 2003) that LPS produced a significant increase in nitric oxide production in uterus at 6, 12, and 24 h after injection reaching a maximum at 6 h. So in the present work, treated mice were killed 6 h after LPS

administration. In mice, the endometrial estroma undergoes dramatic cytologic changes to form a decidua in response to the implanting embryo. After attachment, the embryo becomes embedded in an enlarging mass of decidual tissue, and this tissue is separated from uterine adventitia by an inner circular and an outer longitudinal smooth muscle layer (myometrium). After isolation of each implantation site, the uterus was cut open along the longitude axis. Decidual tissues were separated from myometrium by gently scrapping off the decidua. The authors use the term 'uterus' for the tissue composed of nondecidualized endometrium, myometrium, and adventitia/serosa and the term decidua for the decidualizing stromal cells and the embryo. Uterus and decidua from each implantation site were separated and immediately frozen at –80 °C.

Determination of PGE synthase activity

Enzyme activity in uterine tissue was measured by assessing the conversion of PGH₂ to PGE₂ as in Murakami *et al.* (2000) with minor modifications. Tissues were homogenized in a 0.01 mol/l Tris–HCl buffer (pH=8) and sonicated. After centrifugation, 100 µg total protein were incubated with 2 µg PGH₂ for 1 min at 24 °C in 0.1 ml of 1 mol/l Tris–HCl (pH=8) containing 0.002 mol/l glutathione. After terminating the reaction by the addition of 20 µl of 6 M HCl, samples were dried under vacuum and resuspended to determine the levels of PGE₂ by RIA. Results were expressed as ng PGE₂/mg protein per h.

PGE₂ determination

PGE₂ was quantified by specific RIA. Uteri and deciduas were removed, cleaned, and rinsed in cold PBS. Tissues were weighed and incubated in 2 ml modified Krebs–Ringer bicarbonate solution (0.118 mol/l NaCl, 0.005 mol/l KCl, 0.001 mol/l KH₂PO₄, 0.001 mol/l MgSO₄×7H₂O, 0.025 mol/l NaHCO₃, and 0.011 mol/l glucose) in 95% O₂/5% CO₂ atmosphere at 37 °C for 1 h. PGs were acidified to pH 3 with 1 N HCl and extracted twice with 2 ml ethyl acetate. Samples were subsequently dried under vacuum. These samples were store at –20 °C before analysis for no longer than 3 weeks to minimize degradation of lipids and they were resuspended in RIA buffer (0.007 mol/l K₂HPO₄×3H₂O, 0.003 mol/l KH₂PO₄, 0.154 mol/l NaCl, 0.007 mol/l albumin bovine, and 0.015 mol/l sodium azide, pH=7.4) the day of the assay. PGE₂ antiserum was highly specific and showed low cross-reactivity, <0.1% with other PGs. Intra- and interassay variations were <8%. Values were expressed as pg PGE₂/mg tissue.

Quantification of PGE₂ metabolites

Metabolites were determined in uteri and deciduas by PGE Metabolite EIA Kit (cat. no. 514531.1, Cayman Chemical Co.) according to the manufacturer's recommendations. PGE₂ is not chemically stable and is rapidly converted to its 13,14-dihydro-15-keto metabolite. The PGE₂ metabolite assay kit converts all the immediate PGE₂ metabolites to a single stable derivative that could be easily quantified by EIA. Metabolite levels are reported as pg PGE₂-M/mg tissue.

Table 1 Protocols, doses, routes of administration, and vehicles used for *in vivo* treatments.

Treatment	Dose	Vehicle	Route of administration	Day and hour of injection ^a
LPS	1 µg/g	PBS	I.p.	Day 7/0900 h
Amino-guanidine	6 mg/mouse	PBS	I.p.	Day 6/0900 h and 1300 h
SNAP	3 mg/kg	PBS	I.p.	Day 7/0900 h
Quercetin	10 mg/kg	Corn oil	S.c.	Day 7/0900 h

In all cases, the animals were killed 6 h after LPS injection.

^aCorresponds to the day of gestation in which the animal was injected.

Western blot analysis

Isolated tissues were homogenized (Ultra Turrax, T25 basic, IKA Labortechnik, Staufen, Germany) in RIPA buffer (0.05 mol/l Tris-HCl pH=7.4, Triton X-100 1% (v/v), 0.15 mol/l NaCl, 0.001 mol/l EDTA, and 0.25% w/v sodium deoxycholate) with freshly added protease inhibitor cocktail (10 µg/ml leupeptin, 2 µg/ml aprotinin, 100 µg/ml soybean trypsin inhibitor, 1 mmol/l EDTA, 1 mg/ml benzamidine, 10 µg/ml dithiothreitol, and 1 mg/ml caproic acid). Tissues were sonicated (Ultrasonic Cell Disrupter, Microson, Heat systems, Inc., New York NY, USA) for 30 s, centrifuged at 1500 *g* for 5 min, and protein concentration was determined by the Bradford assay (Bradford 1976). Proteins (100 µg/lane) were separated by electrophoresis in 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked using 5% (w/v) dried nonfat milk and then incubated overnight at 4 °C with the primary antibody, an antisera against mouse 15-PGDH (1:500 containing 3% of dried nonfat milk, diluted in PBS). After washing with 0.1% (v/v) PBS-Tween 20, membranes were incubated for 15 min with the β-actin antibody (1:4000, diluted in PBS). After that, they were washed followed by 1 h incubation with HRP-conjugated anti-rabbit secondary antibody (1:20 000 containing 2% of milk, diluted in PBS) and developed using the ECL system. The corresponding immunoreactive bands were registered with a digital camera Olympus C-5060 and analyzed using the software package Image J (NIH, Bethesda, MD, USA; <http://imagej.nih.gov/ij/>). Results were expressed as the ratio of the optical density of the 15-PGDH band normalized with the optical density of β-actin.

Statistical analysis

Data were analyzed using GraphPad Prism version 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Pairwise comparisons of means were made with Student's *t*-test or Tukey's multiple comparison tests for unequal replicates. Comparisons between means for more than two groups were performed using one-way ANOVA. All values presented in this study represent mean ± S.E.M. of at least three independent determinations. Differences were considered significant when *P* was 0.05 or less. On graphs, dissimilar superscripts (letters) denote significantly different values.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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