Aliment Pharmacol Ther 2003; 18 (Suppl. 1): 113–118.

Effect of rebamipide on prostaglandin receptors-mediated increase of inflammatory cytokine production by macrophages

H. BAMBA*, S. OTA†, A. KATO*, H. MIYATANI*, C. KAWAMOTO*, Y. YOSHIDA* & K. FUJIWARA†
First Department of Internal Medicine, Saitama Medical Center, Saitama Medical School, Saitama; and †Third Department of Internal Medicine, Saitama Medical School, Saitama, Japan

SUMMARY

Background: Rebamipide (Reb) is an anti-ulcer drug, and has unique properties such as anti-inflammatory action. We previously reported that prostaglandins (PGs) dramatically increased vascular endothelial growth factor (VEGF), a known angiogenic factor and a vascular permeable factor, by activated macrophages through specific PGE receptor and peroxisome proliferator-activated receptor γ (PPARγ, a nuclear receptor of PG) mediated process. Effects of PGs on the production of other cytokines such as interleukin (IL)-6 and IL-8 have been controversial.

Aim: To clarify the anti-inflammatory roles of Reb, we examined the effect of Reb on PGE_1- and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (a potent PPARγ ligand, 15d-PGJ_2) -induced increase of VEGF production by macrophages. Additionally, effects of these PGs on the production of IL-6 and IL-8, and modulation of these actions by Reb were studied.

Methods: Phorbol 12-myristate 13-acetate-differentiated U937 cells were used as a human macrophage model (H-Mac). VEGF, IL-6, IL-8 and cAMP were measured by EIA.

Results: Reb suppressed PGE_1-, but not 15d-PGJ_2-, induced increase of VEGF production partially through decrease of cAMP formation. Reb suppressed PGE_1 -, but not 15d-PGJ_2-, induced increase of IL-6 and IL-8 production.

Conclusion: Reb suppresses membrane, but not nuclear PG receptors mediated increase of inflammatory cytokine production, which may be involved in anti-ulcer action of this drug.

INTRODUCTION

Rebamipide (Reb) is an anti-ulcer drug, which does not suppress acid secretion. Mechanisms involved in the action of this drug has not been clearly understood. Possible therapeutic effects of Reb have been attributed to induce endogenous prostaglandins (PGs), to scavenge free radicals, and to increase gastric mucus.[1] Recently, it has been demonstrated that this drug has unique properties such as anti-inflammatory action.[2]

Correspondence to: Dr H. Bamba, 1st Department of Internal Medicine, Saitama Medical Center, Saitama Medical School, 1981 Tsujido, Kamoda, Kawagoe, Saitama 350-8550, Japan.
E-mail: bam@saitama-med.ac.jp

On the other hand, PGs have been shown to modulate gastric functions such as acid suppression, cytoprotection, mucus secretion, bicarbonate secretion, blood flow, and motility. Although PGs are also well-known important mediators of inflammation, effect of PGs on the production of other inflammatory mediators has not been fully understood. Recent studies have shown that physiological effects of PGs appear to be mediated by two distinct pathways involving G protein-coupled cell surface receptors (membrane receptors) and nuclear receptor.[3–5] In most PGs, physiological effects are mediated by membrane receptors.[3] However, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), a metabolite of PGD_2, shows its physiological effects via nuclear receptor, peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is a

recently identified member of the nuclear receptor superfamily of ligand-dependent transcription factors.[4, 5] When a ligand activates this receptor, it forms a heterodimer with the retinoid X receptor-α and binds to specific peroxisome proliferator response element (PPRE) located within the promoters of target genes, thus directly up- or down-regulating their expressions.[6, 7]

Inflammatory process is mediated by guranulocytes, eosinophils, T cells, and monocytes/macrophages. We focused on macrophages, which have been found to play crucial roles in the inflammatory response in many organs including stomach. Macrophages are a potential source of various inflammatory cytokines such as PG, vascular endothelial growth factor (VEGF), interleukins (IL). VEGF is a potent angiogenic, vascular permeability-enhancing cytokine with overexpression in inflammation.[8] We previously reported that PGs dramatically increased VEGF and IL-1β production by activated macrophages through specific PGE receptor and PPAR γ mediated process.[9] Therefore, it is of interest to study the effect of Reb on this process. The effects of PGs on the production of other cytokines have been controversial. While PGE_2 has been reported to up-regulate production of IL-6, another inflammatory cytokine,[10] 15d-PGJ_2 negatively regulated inflammatory cytokine production by activated macrophages.[11, 12]

In the present study, we studied the effects of PGs on the production of these cytokines through membrane and nuclear PG receptors and the modulation of these steps by Reb, to clarify the anti-ulcer action of this drug.

MATERIALS AND METHODS

Chemicals

Reb was kindly donated by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). PGE_1 and 15d-PGJ_2 were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). 8-bromo-cAMP, cholera toxin, phorbol 12-myristate 13-acetate (PMA) and 3-isobutyl-1-methyl xanthine (IBMX) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Cell cultures

U937 DE-4 cells, a subclone of U937, were obtained from RIKEN Cell Bank (Tsukuba, Japan). Cell lines were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD, USA) and incubated at 37 °C in a 5% CO_2 humidified atmosphere.

Induction of differentiation

U937 DE-4 cells were harvested at the logarithmic phase of growth, adjusted to 2×10^6/mL in the presence of 10^{-7} M PMA, and then placed in 24-well culture plates at a density of 10^6 cells/well for VEGF measurements, 35 mm culture dishes at 4×10^6/dish for cAMP measurements. The cells were incubated for 3 days to differentiate into adherent macrophages. This PMA-differentiated U937 was used as a human macrophage model (H-Mac). The cells were then quiescenced for 24 h by incubation in fresh RPMI complete medium before experiments.

Determination of cytokine concentration in conditioned medium

After preparation of H-Mac cells, the culture medium was aspirated. RPMI 1640 containing 1% FBS alone or with test reagents was added to each well. After incubation for 12 h, the conditioned medium was collected and stored at −20 °C. The concentration of human VEGF, IL-6, and IL-8 in medium was determined by enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R & D Systems, Inc., Minneapolis, MN, USA).

Determination of cAMP

H-Mac cells were prepared as described above. This experiment was performed in the presence of 1 mM IBMX. After incubation for 1 h with RPMI 1640 medium containing 1% FBS either with PGE_1, Reb alone, or the combination of both, the medium was decanted and cAMP was extracted from the cells by adding 0.5 mL of ice-cold 5% (v/v) trichloroacetic acid to each culture. After 10 min at 4 °C, the cells were sonicated and centrifuged at $1000 \times g$ for 15 min. The supernatant was extracted twice with 10 × volumes of water-saturated diethyl ether. The cAMP concentration was measured using a cAMP EIA kit (Cayman).

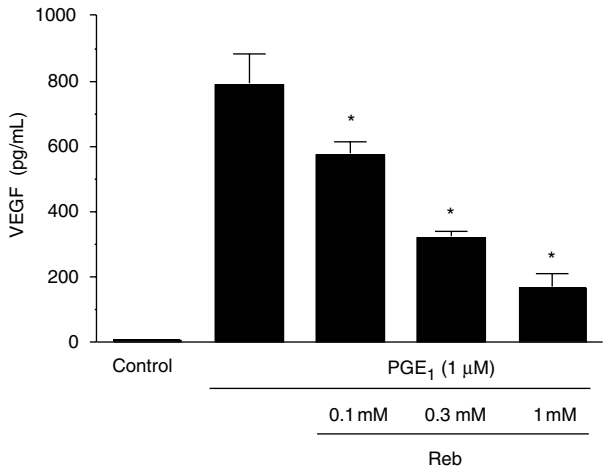

Figure 1. Reb suppressed PGE_1-induced increase of VEGF production by H-Mac in a dose-dependent fashion. Cells were incubated with or without the indicated chemicals for 12 h. VEGF levels were assessed by ELISA. Values are mean ± S.E. of three determinations. *$P < 0.05$ vs. PGE_1 alone.

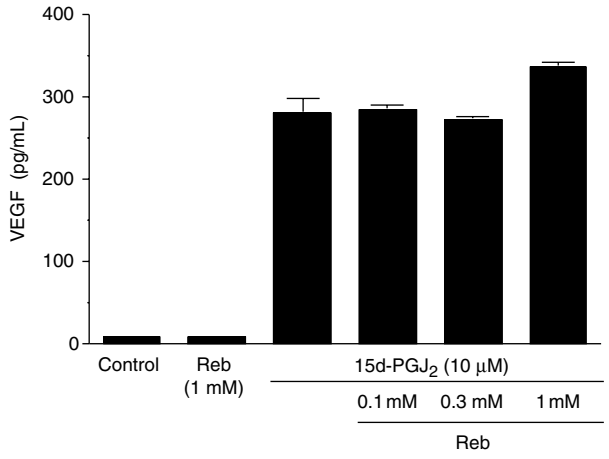

Figure 2. Reb did not have any effects on 15d-PGJ_2 induced increase of VEGF production by H-Mac. Cells were incubated with or without the indicated chemicals for 12 h. VEGF levels were assessed by ELISA. Values are mean ± S.E. of three determinations.

Statistical analysis

Values are presented as mean ± S.E. Comparisons between two groups were performed using Student's *t* -test for grouped or paired data when appropriate. Comparisons among several groups were performed by analysis of variance, followed by Dunnett's test, when appropriate. For all analyses, findings were considered significant when $P < 0.05$.

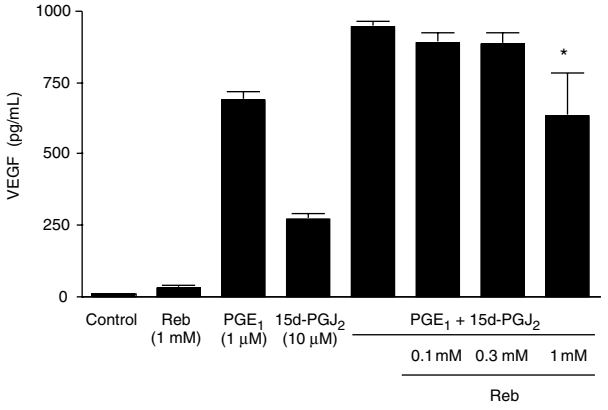

Figure 3. Reb partially blocked the additive increase of VEGF production by combination of PGE_1 and 15d-PGJ_2 in H-Mac. Cells were incubated with or without the indicated chemicals for 12 h. VEGF levels were assessed by ELISA. Values are means ± S.E. of three determinations, *$P < 0.005$ vs. PGE_1 and 15d-PGJ_2.

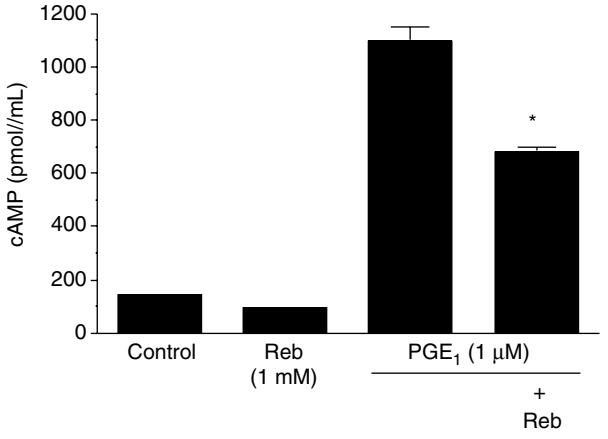

Figure 4. Reb blocked PGE_1-induced increase of VEGF production partially through suppression of endogenous cAMP formation. H-Mac cells were incubated with the indicated chemicals for 1 h in the presence of IBMX. cAMP was determined as described under 'Materials and Methods'. Values are mean ± S.E. of three determinations, *$P < 0.005$ vs. PGE_1 alone.

RESULTS

Effect of Reb on PGs- induced increase of VEGF production by H-Mac

We previously reported that 1 μM of PGE_1 or 10 μM 15d-PGJ_2 significantly increased VEGF production. We also demonstrated that the combination of these two agents additively increased VEGF production. We first examined the effect of Reb on these actions. As shown in Figure 1, Reb suppressed PGE_1-induced increase of

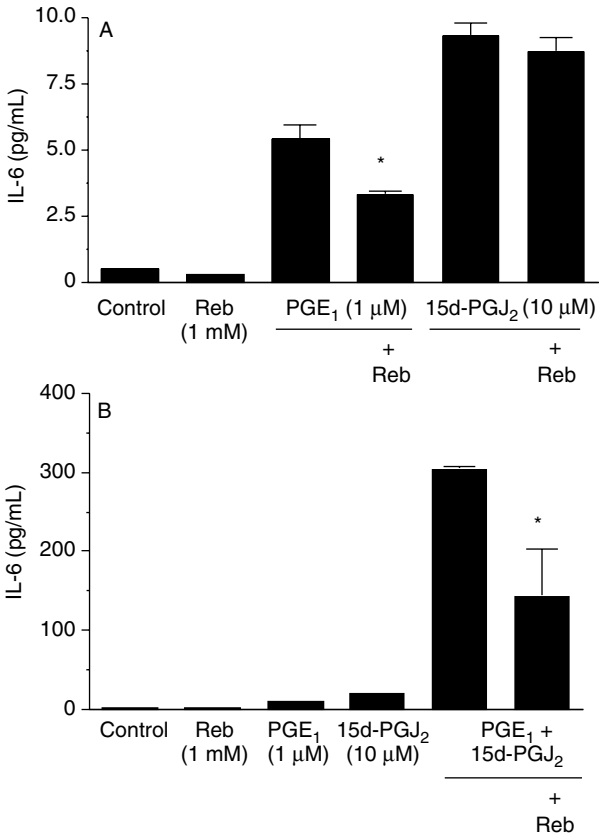

Figure 5. Reb suppressed membrane receptor-, but not nuclear receptor-, mediated increase of IL-6 production by H-Mac. Cells were incubated with or without the indicated chemicals for 12 h. IL-6 levels were evaluated by ELISA. (A) Reb suppressed PGE_1-, but not 15d-PGJ_2-, induced increase of IL-6 production by H-Mac. Values are means ± S.E. of three determinations, $^*P < 0.005$ vs. PGE_1 alone. (B) Reb suppressed the increase of IL-6 production by combination of PGE_1 and 15d-PGJ_2 in H-Mac. Values are mean ± S.E. of three determinations, $^*P < 0.005$ vs. PGE_1 and 15d-PGJ_2.

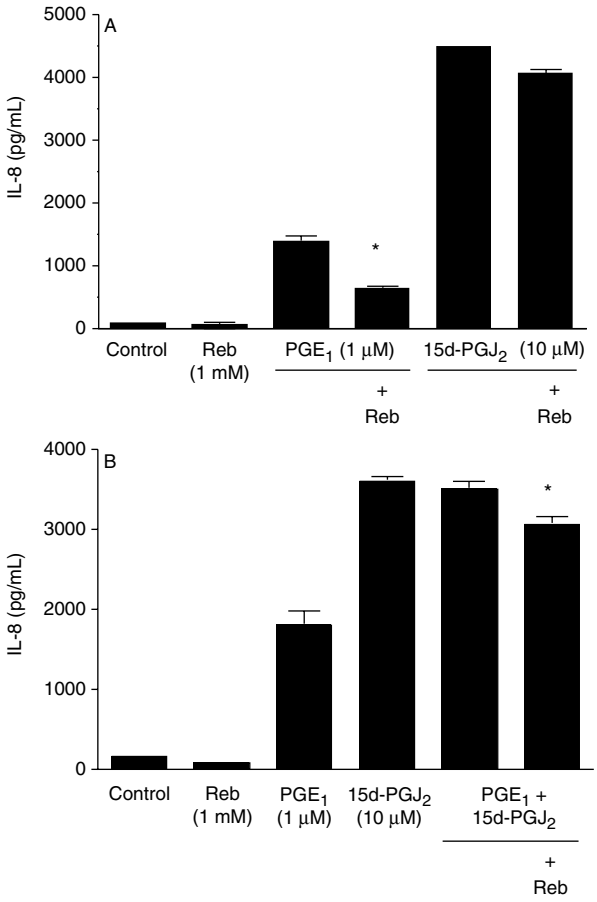

Figure 6. Reb suppressed membrane receptor-, but not nuclear receptor-, mediated increase of IL-8 production by H-Mac. Cells were incubated with or without the indicated chemicals for 12 h. IL-8 levels were evaluated by ELISA. (A) Reb suppressed PGE_1-, but not 15d-PGJ_2-, induced increase of IL-8 production by H-Mac. Values are means ± S.E. of three determinations, $^*P < 0.005$ vs. PGE_1 alone. (B) Reb suppressed the increase of IL-8 production by combination of PGE_1 and 15d-PGJ_2 in H-Mac. Values are mean ± S.E. of three determinations, $^*P < 0.005$ vs. PGE_1 and 15d-PGJ_2.

VEGF production in a dose-dependent manner. In contrast, Reb did not have any effect on 15d-PGJ_2-induced increase of VEGF production (Figure 2). Reb also partially blocked the additive increase of VEGF production by combination of these PGs (Figure 3). These results suggest that Reb may suppress membrane PG receptor mediated process.

Signal transduction system in PGs-induced increase of VEGF production and modulation of this step by Reb

EP_2 and EP_4 membrane PGE receptors are coupled to the adenylate cyclase, inducing the increase of cAMP.[3] We next examined the effects of PGE_1 and Reb on cAMP formation. While PGE_1 significantly increased intracellular cAMP formation, Reb did not have any effects on basal level of cAMP formation. However, Reb partially blocked PGE_1-induced increase of cAMP formation. These results suggest that Reb blocks PGE_1-induced increase of VEGF production partially through suppression of cAMP formation (Figure 4).

Effects of PGE_1 and 15d-PGJ_2 on the production of IL-6 and IL-8, and modulation of these actions by Reb

We further examined the effects of PGs on production of other key inflammatory cytokines, such as IL-6 and IL-8. PGE_1 significantly induced both IL-6 and IL-8

production by H-Mac. 15d-PGJ_2 also significantly increased IL-6 production and dramatically stimulated IL-8 production by H-Mac. Reb suppressed PGE_1-induced increase of both IL-6 and IL-8 production, although Reb did not modulate 15d-PGJ_2-induced increase of IL-6 or IL-8 production (Figures 5A and 6A). Combination of PGE_1 and 15d-PGJ_2 additively increased both IL-6 and IL-8 production. Reb also significantly suppressed the additive increase of IL-6 and IL-8 production by combination of PGE_1 and 15d-PGJ_2 (Figures 5B and 6B). These findings support that Reb may suppress membrane receptors, but not nuclear PG receptors-mediated increase of cytokine production.

DISCUSSION

In the present study, we demonstrated Reb suppressed PGE_1- (a ligand for PGE receptor), but not 15d-PGJ_2- (a ligand for PPARγ), induced increase of inflammatory cytokine including VEGF, IL-6 and IL-8 production using a human macrophages model.

We previously reported that these PGs dramatically increased VEGF production by activated macrophages through membrane receptor such as PGE receptor, and nuclear receptor such as PPARγ mediated process. mRNAs of both EP_2 and EP_4 PGE receptors and PPARγ were expressed in H-Mac. These PGE receptors induce adenylate cyclase-mediated increases in cAMP, an important second messenger in cellular signal transduction.[3] PGE_1 stimulated cAMP formation, and exogenous cAMP and activation of adenylate cyclase by cholera toxin had effects similar to PGE_1 on VEGF production. In contrast, 15d-PGJ_2 did not modulate cAMP formation, whereas 15d-PGJ_2 also dramatically increased VEGF production. The combination of PGE_1 and 15d-PGJ_2 additively increased VEGF production. However, the combination of PGE_1 and 15d-PGJ_2 significantly increased cAMP formation to an extent similar to PGE_1 alone. Therefore, PGs dramatically increased VEGF production by activated macrophages through specific PGE receptor and PPARγ mediated processes.[9] As shown in Figures 1 and 2, Reb suppressed PGE_1-, but not 15d-PGJ_2 -, induced increase of VEGF production. Reb also partially blocked the additive increase of VEGF production by combination of PGE_1 and 15d-PGJ_2 in H-Mac (Figure 3). In addition, Reb partially blocked PGE_1-induced increase of cAMP formation, although Reb did not have any effects on basal level of cAMP formation (Figure 4). Therefore, Reb blocked PGE_1-induced increase of VEGF production partially through suppression of endogenous cAMP formation. However, downstream of signal transduction system might also be involved in the action of Reb, and further studies are required to identify the targets of Reb in this action.

Unexpectedly, PGE_1 and 15d-PGJ_2 drastically increased IL-6 and IL-8 production (Figures 5 and 6). PGE_2 has been reported to up-regulate production of IL-6 which is compatible with our results.[10] Concerning

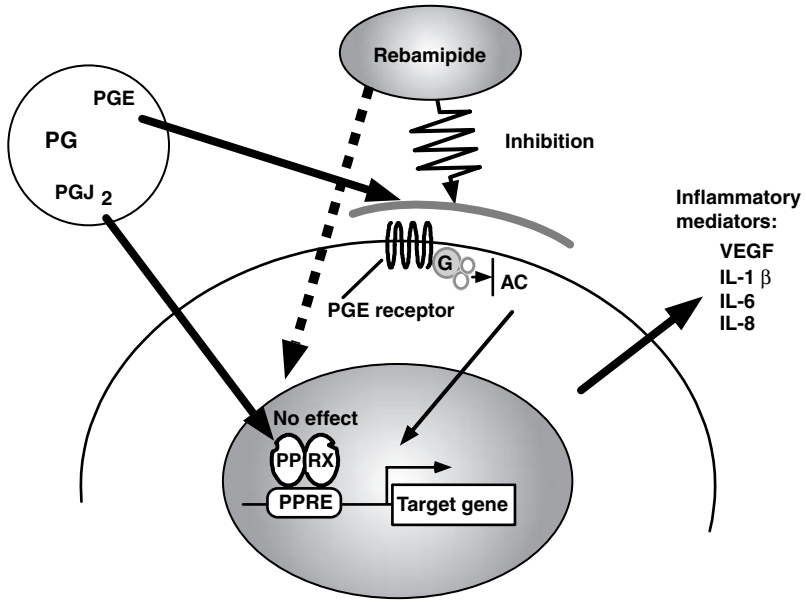

Figure 7. Action of Reb. PGs-induce increase of inflammatory mediators. Reb suppress membrane, but not nuclear PG receptor (PPARγ), mediated increase of inflammatory cytokines. PG, prostaglandin; PPRE, peroxisome proliferator response element; PP, peroxisome proliferator-activated receptorγ; RX, retinoid X receptor-α; G, G protein; AC, adenylate cyclase; VEGF, vascular endothelial growth factor; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8.

the PGJ-series, conflicting results have been reported.[11–14] However, in the present study, 15d-PGJ_2 clearly increased the production of these cytokines. Reb suppressed membrane receptors-, but not possibly PPARγ-, mediated increase of IL-6 and IL-8 production.

In conclusion, Reb suppressed PGE_1, but not 15d-PGJ_2-, induced increase of inflammatory cytokine including VEGF, IL-6 and IL-8 production. Therefore, Reb might show its anti-inflammatory action through suppression of the function of membrane PGE receptors mediated increase of inflammatory cytokine production by activated macrophages (Figure 7).

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the Japanese Ministry of Education, Science and Culture (KAKEN 13670553 to S.O).

REFERENCES

1 Arakawa T, Kobayashi K, Yoshikawa T, Tarnawski A. Rebamipide. Overview of its mechanisms of action and efficacy in mucosal protection and ulcer healing. Dig Dis Sci 1998; 43(Suppl.): 5S–13S.
2 Aihara M, Imagawa K, Funakoshi Y, Ohmoto Y, Kikuchi M. Effect of rebamipide on the several cytokines by human peripheral blood mononuclear cells. Dig Dis Sci 1998; 43: 160S–166S.
3 Coleman RA, Smith WL, Narumiya S. International union of pharmacology classification of prostanoid receptors, properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994; 46: 205–27.
4 Forman BM, Tontonoz P, Chen J, *et al.* 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell 1995; 83: 803–12.
5 Kliewer SA, Lenhard JM, Willson TM, *et al.* Prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell 1995; 83: 813–9.
6 Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. Nature (Lond) 1992; 358: 771–4.
7 Palmer CNA, Hsu MH, Griffin KJ, Johnson EF. Novel sequence determinants in peroxisome proliferator signaling. J Biol Chem 1995; 270: 16114–21.
8 Connolly DT, Olander JV, Heuvelman D, *et al.* Human vascular permeability factor. Isolation from U937 cells. J Biol Chem 1989; 264: 20017–24.
9 Bamba H, Ota S, Kato A, Kawamoto C, Fujiwara K. Prostaglandins up-regulate vascular endothelial growth factor production through distinct pathways in differentiated U937 cells. Biochem Biophys Res Commun 2000; 273: 485–91.
10 Williams JA, Shacter E. Regulation of macrophage cytokine production by prostaglandin E_2. Distinct roles of cyclooxygenase-1 and -2. J Biol Chem 1997; 272: 25693–9.
11 Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. Nature (Lond) 1998; 391: 79–82.
12 Jiang C, Ting AT, Seed B. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature (Lond) 1998; 391: 82–6.
13 Thieringer R, Fenyk-Melody JE, Le Grand CB, *et al.* Activation of peroxisome proliferator-activated γ does not inhibit IL-6 or TNF-α responses of macrophages to lipopolysaccharide *in vitro* or *in vivo*. J Immuol 2000; 641: 1045–54.
14 Fu Y, Luo N, Lopes-Viirella MF. Upregulation of interleukin-8 expression by prostaglandin D2 metabolite 15-deoxy-delta 12,14 prostaglandin J2 (15d-PGJ2) in human THP-1 macrophages. Atherosclerosis 2002; 160: 11–20.