Feedback Control of Cyclooxygenase-2 Expression through PPARγ

Hiroyasu Inoue‡¶, Tadashi Tanabe‡, and Kazuhiko Umesono§

‡Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan.
§Department of Molecular Biology and Genetics, Institute for Virus Research, Kyoto University, Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8397, Japan.

Corresponding Author: Hiroyasu Inoue,
Mailing Address: Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan.
Phone: +81-6-6833-5012 ext. 2588
Fax: +81-6-6872-7485
e-mail: inoue@ri.ncvc.go.jp

Running title: Control of Cyclooxygenase-2 expression by PPARγ
SUMMARY

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme for prostaglandins (PG), plays a key role in inflammation, tumorigenesis, development and circulatory homeostasis. The PGD\(_2\) metabolite 15-deoxy-\(\Delta^{12,14}\) PGJ\(_2\) (15d-PGJ\(_2\)) was identified as a potent natural ligand for the peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)). PPAR\(\gamma\) expressed in macrophages has been postulated as a negative regulator of inflammation and a positive regulator of differentiation into foam cell associated with atherogenesis. Here we show that 15d-PGJ\(_2\) suppresses the lipopolysaccharide (LPS)-induced expression of COX-2 in the macrophage-like differentiated U937 cells but not in vascular endothelial cells. PPAR\(\gamma\) mRNA abundantly expressed in the U937 cells not in the endothelial cells is down-regulated by LPS. In contrast, LPS up-regulates mRNA for the glucocorticoid receptor which ligand anti-inflammatory steroid dexamethasone (DEX) strongly suppresses the LPS-induced expression of COX-2 gene although both 15d-PGJ\(_2\) and DEX suppressed COX-2 promoter activity by interfering with the NF-\(\kappa\)B signaling pathway. Transfection of a PPAR\(\gamma\)-expression vector into the endothelial cells acquires this suppressive regulation of COX-2 gene by 15d-PGJ\(_2\) but not by DEX. A selective COX-2 inhibitor NS-398 inhibits production of PGD\(_2\) in the U937 cells. Taken together, we propose that expression of COX-2 will be regulated by a negative feedback loop mediated through PPAR\(\gamma\), which makes possible a dynamic production of PG, especially in macrophages, and may be attributed to various expression patterns and physiological functions of COX-2.
Cyclooxygenase (COX) has two isoforms, COX-1 and -2. COX-1 is constitutively expressed in most cells, whereas COX-2 is largely absent but induced upon stimulation by inflammatory stimuli such as endotoxin lipopolysaccharide (LPS), suggesting that COX-2 plays a critical role in inflammation (1, 2). However, growing evidence indicates that expression of COX-2 is differently regulated in different types of cells and also plays a key role in tumorigenesis (3), development (4-6) and circulatory homeostasis (7, 8). In fact, three cis-acting elements, that is, NF-κB, NF-IL6 sites, and cyclic AMP response element (CRE), are differently involved in the COX-2 promoter activity in different cells (9, 9-17). Anti-inflammatory steroid dexamethasone (DEX) suppresses COX-2 expression in macrophage-like differentiated U937 cells (13), but not in bovine arterial endothelial cells (BAEC) (16). This cell-type specific regulation may be physiologically important since thromboxane (TX) A₂ produced by macrophages (18) has opposite effects of prostacyclin (PGI₂) produced by vascular endothelial cells. We have recently reported that this different effect of DEX is partly explained by different expression levels of glucocorticoid receptor (GR) between them (16). Moreover, expression of PGI₂ and TXA₂ synthases are inversely regulated in resident and activated peritoneal macrophages (19) where production of PGD₂ and PGE₂ is also inversely regulated (20), which suggests complex regulation of COX-2 expression as well as its physiological roles at different activated stages of macrophages.

The peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-dependent transcription factor belonging to the family of nuclear receptors that includes the estrogen receptors, thyroid hormone receptors and glucocorticoid receptors (GR) (21). The PGD₂ metabolite 15-deoxy-Δ¹²,¹⁴ PGJ₂ (15d-PGJ₂) was identified as a potent natural ligand for the PPARγ (22, 23). PPARγ expressed in macrophage has been postulated as a negative regulator of inflammation (24, 25) and a positive regulator of differentiation into foam cell associated with atherogenesis (26, 27). Recently, induction of COX-2 by 15d-PGJ₂ was reported in immortalized epithelial and colorectal cancer cells (28, 29), although 15d-PGJ₂ suppressed COX-2 expression in fetal hepatocytes (30). The molecular mechanisms that underlie...
different regulation of COX-2 expression remain to be elucidated.

In the present study, we investigated the different effect of 15d-PGJ2 on expression of COX-2 gene between macrophage-like differentiated U937 cells and BAEC. We provide evidence that a unique expression pattern of PPARγ is involved in this different effect. Especially in the U937 cells, LPS down-regulates PPARγ mRNA but up-regulates GR mRNA although both 15d-PGJ2 and DEX suppressed COX-2 expression, at least, by interfering the NF-κB signaling pathway. With additional evidences, we propose that expression of COX-2 will be regulated by a negative feedback loop mediated through PPARγ. This makes possible a dynamic production of PG especially in macrophages.

MATERIALS AND METHODS

Cell culture---U937 cells (10) and BAEC (11) were grown in RPMI 1640 and DMEM media, respectively, supplemented with 10% fetal calf serum (Flow), 50 µM 2-mercaptoethanol, 100 units/ml penicillin and 100 µg/ml streptomycin. For differentiation into monocyte/macrophage, U937 cells were treated with 100 nM TPA and allowed to adhere for 48 h, after which they were fed with TPA-free medium and cultured for 24 h prior to use.

Determination of PG synthesis---The TPA-differentiated U937 cells (5 x 10^5 cells/well) were cultivated on 12-well tissue culture plates with 1 ml of the culture medium. After a further 24-h of incubation, the relevant reagents were added to the medium. After 12 h of incubation, the culture medium was removed and subjected to enzyme immunoassays for PGE_2 and PGD_2 (Cayman). PGD_2 was measured as its methyl oximes after derivatization with methoxamine.

RNA analysis---Total RNA was isolated using the acid guanidinium thiocyanate procedure. RNAs were then subjected to electrophoresis. The cDNA probes used were the 1.5-kb insert of pHEPSII17 for COX-2 (31), the 3-kb fragment of pRShGRα digested with Kpn I/Xho I for GR (32), the entire coding sequences for human PPARγ from the expression vector and the cDNA insert (nucleotide 61-950) for glyceraldehyde-3-phosphate dehydrogenase (31). The
levels of mRNA were calculated on the basis of hybridization signals as measured by an imaging analyzer BAS 5000 (Fuji Photo Film Co., Tokyo). RT-PCR analysis was performed using KOD DNA polymerase (Toyobo, Osaka) as described previously (16). The primer pair for PPARγ amplification was designed to anneal to both human (33) and bovine (34) sequences had the following sequence, 5'-CCAAAGTGCAATCAAAGTGGAGCC-3' and 5'-GCAGGCTCTTTAGAAAAACTC-CCTTG-3'. The cycling conditions were as follows: 3 min at 96 °C, followed by 30 cycles of 94 °C, 15 s; 57 °C, 2 s; 74 °C, 30 s. The primer pair for human PGD$_2$ synthase (35) had the following sequence, 5’-CCTTGGGCAGAGAAAAAGC-AAG-3’ and 5’-AACATGGATCAGCTAGAGTTT-GG-3’. The cycling conditions were as follows: 3 min at 96 °C, followed by 30 cycles of 94 °C, 15 s; 58 °C, 2 s; 74 °C, 15 s.

**Western Blot Analysis**---Cell lysates (10$^5$ cell equivalents) were subjected to SDS-polyacrylamide gel electrophoresis on 10% gels. The separated proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore). The membranes were probed with the human COX-2 antisera (IBL, Gunma, Japan) and visualized using the ECL Western blot analysis system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Transcription assays---U937 cells stably transfected with a COX-2 (nucleotide -327/+59) luciferase reporter containing NF-kB site alone and pCB6 containing a neomycin-resistant gene were made by electroporation as described previously (13). BAEC was transfected using Trans ITTM-LT-1 (Mirus) (16). An each 0.2 µg COX-2 reporter vector pHES2(-327/+59) (10), pRShGRα or pCMX-hPPARγ1, and 0.02 µg pSV-βgal (Promega) were used for transfection of each 24-well. pCMX-hPPARγ1 was human PPARγ1 expression vector under control of a CMV promoter made by Dr. S. Osada (Kyoto University). Luciferase and β-galactosidase activities were determined and luciferase activity was normalized to the β-galactosidase standard in BAEC (11), whereas that was normalized with protein concentration in the U937 cells (13).

**RESULTS**

5
15d-PGJ2 inhibits expression of COX-2 in U937 cells but not in BAEC---To determine effects of 15d-PGJ2 on expression of COX-2 gene, we performed northern blot analysis using RNA derived from the differentiated U937 cells. LPS-induced expression of COX-2 mRNA (Fig. 1A and 1B) and production of PGE2 (Fig. 1C) were suppressed by 15d-PGJ2 in the U937 cells. Suppressive effect of 15d-PGJ2 was a dose-dependent manner (Fig. 1A), and milder than that of DEX, i.e., 10 µM 15d-PGJ2 showed 50-60% suppression (Figs. 1B & 2A) whereas 100 nM DEX showed over 70% (13). This was also confirmed by western blot analysis of COX-2 protein (Fig. 1D). In contrast, in BAEC, 15d-PGJ2 showed no effect of the LPS-induced COX-2 mRNA expression (Fig. 2A). Similar result was also obtained in human umbilical vein endothelial cells (HUVEC) (data not shown).

Down-regulation of PPARγ by LPS in U937 Cells---DEX-mediated suppression of COX-2 expression is modulated by GR, which will be attributed to distinct effect of DEX on COX-2 expression between macrophages and endothelial cells (16). Similarly, we determined whether expression of PPARγ accounts for the different effects of 15d-PGJ2. Expression of PPARγ mRNA was observed in the differentiated U937 cells (33) as well as monocytes and macrophages (27) and that, was down-regulated by treatment of LPS in a time-dependent manner (Fig. 3A and 3C). This down-regulation was not observed by treatment with 15d-PGJ2 alone (data not shown). In contrast, no PPARγ mRNA was detected in BAEC (Fig. 3A and 3B) and HUVEC although PPARδ mRNA was constitutively expressed in both cells as well as the U937 cells (data not shown). In aortic smooth-muscle cells (36), the PPARα activators inhibit the inflammatory response. However, in the U937 cells as well as activated macrophages (24), no expression of PPARα was observed by northern blot analysis, and that, a selective PPARα activator Wy-14643 (100 µM) showed no effect on COX-2 mRNA expression in the U937 cells (data not shown).

Inverse expression of PPARγ and GR by LPS in U937 cells---As described previously, suppressive effect of 15d-PGJ2 on COX-2 expression was milder than that of DEX in the U937 cells. To address this question, we examined expression levels of GR after various
treatments (Fig. 4). LPS increased GR mRNA about two-fold, which shows inverse expression pattern between GR and PPARγ. Moreover, DEX partly restored the suppressive expression of PPARγ by LPS. This inverse expression pattern between GR and PPARγ will be partly explained the milder suppressive effect of 15d-PGJ₂ than that of DEX, and that, suggesting that different roles of GR and PPARγ on COX-2 expression.

**Involvement of PPARγ on COX-2 expression**---Next, we examined effect of 15d-PGJ₂ on the COX-2 promoter activity. The human COX-2 promoter region (-327/+59) contains the NF-κB, NF-IL6 sites and CRE (31). In the differentiated U937 cells expressing GR and PPARγ, the NF-κB site is involved in both LPS-induced expression of the COX-2 gene and its suppression by DEX (13). Similarly, 15d-PGJ₂ suppressed the COX-2 transcription mediated through the NF-κB site in a dose-dependent manner (Fig. 5). On the other hand, in BAEC expressing no detectable levels of GR (16) and PPARγ (Fig. 3A and 3B), C/EBPδ (also known as NF-IL6β) activates COX-2 transcription mainly through CRE whereas the NF-κB and NF-IL6 sites also contribute to the COX-2 expression (11). Transient transfection assay using the COX-2 promoter (-327/+59) showed that 15d-PGJ₂ did not suppress the COX-2 promoter activity in BAEC (Fig. 6), which is consistent with no suppression of COX-2 mRNA by 15d-PGJ₂ (Fig. 2). However, by coexpression of PPARγ, BAEC acquired the suppressive regulation of COX-2 gene by 15d-PGJ₂ but not by DEX, whereas by coexpression of GR (16), acquires more suppressive regulation by DEX than that by 15d-PGJ₂ (Fig. 6), indicating the involvement of PPARγ in regulation of COX-2 expression by 15d-PGJ₂.

**Suppression of PGD₂ Production by NS398**---In the presence of albumin or serum, PGD₂ is metabolized to PGJ₂ and ∆¹²-PGJ₂, natural ligands for PPARγ (22, 23), and that, these PGD₂ metabolites actively transport to cellular nuclei (37). Therefore, there is a possibility that COX-2 expression is self-regulated by PGD₂ metabolites, which is produced in a COX-2 dependent manner. To examine this possibility, we investigated whether U937 cells produce PGD₂ in a COX-2 dependent manner. EIA assay showed that U937 cells produced PGD₂, and a COX-2 selective inhibitor NS398 suppressed this production (Fig. 7). Moreover, PGD₂
synthase mRNA was detected by RT-PCR analysis of the U937 cells (data not shown). These results are also consistent with previous reports in bone-marrow derived macrophages (19) and specialized antigen-presenting cells (38).

**DISCUSSION**

The present study has shown that 15d-PGJ₂ suppressed LPS-induced COX-2 mRNA in macrophage-like differentiated U937 cells, but not in vascular endothelial cells. This difference comes from different expression pattern of PPARγ, that is, much higher expression in the U937 cells (Fig. 3) and acquisition of the 15d-PGJ₂-sensitivity on the COX-2 expression by coexpression of PPARγ into BAEC (Fig. 6). Moreover, LPS down-regulates PPARγ mRNA but up-regulates GR although both suppressed COX-2 promoter activity by interfering with the NF-κB signaling pathway (13) (Fig. 5). On the other hand, U937 cells as well as macrophages (19) produces PGD₂ in a COX-2 dependent manner, and PGD₂ is spontaneously converted to PGJ₂ derivatives by non-enzymatic dehydration (37). Taken together, we propose that PGD₂ metabolites such as 15d-PGJ₂ work an intracellular signaling mediator which retains the low expression level of COX-2 by negative feedback loop mediated through PPARγ in macrophages (Fig. 8). After LPS-treatment, up-regulation of COX-2 was coincident with down-regulation of PPARγ (Fig. 3), which canceled the negative feedback loop. Simultaneously rapid increase of PGE₂ (Fig. 1) was observed and cAMP enhanced the COX-2 transcription by LPS in the U937 cells (13) suggesting that COX-2 expression will be enhanced by a positive feedback loop (20) mediated through PG receptors. In fact, existence of PGE receptor subtypes EP2 and EP4 increasing an intracellular cAMP level were reported in murine macrophage-like cell line, J774.1 (39). This positive feedback loop can be suppressed by DEX since LPS up-regulates GR mRNA and will increase the sensitivity to DEX (Fig. 4). Moreover, the possibility that COX-2 has anti-inflammatory properties at the later phase of a carrageenin-induced pleurisy was recently reported (40), which will be also explained by the negative feedback regulation of COX-2 by PPARγ.
PPARγ is activated by a range of synthetic and naturally occurring substances, including antidiabetic thiazolidinediones, polyunsaturated fatty acids, PGD₂ metabolites, components of oxidized LDL and 12/15-lipoxygenase products (41). Among them, rosiglitazone (BRL49653), the most potent synthetic ligand for PPARγ, did not suppressed LPS-induced expression of COX-2 mRNA in U937 cells (data not shown). Interestingly, in spite of the stronger binding activity of rosiglitazone in vitro, several reports point out the higher biological activity of 15d-PGJ₂ compared to rosiglitazone (42). In this context, 15d-PGJ₂ but not PPARγ agonists such as troglitazone is recently reported as a direct inhibitor of IκB kinase which is responsible for the NF-κB activation (43), suggesting that some biological effects of 15d-PGJ₂ are independent of PPARγ. On the other hand, a somatic PPARγ mutation R288H showed a normal response to synthetic ligands but greatly decreased response to natural ligand 15d-PGJ₂ (44), implying the different responses of PPARγ between different ligands. Moreover, the subtype U937 cells expressing no detectable level of PPARγ shows a significant amount of COX-2 mRNA in the inactivated stage but no induction of COX-2 mRNA by LPS (manuscript in preparation), suggesting the involvement of PPARγ in COX-2 expression. Further studies are necessary to elucidate these different effects between 15d-PGJ₂ and synthetic PPARγ ligands.

TPA-differentiated U937 cells would be assumed as responsive macrophages because of similar expression patterns of COX-2 and TXA₂ synthase mRNAs in casein-elicited peritoneal macrophage (19). However, expression of PPARγ but not PPARα is observed in both undifferentiated and differentiated U937 cells, which is different from the report that PPARγ is induced upon differentiation into macrophages whereas PPARα is already present in undifferentiated monocytes (45). This discrepancy may be attributed to heterogeneity of macrophages (19).

COX-2 expression is regulated not only in cell-type specific but also species-specific manner. In fact, the delayed induction of COX-2 by gonadotropin was reported in bovine granulosa cells but not in the rat cells, however; the induction was observed in both species.
Similarity of nucleotide sequence of the COX-2 promoter region between bovine and human was higher than that between bovine and rat, and that, cis-acting elements for NF-κB, NF-IL6 sites, and CRE are conserved among human, bovine, rat and mouse COX-2 promoter regions. No suppression of 15d-PGJ₂ on the LPS-induced COX-2 mRNA and no detectable level of PPARγ mRNA were observed in HUVEC as well as BAEC. Therefore, there will be not so much difference on the regulation of COX-2 expression at least between human and bovine endothelial cells.

PPARγ and GR mRNAs are inversely regulated by LPS in the U937 cells (Fig. 4) although both 15d-PGJ₂ and DEX suppressed COX-2 promoter activity by interfering with the NF-κB signaling pathway (Fig. 5). Ligands for PPARs and DEX are reported to enhance COX-2 expression in some carcinoma cells (28, 29) and amnion cells (47), respectively. These different effects on COX-2 expression may explain by different regulated expression of PPARs, steroid hormone receptors and C/EBPs. In this context, estrogen-induced production of a PPAR ligand was reported in a PPARγ-expressing tissue, in which induced conversion of PGD₂ to a metabolite was observed (48). Interestingly, a precise transcriptional network among these transcription factors is important for adipocyte differentiation. Therefore, it will be interesting to find each relationship between COX-2 and the transcriptional network in physiological and pathophysiological functions.

Acknowledgements--- We acknowledge the technical assistance of S. Bandoh, T. Sugimoto and Y. Miyamoto, and thank Dr. S. Osada (Kyoto University) for human PPAR expression vectors, Dr. M. Masuda (National Cardiovascular Center) for providing BAEC and Drs. T. Masaki and T. Sasaguri (National Cardiovascular Center) for helpful discussion.
REFERENCES

1. Smith, W. L., Garavito, R. M., and DeWitt, D. L. (1996) *J. Biol. Chem.* **271**, 33157-33160

2. Herschman, H. R. (1996) *Biochim. Biophys. Acta* **1299**, 125-140

3. Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trazakos, J. M., Evans, J. F., and Taketo, M. M. (1996) *Cell* **87**, 803-809

4. Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, H. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., Lee, C. A., and Smithies, O. (1995) *Cell* **83**, 473-482

5. Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N.R., Eng, V. M., Collins, R. J., Czerniak, P. M., Gorry, A. G. and Trzaskos, J. M. (1995) *Nature*, **378**, 406-409

6. Lim, H., Paria, B. C., Das, S. K., Dinchuk, J. E., Langenbach, R., Trzaskos, J. M., and Dey, S. K. (1997) *Cell* **91**, 197-208

7. Vane, J. R., Bakhel, Y. S., and Botting, R. M. (1998) *Annu. Rev. Pharmacol. Toxicol.* **38**, 97-120

8. Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L.S., Van De Putte, L. B., Lipsky, P. E. (1998) *FASEB J.* **12**, 1063-73

9. Sirois, J., Levy, L., Simmons, D. L., and Richards, J. S. (1993) *J. Biol. Chem.* **268**, 12199-12206

10. Inoue, H., Nanayama, T., Hara, S., Yokoyama, C., and Tanabe, T. (1994) *FEBS Lett.* **350**, 51-54

11. Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995) *J. Biol. Chem.* **270**, 24965-24971

12. Yamamoto, K., Arakawa, T. Ueda, N., and Yamamoto, S. (1995) *J. Biol. Chem.* **270**, 31315-31320

13. Inoue, H., and Tanabe, T. (1998) *Biochem. Biophys. Res. Commun.* **244**, 143-148
14. Newton, R., Hart, L. A., Stevens, D. A., Bergmann, M., Donnelly, L. E., Adcock, I.M., and Barnes, P. J. (1998) *Eur. J. Biochem.* **254**, 81-89

15. Kim, Y., Fischer, S. M. (1998) *J. Biol. Chem.* **273**, 27686-27694

16. Inoue, H., Umesono, K., Nishimori, T., Hirata, Y., and Tanabe, T. (1999) *Biochem. Biophys. Res. Commun.* **254**, 292-298

17. Matsuura, H., Sakaue, M., Subbaramaiah, K., Kamitani, H., Eling, T. E., Dannenberg, A. J., Tanabe, T., Inoue, H., Arata, J., and Jetten, A. M. (1999) *J. Biol. Chem.* **274**, 29138-29148

18. Bienkowski, M. J., Petro, M. A., and Robinson, L. J. (1989) *J. Biol. Chem.* **264**, 6536-6541

19. Kuwamoto, S., Inoue, H., Tone, Y., Izumi Y., and Tanabe, T. (1997) *FEBS lett.* **409**, 242-246

20. Fournier, T., Fadok, V., and Henson, P. M. (1997) *J. Biol. Chem.* **272**, 31065-31072

21. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. (1995) *Cell* **83**, 835-839

22. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) *Cell* **83**, 803-812

23. Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) *Cell* **83**, 813-819

24. Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., and Glass, C. K. (1998) *Nature* **391**, 79-82

25. Jiang C., Ting, A. T., and Seed, B. (1998) *Nature* **391**, 82-86

26. Nagy, L., Tontonoz, P., Alvarez, J.G., Chen, H., and Evans, R. M. (1998) *Cell* **93**, 229-240

27. Tontonoz, P., Nagy L., Alvarez, J. G., Thomazy, V. A., and Evans, R. M. (1998) *Cell* **93**, 241-252

28. Meade, E. A., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1999) *J. Biol.*
Chem. **274**, 8328-8334

29. Chinery, R., Coffey, R. J., Graves-Deal R., Kirkland, S. C., Sanchez, S. C., Zackert W. E., Oates, J. A., and Morrow, J. D. (1999) *Cancer Res.* **59**, 2739-2746

30. Callejas, N. A., Castrillo, A., Bosca, L., Martin-Sanz, P. (1999) *J. Pharmacol. Exp. Ther.* **288**, 1235-1241

31. Kosaka, T., Miyate, A., Ihara, H., Haru, S., Sugimoto, T., Takeda, O., Takahashi, E., and Tanabe, T. (1994) *Eur. J. Biochem.* **221**, 889-897

32. Giguère, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans R. M. (1986) *Cell* **46**, 645-652

33. Greene M. E., Blumberg, B., McBride, O. W., Yi, H. F., Kronquist, K., Kwan, K., Hsieh, L., Greene, G., and Nimer S. D. (1995) *Gene Express.* **4**, 281-299

34. Sundvold, H., Brzozowska, A., and Lien, S. (1997) Biochem. Biophys. Res. Commun. **239**, 857-861

35. Kanaoka Y, Ago, H., Inagaki, E., Nanayama, T., Miyano, M., Kikuno, R., Fujii, Y., Eguchi, N., Toh, H., Urade, Y., and Hayaishi, O. (1997) *Cell* **90**, 1085-1095

36. Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P., Delerive, P., Fadel, A., Chinetti, G., Fruchart, J.-C., Najib, J., Maclouf, J., and Tedgui, A. (1998) *Nature* **393**, 790-793

37. Fukushima, M. (1992) *Prostaglandins Leukot. Essent. Fatty Acids* **47**, 1-12

38. Urade, Y., Ujihara, M., Horiguchi, Y., Ikai, K., and Hayaishi, O. (1989) *J. Immunol.* **143**, 2982-2989

39. Katsuyama, M, Ikegami, R., Karahashi, H., Amano, F., Sugimoto, Y., and Ichikawa, A. (1998) *Biochem. Biophys. Res. Commun.* **251**, 727-731

40. Gilroy, D. W., Colville-Nash, P. R., Chivers, W. J., Paul-Clark, M. J., and Willoughby, D. A. (1999) *Nature Med.* **5**, 698-701

41. Huang, J. T., Welch, J. S., Ricotte, M., Binder, C. J., Willson, T. M., Kelly, C., Witztum, J. L., Funk, C. D., Conrad, D., and Glass, C. K. (1999) *Nature* **400**, 378-382

13
42. Spiegelman, B. M. (1998) *Cell* **93**, 153-155

43. Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., and Santoro, M. G. (2000) *Nature*, **403**, 103-118

44. Sarraf, P., Mueller, E., Smith, W. M., Wright, H. M., Kum, J. B., Aaltonen, L. A., de la Chapelle, A., Spiegelman, B. M., Eng, C. (1999) *Mol. Cell* **3**, 799-804

45. Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J., and Staels, B. (1998) *J. Biol. Chem.* **273**, 25573-25580

46. Liu, J., Antaya, M., Boerboom, D., Lussier, J. G., Silversides, D. W., and Sirois, J. (1999) *J. Biol. Chem.* **274**, 35037-35045

47. Zakar, T., Hirst, J. J., Mijovic, J. E., and Olson, D. M. (1995) *Endocrinology* **136**, 1610-1619

48. Ma, H., Sprecher, H. W., and Kolattukudy, P. E. (1998) *J. Biol. Chem.* **273**, 30131-30138
FOOTNOTES

*This work was supported in part by grants from the Ministry of Health, and Welfare and the Ministry of Education, Science, Culture and Sports of Japan, the Japan Cardiovascular Research Foundation, the Yamanouchi Foundation for Research on Metabolic Disorders. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶To whom correspondence should be addressed: Tel.: +81-6-6833-5012 (ext. 2588); Fax: +81-6-6872-8092; E-mail: inoue@ri.ncvc.go.jp.

§Deceased. This paper is dedicated to the memory of Kazuhiko Umesono, our friend and esteemed collaborator.

The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin(s); LPS, lipopolysaccharide; DEX, dexamethasone; BAEC, bovine arterial endothelial cell(s); GR, glucocorticoid receptor; TX, thromboxane; PPARγ, peroxisome proliferator-activated receptor-γ; 15d-PGJ2, 15-deoxy-Δ12,14 PGJ2; NF-IL6, nuclear factor for interleukin-6 expression; NF-κB, nuclear factor κB; TPA, 12-O-tetradecanoylphorbol-13-acetate; bp, base pair(s); PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells.
LEGENDS TO FIGURES

Fig. 1. Inhibition of COX-2 gene expression and production of PGE$_2$ by 15d-PGJ$_2$ in differentiated U937 cells. A, macrophage-like differentiated U937 cells were treated for 5 h with LPS in the presence or absence of the indicated concentrations of 15d-PGJ$_2$. Total RNA (10 µg) was isolated from the U937 cells, and subjected to Northern blot analysis using specific COX-2 and GAPDH cDNA probes. B, time course of COX-2 mRNA expression in the U937 cells treated with LPS in the presence or absence of 10 µM 15d-PGJ$_2$. Relative amount of COX-2 mRNA was measured by an image-analyzer after normalization with that of GAPDH. Values represent the means ± standard deviations of three separate dishes. C, PGE$_2$ in the culture medium was measured by enzyme immunoassays after treatment of the cells with LPS (10 µg/ml) and/or DEX (100 nM) or 15d-PGJ$_2$ (10 µM) for 12 h. Values represent the means ± standard deviations of three separate wells. D, cells treated with reagents described in C were collected, and proteins were examined by Western blot analysis using antisera specific for COX-2. Similar results were obtained in two additional experiments.

Fig. 2. Different effect of 15d-PGJ$_2$ between differentiated U937 cells and BAEC. Macrophage-like differentiated U937 cells and BAEC were treated with LPS for 5 h in the presence or absence of 10 µM 15d-PGJ$_2$. Isolated total RNA (10 µg) was examined by Northern blot analysis for expression of COX-2 mRNA. Relative amount of COX-2 mRNA was measured by an image-analyzer after normalization by that of GAPDH, and indicated as 100% without 15d-PGJ$_2$ since expression of COX-2 mRNA was very low in both cells without the LPS-treatment. Results represent the mean ± standard deviations of three separate dishes. GAPDH expression level in the U937 cells is higher than that in BAEC although ethidium bromide-staining intensities of 28S RNA were equal between them as measured by an imaging analyzer FLA2000. Similar result was also obtained when using a bovine COX-2 cDNA probe instead of the human probe.
**Fig. 3. Different expression patterns of PPARγ mRNA between U937 cells and BAEC.**

Macrophage-like differentiated U937 cells and BAEC were treated with LPS for 5 h in the presence or absence of 10 μM 15d-PGJ2. **A,** isolated total RNA (10 μg) was examined by Northern blot analysis using radiolabeled probes for COX-2, PPARγ, COX-1 and GAPDH, respectively after stripping each probe in this order. **B,** RNA samples (1 μg each) extracted from U937 cells and BAEC were subjected to RT-PCR to confirm the relative expression levels of PPARγ, as described under “Materials and methods.” **C,** U937 cells were treated with LPS (10 μg/ml), and at the times indicated total RNA was isolated and examined by Northern blot analysis using radiolabeled probes for PPARγ and GAPDH. Relative amount of PPARγ mRNA was measured by an image-analyzer after normalization by that of GAPDH, and indicated as 100% before treatment with LPS. Values represent the means ± standard deviations of three separate dishes. This down-regulation of PPARγ mRNA was observed by treatment with LPS but not with 15d-PGJ2 (10 μM) alone. Similar results were obtained in two additional experiments.

**Fig. 4. Inverse expression patterns of PPARγ and GR in the differentiated U937.**

Macrophage-like differentiated U937 cells were treated with the indicated reagents for 5 h. Isolated total RNA (10 μg) was examined by Northern blot analysis using radiolabeled probes for COX-2, PPARγ, GR and GAPDH, respectively after stripping each probe in this order. Relative amount of PPARγ (A), GR (B) and COX-2 (C) mRNAs were measured by an image-analyzer after normalization by that of GAPDH. Values represent the means ± standard deviations of three separate dishes. Similar results were obtained in two additional experiments.
Fig. 5. Suppressive effect of 15d-PGJ₂ on COX-2 transcription in the differentiated U937 cells. U937 cells stably transfected with a -327/+59 COX-2 reporter gene phPES2(CRM, ILM) consisting of only NF-κB site were differentiated into the macrophage-like cells as described under “Materials and Methods.” The cells were treated with LPS (10 μg/ml) and the indicated concentrations of 15d-PGJ₂ and analyzed for luciferase activity 5 h later. Results are represented as fold increases in luciferase activity per microgram protein relative to the control. Values represent the means ± standard deviations of three separate wells. Similar results were obtained in two additional experiments.

Fig. 6. BAEC transfected with a PPARγ-expression vector acquire the ability to suppress COX-2 transcription after treatment with 15d-PGJ₂ but not with DEX. A, BAEC were transfected with a COX-2 reporter vector phPES2(-327/+59) along with either human PPARγ expression vector (pCMX-hPPARγ1) or human GR expression vector (pRShGRα) and with pSV-βgal as an internal control for the transfection. Forty-eight hours after transfection, the cells were incubated for 5 h with no stimulant or 1 μg/ml LPS in the presence or absence of 10 μM 15d-PGJ₂ or 100 nM DEX. Values represent the means ± standard deviations of three separate wells. Similar results were obtained in two additional experiments.
**Fig. 7. Production of PGD<sub>2</sub> through COX-2 pathway in U937 cells.** PGD<sub>2</sub> in culture medium was measured by enzyme immunoassay after cells were treated with LPS (10 µg/ml) and/or NS-398 (1 µM) for 16 h and then stimulated with the indicated concentrations of arachidonic acid for 1 h. Values represent the means ± standard deviations of three separate wells. Similar results were obtained in two additional experiments. Concentration of PGD<sub>2</sub> was not changed by addition of 10 µM arachidonic acid alone.

**Fig. 8. Control of COX-2 expression by positive and negative feedback loops mediated through PPARγ and PG receptors in macrophages.** In the inactivated stage, macrophages produce PGD<sub>2</sub> metabolites via cyclooxygenase pathway using COX-2. PGD<sub>2</sub> metabolites inhibit COX-2 transcription partly mediated through PPARγ by interfering with the NF-κB pathway. Due to suppression of COX-2 expression, production of PGD<sub>2</sub> decreases. A low level COX-2 expression is retained in this negative feedback loop. In the activated stage by stimulation with endotoxin LPS, up-regulation of COX-2 is coincident with down-regulation of PPARγ, which removes the negative feedback loop. A large amount of PGE<sub>2</sub> by the induction of COX-2 expression activates PGE receptor subtypes EP2 and EP4, which increase an intracellular cAMP level and enhance COX-2 transcription. This positive feedback loop can be removed by DEX, which activates GR and suppresses COX-2 expression, and may return to the inactivated stage. In fact, LPS up-regulates GR mRNA.
Inoue et al., Figure 1

A

- - 0.1 1 10 15d-PGJ₂ (µM)
- + + + + LPS (10 µg/ml)

COX-2
GAPDH

B

Relative Amount of COX-2 mRNA

LPS  LPS+15d-PGJ₂ (h)
0 1 2 3 4

COX-2
GAPDH

C

Production of PGE₂ (ng/ml)

LPS  DEX  15d-PGJ₂
- + + + - - +

D

COX-2

Inoue et al., Figure 1
Inoue et al. Fig. 2
COX-1
GAPDH
PPARγ
BAECU937
LPS

15d-PGJ2

PPARγ
RT-PCR
U937 BAEC
GAPDH
COX-1
PPARγ
LPS (h)

Relative Amount of PPARγ mRNA

0 0 25 50 75 100
(%)

0 1 2 3
LPS (h)

Inoue et al. Fig. 3
LPS (10 µg/ml) − + + +
DEX (100 nM) − − + −
15d-PGJ$_2$ (100 µM) − − − +

**GR**

**GAPDH**

---

**Inoue et al. Fig. 4**
Inoue et al. Fig. 5

NF-κB

phPES2 (CRM, ILM)

Luciferase

Relative Luciferase Activity

LPS (10 µg/ml)

- + + + + + +

15d-PGJ2 (µM)

- - 0.6 1.3 2.5 5 10
Inoue et al. Fig. 6
Figure 7: Graph showing the effects of LPS, NS398, and arachidonic acid on the production of PGD2 (pg/ml). The graph indicates a significant increase in PGD2 production with the addition of arachidonic acid at 10 µM, particularly in the presence of LPS and NS398.

Inoue et al. Fig. 7
COX-2 expression is regulated by EP2/4, NF-κB, PPARγ, PGE2, and PGD2 metabolites. In the inactivated stage (Negative Feedback Loop), COX-2 expression is inhibited by PPARγ and NF-κB. In the activated stage (Positive Feedback Loop), COX-2 expression is activated by PGE2 and EP2/4, leading to increased production of PGE2 and PGD2 metabolites. Inoue et al., Figure 8.
