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Inhibition of Inducible Nitric Oxide Synthase by Peroxisome Proliferator-Activated Receptor Agonists: Correlation with Induction of Heme Oxygenase 1

Paul R. Colville-Nash, Saima S. Qureshi, Dean Willis, and Derek A. Willoughby

Genetic knock-out in mice of peroxisome proliferator-activated receptor-α (PPARα) can prolong inflammation in response to leukotriene B4. Although cyclooxygenase 2 has been shown to be induced by PPAR activation, the effect of PPAR agonists on the key inflammatory enzyme systems of nitric oxide synthase (NOS) and stress proteins has not been investigated. The effect on these of naturally occurring eicosanoid PPAR agonists (leukotriene B4 and 8(5)-hydroxyeicosatetraenoic acid, which are PPARα selective; PGA2, PGD2, PGJ2, and Δ12PGJ2, which are PPARγ selective) and the synthetic PPARα agonist Wy14,643 was examined in activated RAW264.7 murine macrophages. Leukotriene B4 and 8(5)-hydroxyeicosatetraenoic acid stimulated nitrite accumulation, indicative of enhanced NOS activity. PGA2, PGD2, PGJ2, Δ12PGJ2, and Wy14,643 reduced nitrite accumulation, with Δ12PGJ2 being the most effective. The mechanism behind this reduction was examined using Western blotting. Inhibition of nitrite accumulation was associated with a fall in inducible NOS protein and an induction of heme oxygenase 1, correlating both dose dependently and temporally. Other proteins examined (cyclooxygenase 2, heme oxygenase 2, heat shock protein 70, and glucose-regulated protein 78) were unaffected. The data suggest that naturally occurring PPAR agonists can inhibit the inducible NOS enzyme pathway. This inhibition may be mediated by modulation of the stress protein, heme oxygenase 1. Thus, the generation of eicosanoid breakdown products during inflammation may contribute to its eventual resolution by activation of the PPAR system. This system may thus represent a novel target for therapeutic intervention in inflammatory disease.


The inflammatory response involves the sequential activation of a number of signaling pathways, resulting in the production of a variety of mediators that act both locally and systemically. Since the demonstration that part of the anti-inflammatory activity of aspirin stems from inhibition of cyclooxygenase (COX)3 (1) and a reduction in the formation of PG, there have been enormous advances in the understanding of this pathway, as well as the discovery of a role for a variety of other pathways in inflammation, such as nitric oxide synthase (NOS; particularly the inducible isofom (iNOS) (2)) and nitric oxide (NO), the stress proteins (heat shock proteins (HSPs)) (3), and the heme oxygenase (HO) (4) pathway. Much interest is now focused on the interactions between these systems, their potential role in modulating inflammation, and their role as therapeutic targets for intervention in inflammation and other forms of disease.
anti-inflammatory. However, this enhanced degradation of lipid mediators has not been demonstrated in this model, and a variety of other inflammatory mechanisms such as those suggested above may also be important in the effects attributable to PPAR activation.

To date, the effect of cyclopentenone PGs and other PPAR agonists on iNOS in macrophages, a key cell and enzyme system in inflammation and other pathologies, has not been described. In this study, we demonstrate that $\Delta^{12}$PGJ$_2$, a PPAR$\gamma$ agonist, is a potent inhibitor of iNOS activity and protein expression in stimulated RAW264.7 macrophages. Protein expression and inhibitor studies implicate activation of HO-1 in this phenomenon.

Materials and Methods

Culture and stimulation of murine RAW264.7 macrophages

RAW264.7 macrophages (European Collection of Animal Cell Cultures) were cultured as described previously (16). Cells were passaged and grown to 90% confluence in 96-well plates (Greiner) for analysis of nitrite production and viability or in 75-cm$^2$ culture flasks (Greiner, Stonehouse, U.K.). Western blot analysis of protein expression. Macrophages were stimulated for 18 h (unless otherwise indicated) by addition of 100 U/ml murine rIFN-\gamma (Sigma Chemical, Poole, U.K.) and 0.1 $\mu$g/ml LPS (LPS, Escherichia coli serotype 0111:B4; Sigma Chemical). Concurrently administered PPAR agonist compounds were dissolved in methyl acetate such that the final concentration of solvent in medium was 0.1% by volume, with solvent alone as control. At this concentration, the solvent has no effect on cellular functions examined in this study (data not shown). Ethanolic cosanoids were purchased from Biomol (Plymouth Meeting, PA), and Wy14,643 was purchased from Caymen Chemical (Ann Arbor, MI). Zinc deuteroporphorpin (ZnDPP; Porphyrin Products, Logan, UT) was initially dissolved at 100$\times$ the required concentration in 0.1 M sodium hydroxide before dilution with appropriate media.

Nitrite assay

Nitrite accumulation in culture medium was used as a measure of cellular NO synthesis (17). This was assessed spectrophotometrically by addition of 100 $\mu$l of Griess reagent (1% sulfanilamide and 0.1% $N$-(1-naphthyl)ethylenediamine in 5% $\alpha$-phosphoric acid; Sigma Chemical) to an equal volume of sample medium in 96-well plates. Optical densities were measured by dual wavelength analysis (OD 570–630 nm) in a plate reader (Biotek EL-310; Biotek Instruments, Winooski, VT); sodium nitrite was used as standard.

Cell viability assay

Cell viability was assessed by measuring the ability of macrophages to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium (MTT) at the concentration of 100 $\mu$g/ml or below. A 51% decrease at 10 $\mu$g/ml and an 81% reduction at the highest nontoxic dose of 30 $\mu$g/ml are breakdown products of HO-1 (4).

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Results

Effects of various PPAR agonists on viability and iNOS activity in stimulated RAW264.7 macrophages

Stimulation of macrophages with IFN-\gamma and LPS routinely increased nitrite levels in medium by $\geq$12-fold after 18 h, compared with nonstimulated cultures (data not shown). Viability of cell cultures (Fig. 1) treated with up to 10 $\mu$g/ml of LTB$_4$, 8(5)-hydroxy-eicosatetraenoic acid (8(5)-HETE), PGA$_2$, PGD$_2$, and PGJ$_2$ was not affected. However, Wy14,643 was found to be toxic at a concentration of 100 $\mu$g/ml, although no effect on viability was observed at 30 $\mu$g/ml or below. $\Delta^{12}$PGJ$_2$ reduced viability at 10 $\mu$g/ml. Only data derived from nontoxic doses of compounds was considered, therefore, in subsequent analysis of the results.

LTB$_4$ and 8(5)-HETE significantly increased nitrite accumulation in culture medium at higher dosages of 3 $\mu$g/ml (38% rise) and 10 $\mu$g/ml (36%), respectively (Fig. 1). In contrast, Wy14,643 dose dependently reduced nitrite accumulation, causing a significant 60% decrease at the highest nontoxic dose of 30 $\mu$g/ml.

At doses shown to be nontoxic, all PGs tested proved capable of inhibiting iNOS activity at the higher doses investigated, indicated by a reduction in nitrite accumulation in test medium (Fig. 2). PGA$_2$ at doses up to 1 $\mu$g/ml significantly increased nitrate production. Above this level, nitrite production was reduced, with a 94% reduction at 10 $\mu$g/ml. PGD$_2$ did not elevate nitrite accumulation at low doses, but dose dependently reduced nitrite accumulation. This reached significance at 1 $\mu$g/ml, and nitrite levels fell by 51% at 10 $\mu$g/ml. PGJ$_2$ elicited a response similar to that of PGD$_2$, although significance was not reached until 3 $\mu$g/ml, with a 69% fall at 10 $\mu$g/ml. $\Delta^{12}$PGJ$_2$ was the most potent inhibitor of nitrite accumulation, with reaching significance at 30 ng/ml, and an 81% reduction at the highest nontoxic dose of 3 $\mu$g/ml. For subsequent analysis of changes in protein expression in response to PPAR agonist treatment, the response to the most potent inhibitor of iNOS, $\Delta^{12}$PGJ$_2$, was investigated.

Western blot analysis of the dose-dependent effects of $\Delta^{12}$PGJ$_2$ on stimulated RAW264.7 macrophages

Stimulation of macrophages with IFN-\gamma and LPS markedly increased iNOS protein expression at 18 h poststimulation (Fig. 3). At doses up to 0.3 $\mu$g/ml, $\Delta^{12}$PGJ$_2$ did not affect this level visibly. Above this dosage, however, iNOS protein levels fell markedly, with little expression visible, compared with stimulated control cultures at the highest nontoxic dose of 3 $\mu$g/ml. These changes in iNOS expression correlated with changes in the levels of HO-1 present in these macrophages. Thus, at low doses, induction of HO-1 protein expression was evident, but increased markedly at concentrations of $\Delta^{12}$PGJ$_2$ of 0.3 $\mu$g/ml and above. Additional lower m.w. bands seen at 3 $\mu$g/ml and 10 $\mu$g/ml are breakdown products of HO-1 (4).

COX-2 protein levels were increased by stimulation of macrophages with IFN-\gamma and LPS, but although minor variations in protein levels were observed, no consistent effect on protein expression was seen in the presence of $\Delta^{12}$PGJ$_2$ compared with
stimulated control cultures, with no evidence of a dose-dependent effect (data not shown). Expression of HO-2, HSP70, and GRP78 (Fig. 3) were also unaffected either by stimulation or by treatment with D<sub>12</sub>PGJ<sub>2</sub>.

**Western blot analysis of the time-dependent effects of 3 µg/ml Δ<sup>12</sup>PGJ<sub>2</sub> on stimulated RAW264.7 macrophages**

Analysis of the time course of protein expression was conducted using the highest nontoxic dose of Δ<sup>12</sup>PGJ<sub>2</sub>, 3 µg/ml. In non-PG-treated cultures, stimulation with IFN-γ and LPS increased iNOS expression (Fig. 4), this effect being evident at 6 h, greatest at 12 h, and slightly reduced at 24 h. HO-1 was similarly induced (Fig. 4), with a longer delay before protein expression was enhanced, this being evident at 12 h and most notable at 24 h.

Treatment with Δ<sup>12</sup>PGJ<sub>2</sub> caused a retardation in iNOS expression, with increased expression first evident at 9 h poststimulation, and the greatest response seen at 24 h (Fig. 4). This correlated with an earlier induction of HO-1, with much increased expression of this protein seen at 6 through 12 h poststimulation. This increased expression was lost at 24 h poststimulation in Δ<sup>12</sup>PGJ<sub>2</sub>-treated cultures.

In keeping with the results reported above, expression of COX-2 was increased by stimulation with IFN-γ and LPS, this being evident at 3 h and maintained through to 24 h. Variation in protein expression between control and treated cultures was seen, but no consistent pattern in the temporal changes was observed over the three experiments, and it was concluded that COX-2 expression was unaffected by the presence of Δ<sup>12</sup>PGJ<sub>2</sub> (data not shown). As seen previously, neither stimulation nor the presence of the PG affected the expression of HO-2, HSP70, and GRP78 at any time point examined (Fig. 4).

**The effect of ZnDPP on the inhibition of nitrite release by Δ<sup>12</sup>PGJ<sub>2</sub>**

Given the correlation between iNOS expression and HO-1 seen in the Western blot analysis, the effect of including a selective HO inhibitor, ZnDPP, was examined. As shown previously, addition of Δ<sup>12</sup>PGJ<sub>2</sub> at doses up to 3 µg/ml was without effect on viability of macrophages stimulated with IFN-γ and LPS (Fig. 5). Addition of ZnDPP alone, at concentrations up to 30 µM, to stimulated cultures did not affect viability nor change nitrate accumulation in the
medium (data not shown). In cultures treated with Δ^{12}PGJ₂, ZnDPP at 30 μM similarly did not affect viability (Fig. 5).

In cultures treated with PG alone, nitrite accumulation was dose-dependently reduced, as seen previously (Fig. 1), with a 79% reduction seen at 3 μg/ml of Δ^{12}PGJ₂. Inclusion of ZnDPP partially reversed these effects, the inhibitory effect of Δ^{12}PGJ₂ being approximately halved (43% reduction in nitrite accumulation with 3 μg/ml Δ^{12}PGJ₂, compared with stimulated controls in the presence of this inhibitor).

Discussion

This study represents the first work showing that naturally occurring prostaglandin PPARγ agonists are able to inhibit iNOS activity, measured by a reduction in nitrite accumulation and iNOS protein expression in macrophages, an effect that correlates with activation of HO-1.

iNOS, inducible HO, the stress proteins, and the inducible COX-2 are several key physiologic and pathophysiologic systems that have stimulated much interest in recent years (2, 19). In particular, the interplay between these systems has been the subject of much debate, not only because of the possibilities of using them as therapeutic targets, but also the possible ramifications of interfering with any one of them. The recent discovery of the possibility of peroxisomal control of inflammation (15), by modulating the destruction of key mediators of the inflammatory response such as the leukotrienes and other prostanoids, has offered another possibility to the anti-inflammatory armory currently under development. To date, the effects of many of the PPAR agonists on iNOS, a key enzyme system in inflammation and other pathologies, have not been reported.

Initial studies investigated the role of the PPARs on the production of NO by stimulated RAW264.7 macrophages, which are used in many in vitro studies (20). This was conducted using a variety

FIGURE 3. Western blot analysis of the dose-dependent effects of Δ^{12}PGJ₂ on stimulated RAW264.7 macrophages. RAW264.7 murine macrophages (controls) were stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS (stimulated controls) for 18 h in the absence or presence of varying concentrations of Δ^{12}PGJ₂. Expression of iNOS, HO-1, HO-2, HSP70, and GRP78 were assessed at this time by Western blotting.

FIGURE 4. Western blot analysis of the time-dependent effects of Δ^{12}PGJ₂ on stimulated RAW264.7 macrophages. RAW264.7 murine macrophages were stimulated with 100 U/ml IFN-γ and 100 ng/ml LPS at 0 h in the presence or absence of 3 μg/ml Δ^{12}PGJ₂. At various times poststimulation (h) indicated above, Western blot analysis of iNOS, HO-1, HO-2, HSP70, and GRP78 was performed.
of naturally occurring eicosanoids, LTB₄, 8(5)-HETE, PGA₂, PGD₂, PGJ₂, and PGA₁₂, as well as the PPARα-selective compound Wy14,643 to screen for their effects on nitrite production, indicative of activity of the iNOS pathway. LTB₄ and 8(5)-HETE, which activate PPARα (15) and are described as poor activators of PPARγ, showed no inhibitory activity, but at high concentrations caused a stimulation of nitrite accumulation. In the case of LTB₄, this has been demonstrated for human neutrophils using a lower dose of 0.1 μM (21). In RAW264.7 macrophages, LTB₄ has been reported to have no effect on iNOS activity, although a concentration of only 1 nM was investigated (22). The ability to inhibit nitrite accumulation of some of the other compounds examined was shown not to be related to their cytotoxic potential, although this phenomenon was evident at higher concentrations. Wy14,643, also described as PPARα selective (23), only inhibited nitrite accumulation, unlike LTB₄ and 8(5)-HETE. It is therefore possible that the stimulatory effects of these latter two are not related to PPARα activation. Inhibition with Wy14,643 was, however, effective only at much higher concentrations than the PGs tested. It is known, however, that Wy14,643 is a less efficient PPARα-binding ligand and is capable of also activating PPARγ at the concentrations used here to inhibit nitrite accumulation (24). In contrast, the cyclopentenone PGs were effective inhibitors. The cyclopentenone PGA₁₂ inhibited nitrite accumulation at higher concentrations, but at lower concentrations PGA₁₂ increased nitrite accumulation. The reason behind this effect is unclear. However, PGE₂, the precursor of PGA₁₂, has been shown to both increase iNOS activity at low concentrations and inhibit them at high concentrations in a similar fashion (25). PGD₂ and its metabolites of the PGJ₂ series are selective activators of the PPARγ receptor pathway (26) and were demonstrated to inhibit iNOS. The data presented in this study suggest, therefore, that in these macrophages, it is PPARγ activation that is responsible for the effects described. This adds to the previously published in vivo data suggesting that PPARα activation is also an important pathway for anti-inflammatory activity (15).

Further studies into the mechanism behind this inhibition used the technique of Western blotting to examine the levels of protein expressed by these macrophages in response to treatment with Δ¹²PGJ₂, the most potent inhibitor of nitrite accumulation activity investigated. Previously published work has revealed a link between these PGs and the induction of the stress response in a variety of cell lines (5, 12). We have previously published the observation that control of HO-1 activity can also affect the outcome of inflammation (4). In this study, it was shown that induction of HO-1 could markedly suppress an acute inflammatory reaction. In addition, work within this department has shown that the iNOS and HO-1 pathways exhibit a degree of interdependency (27).

Examination of HO-1 expression elicited by Δ¹²PGJ₂ revealed a correlation between the degree of inhibition of iNOS and the extent of induction of HO-1. Other markers of the stress response, HSP70 and GRP78, were unaffected by this treatment. Although HSP70 has been shown to be induced by these PGs in human lines, this feature has been reported absent in murine cell lines (28). Similarly, levels of HO-2, which has been described as a constitutively expressed protein (29), were unchanged. There was no correlation between the inhibition of iNOS and the expression of COX-2, although cyclopentenone PGs have been shown to induce this enzyme in murine liver cell lines (30). This induction could not be demonstrated in the current study using murine macrophages. The possibility that induction of COX-2 may play a role in the anti-inflammatory properties of these PGs and/or PPAR activation remains to be investigated. One possible mechanism in which this may be important may involve the generation of these “anti-inflammatory prostaglandins” at later time points in inflammation. In mast cells, COX-2 has been shown to exhibit different modes of arachidonic acid acquisition compared with COX-1 and is responsible for late phase, sustained release of PGD₂ (31). This is the precursor for the PGJ series, which are formed spontaneously from PGD₂ in biologic fluids in the presence of albumin (32), and may represent a negative feedback loop for inflammation control by eicosanoid breakdown products. In the carrageenan pleurisy, Western blotting reveals two distinct peaks in protein expression. An early peak is associated with PGE₂ production, while a late peak is also found that is not associated with PGE₂ elaboration (D.W., unpublished observations). The role of this latter peak in this model is the subject of current study, but it is possible that this peak may represent a new function for COX-2 during resolution by producing prostanoids that may help to terminate inflammation.

The time courses of these changes in the level of these proteins were constructed to attempt to further correlate any changes seen. In non-PG-treated cells, iNOS protein was evident at 6 h and peaked at 18 h, with a fall at 24 h, in agreement with previously published data (20). Inducible HO protein levels did not rise until 24 h; this correlated with the fall in iNOS protein expression seen at the same time point. In PG-treated cells, the expression of HO-1 was increased and also temporally shifted such that expression was seen much earlier, at 6 to 9 h. In keeping with a possible link
between the two systems, iNOS induction was retarded until the HO-1 levels were reduced at 24 h. This latter finding has potential importance for the development of a therapeutic approach, due to the short period of suppression of iNOS seen in these cultures. However, it is known that the half-life of these PGs in vitro is extremely short, and it is possible that HO-1 expression was maintained only for as long as they were present, with later escape of the iNOS response due to the greater stability of the stimulus for iNOS in the culture system used. Again, no correlation was seen between the inhibition of iNOS and the expression of HSP70, GRP78, HO-2, or COX-2.

Given the possible link demonstrated between the induction of HO-1 and the inhibition of iNOS, the effect of ZnDPP, a specific HO-1 inhibitor (33), was examined in the whole cell system used initially. ZnDPP was shown to have no effect on cell viability nor on iNOS activity in this system in the absence of treatment with the PG. However, when ZnDPP was included with the PG, the inhibitory effect of the PG was reduced to half that seen in the presence of the PG alone, suggesting that at least part of this inhibitory activity was due to activation of HO-1. The remainder may represent a more direct action of the PG on iNOS induction, although to date no peroxisome proliferator response element has been described on the iNOS promoter.

The precise mechanism whereby the interaction between HO-1 and iNOS is effected is unknown and will be the subject of further work. It has been shown that the antiproliferative cyclopentenone PGA1 activates HSF, which subsequently induced the expression of HSP70 and HSP90 genes (5, 12). Activation of HSF could potentially modulate a variety of gene products that possess a heat shock element. Thus, activation of the heat shock element up-regulates the production of HO-1 (34) and other stress proteins such as HSP70 and HSP90, which may be anti-inflammatory, but also inhibits production of other pro-inflammatory cytokines such as IL-1 (35). With particular reference to iNOS activation, it is well documented that the heat shock response (36) and HSP70 (37) are capable of inhibiting this enzyme system, the latter by decreasing NFkB activation. These two enzyme systems are also coinduced in macrophages (38), HO-1 induction having been suggested as protective against the harmful effects of nitric oxide production. Although HSP70 was not induced in these murine cells by these PGs, in other mammalian systems and in man, such a mechanism may be of import. HO-1 activation may have further effects on inflammation via the production of heme breakdown products (4); these include CO, which may inhibit iNOS activity by binding to and inactivating the heme moiety on the enzyme; biliverdin and bilirubin, which may scavenge product by direct antioxidant effects; and iron, which will affect cellular redox potentials. Elevation in anti-oxidant molecules may also stabilize inhibitory factor κB-NFkB complexes and lead to a reduction in active NFkB, a factor that is required for iNOS induction (39). Work is in progress on these possibilities.

Although the levels of cyclopentenone PGs used in this study are high in comparison with most studies using conventional eicosanoids, it is known that there are selective uptake mechanisms for these PGs in cells, which can enhance their intracellular concentration (40). In addition, assays in vivo show that micromolar concentrations (40). In addition, assays in vivo show that micromolar concentrations (40). In addition, assays in vivo show that micromolar concentrations (40). In addition, assays in vivo show that micromolar concentrations (40).

References


