Peroxisome Proliferator-activated Receptor \(\gamma\) Ligands Regulate Myeloperoxidase Expression in Macrophages by an Estrogen-dependent Mechanism Involving the \(–463GA\) Promoter Polymorphism* 

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Alan P. Kumar, F. Javier Piedrafita, and Wanda F. Reynolds‡

From the Sidney Kimmel Cancer Center, San Diego, California 92121

A functional myeloperoxidase (MPO) promoter polymorphism, \(–463GA\), has been associated with incidence or severity of inflammatory diseases, including atherosclerosis and Alzheimer’s disease, and some cancers. The polymorphism is within an Alu element encoding four hexamer repeats recognized by nuclear receptors (AluRRE). Here we show that peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) agonists strongly regulate MPO gene expression through the AluRRE. Opposite effects were observed in granulocyte/macrophage colony-stimulating factor (GMCSF)- versus macrophage colony-stimulating factor (MCSF)-derived macrophages (M\(\phi\)): Expression was markedly up-regulated (mean 26-fold) in MCSCF-M\(\phi\) and down-regulated (34-fold) in GMCSF-M\(\phi\). This was observed with rosiglitazone and three other PPAR\(\gamma\) ligands of the thiazolidinedione class, as well as the natural prostaglandin metabolite 15-deoxy-\(\Delta^{2,14}\) prostaglandin J\(2\). The selective PPAR\(\gamma\) antagonist, GW9662, blocked both the positive and negative effects on MPO expression. Gel retardation assays showed PPAR\(\gamma\) bound hexamers 3/4, and estrogen receptor-\(\alpha\) bound hexamers 1/2, with \(–463A\) in hexamer 1 enhancing binding. Estrogen blocked PPAR\(\gamma\) effects on MPO expression, especially for the A allele. Charcoal filtration of fetal calf serum eliminated the block of PPAR\(\gamma\), whereas replenishing the medium with 17\(\beta\)-estradiol reinstated the block. These findings suggest a model in which estrogen receptor binds the AluRRE, preventing PPAR\(\gamma\) binding to the adjacent site. The positive and negative regulation by PPAR\(\gamma\) ligands, and the block by estrogren, was also observed in transgenic mice expressing the G and A alleles. The mouse MPO gene, which lacks the primate-specific AluRRE, was unresponsive to PPAR\(\gamma\) ligands, suggesting the human MPO transgenes will enhance the utility of mouse models for diseases involving MPO, such as atherosclerosis and Alzheimer’s.

Myeloperoxidase (MPO)\(^2\) is an abundant heme enzyme in neutrophils and monocytes and can be expressed in reactive macrophages. MPO plays a key role in the innate immune system as a microbicidal agent (1). Upon ingestion of microbes by neutrophils or monocyte-macrophages, azurophilic granules containing MPO fuse with the phagosome, releasing the enzyme, which reacts with chloride and superoxide-generated hydrogen peroxide to produce hypochlorous acid (HOCl), a toxic oxidant (2). The MPO-HOCl pathway also generates reactive nitrogen species (3) and tyrosyl radicals (4). The inadvertent release of MPO and its reactive byproducts at inflammatory sites can damage bystander cells, implicating MPO in oxidative damage at atherosclerotic lesions, Alzheimer’s plaques, and some cancers (5).

Several lines of evidence link MPO to oxidative damage in atherosclerosis. First, MPO and the end products of MPO-generated oxidants are detected in lesions, colocalizing with foam cell macrophages (6–9). Second, MPO levels in circulating leukocytes and serum are higher in individuals with coronary artery disease (CAD) (10, 11). Third, individuals with inherited MPO deficiencies have less cardiovascular disease (12). Fourth, a functional MPO promoter polymorphism has been associated with increased incidence of CAD (13, 14) and severity of atherosclerosis (15, 16). MPO may contribute to atherosclerosis through its ability to oxidize LDL (7), causing aggregation that enhances uptake through macrophage scavenger receptors (17), leading to lipid-laden foam cell macrophages, a hallmark of atherosclerotic lesions (18).

MPO is highly expressed in promyelocytes (19), with expression sharply decreasing as these precursors mature along the granulocyte or monocyte lineages. MPO protein is retained, stored in cytoplasmic vesicles. When monocytes differentiate to tissue macrophages, MPO protein is no longer present, yet the gene can be reactivated in subsets of reactive macrophages. For example, quiescent brain macrophages, microglias, lack MPO, yet \(\beta\)-amyloid, a component of Alzheimer’s plaques, is able to induce MPO gene expression, and MPO is abundant in reactive microglia surrounding plaques (20), and in foam cell macrophages at atherosclerotic lesions (21). It is important to understand the transcription factors and regulatory pathways that
PPARγ regulates MPO gene expression.

**EXPERIMENTAL PROCEDURES**

Isolation and Culture of Human Peripheral Blood Mononuclear Cells (PBMC)—PBMC were isolated from 500 ml of whole blood from healthy donors. A blood bank provided the cells without personal identifiers as a 50-ml concentrate obtained by low speed centrifugation. A single AA genotype donor was identified, and provided 20 ml of blood. Collection of samples from human donors and anonymous samples from the blood bank was approved by our affiliated institutional review committee. The concentrated leukocytes in 20-ml volume were layered over 20 ml of Ficoll Hypaque (Lymphoprep, Axis Shield) and centrifuged for 30 min at 900 × g. The interphase cell layer was again centrifuged over Lymphoprep, and the interphase layer was diluted with three volumes of RPMI medium 1640 (Invitrogen) and collected by centrifugation for 30 min at 900 × g. The cell isolate includes monocytes, macrophages, and neutrophils, and is largely depleted of neutrophils. The cells were resuspended in 40 ml of RPMI medium supplemented with 200 μM l-glutamine, 10,000 units/ml penicillin G, 10,000 μg/ml streptomycin, nonessential amino acids (Irvin Scientific), 1X Fungizone (Invitrogen), and 10% fetal calf serum (HyClone). The human serum for each donor was heat-inactivated (55 °C, 30 min) and added to the culture medium at 10% volume. The cells were plated in 24-well tissue culture plates (10th cells/well) in 400-μl volume with human GMCSF (Sigma) (10 ng/ml) or human M-CSF (R&D Systems) (10 ng/ml) in a humidified CO2 incubator at 37 °C. The cells were incubated for 24 h prior to addition of PPARγ ligands for an additional 24 h, followed by harvesting.

In 20 μl of Tissue Culture of Monocyte-derived Macrophages—To obtain monocyte-derived macrophages, PBMC were seeded at 106 cells/ml in 24-well plates in RPMI with 10% FCS and 10% autologous human serum, along with recombinant human GMCSF (10 ng/ml) or MCSF (10 ng/ml). Where indicated in the figures, 17β-estradiol (Sigma) (10−7 M) was added. On days 2 and 3, the medium was supplemented with 100 μl of fresh medium containing GMCSF or MCSF (10 ng/ml). On days 4−6, medium was exchanged daily, without human serum, with GMCSF at 10 ng/ml or MCSF at 10 ng/ml, and where indicated, 17β-estradiol (10−7 M). On day 7, fresh medium with rosiglitazone or other ligands was added for an additional 24 h prior to harvesting. The resultant cell population was uniformly adherent, with macrophage morphology, and 98% were positive for CD14 by immunostaining.

**RNA Isolation and Quantitation by TaqMan Real-Time PCR—**Total RNA was isolated from cells by TRIzol reagent (Invitrogen). Medium was aspirated and 400 μl of TRIzol added directly to the adherent macrophages in 24-well plates. For PBMC, TRIzol was added to combined nonadherent and adherent cells. The RNA was reverse transcribed with the Omniscript RT kit (Qiagen) and random hexamer primers. Five μl of the 20-μl cDNA reaction volume was used in real-time quantitative PCR using ABI PRISM 7900 (PerkinElmer Applied Biosystems) using TaqMan Master mix and primers. Normalization was to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for human RNA and β2-microglobulin for mouse RNA. Fluorescence was measured with the Sequence Detection Systems 2.0 software.

Probes and primers were designed by ABI Primer Express software and obtained from PE Biosystems. To prevent amplification of genomic DNA, primer sequences were designed to cross exon-intron boundaries. PCR was performed in multiplex (both target and endogenous control amplified in the same reaction) with distinct fluorescent dyes. The sequences for primers and probes used in this study are as follows: human MPO, forward (5'-TTTGCACACTGTCCAGATG-3'), reverse (5'-CGGTGTCGCTCCGGAATGTA-3'), and probe (5'-CCGTTCCAGTG- AGATGGCCGCCAAGCC-3'); human GAPDH, forward (5'-GGCCTGCCGTTGGCCTCGA-3'), reverse (5'-CCACGAGCCCATGATGGA-3'), and probe (5'-CAAAGGGTGGT CTGGAGG-3'); mouse PPARγ, forward (5'-AAC ATGACGGCAGCGCCAGG-3'), reverse (5'-AGC CCCAAAGAGGCTC-3'), and probe (5'-CTCTCAGGATACATCG-3'). The endogenous control for mouse mRNA was β2-microglobulin. Forward primer was 5'-CCAGGCCAAAGAGGCTC-3', reverse primer was 5'-CTGGATTTGGTAAACCAAGGCTC-3', and probe was 5'-TGATGCTGGTGCACATCATGCTGGCTCA-3'.

Primers and probe for human glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), PPARγ, ERO, and CD36 were purchased as kits from Applied Biosystems (Assays on Demand).

Measurement of MPO Enzyme Activity—Bone marrow cells (106) were harvested in 50 ml of PBS containing 10% fetal calf serum (Invitrogen), and resuspended in 60 μl of PBS containing Triton X-100 (0.2%) and phenylmethylsulfonyl fluoride (1 mM). Protein concentrations were determined using Bio-Rad Protein Assay. MPO activity was measured colorimetrically by oxidation of substrate guaiacol, monitored by absorbance at 470 nm. A reaction volume of 200 μl contained 5 μl of cell extract, sodium phosphate buffer, pH 7.0 (100 mM), 13 μM guaiacol, 2 mM 3-amino-9-aminonaphthalene sulfonic acid in optical grade water, and 0.7 μM guaiacol. The reaction was measured over 5 min using a 96-well plate reader (Spectramax Plus, Molecular Devices). The rate in milliunits/ml/min was calculated from the slope for the initial 2 min.

To measure MPO activity in 7-day macrophages, adherent macrophages were cultured in 6-well plates, washed twice with PBS, and treated for 5 μl of PBS containing Triton X-100 (0.2%) and phenylmethylsulfonyl fluoride (1 mM).

Western Blot Analysis—Cells were lysed in SDS sample buffer, and equal amounts of total protein were electrophoresed on a 4–20% gradient denaturing SDS-PAGE gels and transferred to polyvinylidene...
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difluoride membrane. Blots were blocked in 5% nonfat dry milk in Tris-buffered saline containing 1% Tween 20 (TBS-T) for 1 h, and incubated with primary antibodies for 1 h at room temperature. Blots were then washed in TBS-T twice at 15-min intervals, and incubated with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. After two subsequent washes in TBS-T, the blots were developed with ECL reagent (Amersham Biosciences). Detection of MPO Genotype by Allelic Discrimination Assay—DNA was isolated from leukocytes by proteinase K digestion followed by phenol extraction and ethanol precipitation. DNA (100 ng) was used in the ABI7900 allelic discrimination assay using dual fluorophore probes, which discriminate between alleles based on the single base mismatch. The ABI7900 allelic discrimination assay using dual fluorophore probes, which discriminate between alleles based on the single base mismatch. Primers and probes, designed using the Primer Express 2.0 software (Applied Biosystems), defined the 5′-GCTAAA-3′ oligonucleotide probe. These fluorophore-labeled probes, which discriminate between alleles based on the single base mismatch, were developed with ECL reagent (Amersham Biosciences). Primer and probe sequences included the following: forward primer, 5′-AATTTGCGTATGCTATAA-3′; reverse primer, 5′-GCCAGCTGTTCGTTGAAACTC-3′; −463A-specific probe, 5′-FAM-TACACCTGGCTCAGMGB; −463G-specific probe, 5′-VIC-TACACCGCCCTCA MGB. Probes were labeled at the 5′ end with the fluorophores FAM or VIC, and are stabilized by a minor groove binding moiety (MGB). End point allelic specific fluorescence was measured on the ABI Prism 7900 using Sequence Detection Systems 2.0 software for allelic discrimination.

Generation of MPO G and A Transgenic Mice—Transgenic mice carrying the human G or A alleles were created by microinjection of C57BL/6J embryos with a 32-kb DNA fragment into mouse eggs. One founder was obtained for each allele. A single founder was obtained for each allele.

Isolation of Mouse Bone Marrow Cells and Cell Culture—Bone marrow cells were plated at a density of 10⁵ cells/well in 24-well plates in RPMI with mouse GMCSF (R & D Systems) (10 ng/ml) or mouse M-CSF (R & D Systems) (10 ng/ml). Cells were incubated for 24 h prior to addition of PPARγ ligands for another 24 h in the continued presence of GMCSF or M-CSF.

To obtain bone marrow-derived macrophages, bone marrow cells were incubated for 24 h in 25-cm² flask containing 5 ml of RPMI with GMCSF at 5 ng/ml or M-CSF at 5 ng/ml. Non-adherent cells were seeded at 10⁶ cells/400 µl/well in 24-well plates and incubated for 7 days. The medium was supplemented on days 2 and 3 with 100 µl of fresh medium with GMCSF or M-CSF (10 ng/ml). The medium was exchanged on day 4, 5, and 6 to remove nonadherent cells. Where indicated, ε-estradiol was present throughout at 10⁻⁷ M. Adherent cells at day 7 were homogeneous in morphology, consisting of mononuclear cells with abundant cytoplasm.

Immunohistochemistry—Macrophages were briefly fixed in methanol and 4% paraformaldehyde, blocked with 10% goat normal serum and 1% BSA in TBS for 24 h, incubated with primary antibody as above, followed by biotinylated secondary Ab and detected with peroxidase chromogen kits (Dako, Carpinteria, CA). Immunohistochemical staining was visualized with confocal microscopy. For nonfluorescent detection, cells were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature. Blots were incubated with primary antibodies for 1 h at room temperature.

RESULTS

PPARγ-RXR Heterodimer Binds to the AluRRE in the MPO Promoter, Adjacent to the −463GA Polymorphism and an Estrogen Receptor Binding Site—The Alu upstream of the MPO gene is typical of the major class of Alu elements (Sx) (47) with hexamer spacing of 2-4-2 bp (36) (Fig. 1A). To test for binding by PPARγ to the MPO AluRRE, gel retardation assays were carried out with PPARγ and an estrogen receptor and its ligands programmed with in vitro synthesized mRNA. Synthesized receptors were incubated with [32P]-labeled oligonucleotides (45 bp) including the four hexamers with 6-7 bp of flanking sequences and either −463G or −463A at position 5 of hexamer 1 (Fig. 1A). PPARγ bound the AluRRE with either −463G or −463A, requiring co-presence of RXR (Fig. 1B). To further define the binding site, we tested oligonucleotides including the individual hexamer pairs and found that PPARγ binds to hexamers 3/4 (Fig. 1C), but not hexamers 1/2 or 2/3 (data not shown). The presence of PPARγ in the retarded complex was demonstrated by incubating the proteins with anti-PPARγ antibodies, which blocked complex formation (Fig. 1D). Binding of PPARγ to direct repeat hexamers with 2-bp spacing, though not optimal, has been reported previously (48).

In an earlier study, we found that estrogen receptor α (ERα) binds selectively to an oligonucleotide including −463A (38). In the present study, the 45-bp oligonucleotide included 6–7 bp of flanking sequences. Under these conditions, purified baculovirus-generated recombinant ERα (Panvera) bound the AluRRE with either −463G or −463A, in the presence of 3-bp flanking sequence, ERα bound preferentially to the A allele sequence (Fig. 1F). When the sequence was further restricted to include one or two bases of flanking sequence, ERα bound exclusively to the −463A sequence (Fig. 1G). This indicates that ERα was able to bind either the G or A allele sequence in the context of the longer oligonucleotide, but preferentially bound to the A allele when the available sequence was restricted. The hexamer 1/2 region lacks the consensus ERα binding site, which is a palindromic repeat with 3-bp spacing (PR3); however, ERα has been previously found to bind to half-sites with atypical spacings (49). Consistent with these binding assays, an earlier study showed that Alu elements can function as estrogen response elements (50).

PPARγ Ligands Have Either Positive or Negative Impact on MPO Expression in Human Peripheral Blood Leukocytes—To investigate the potential impact of the upstream PPARγ binding site, we assessed the effects of PPARγ ligands on MPO expression in PBMC. PBMC were isolated by Ficoll-Hypaque density gradient. The mononuclear cells were placed in culture medium with 10% autologous serum and 1% FCS for 24 h with RS, a representative of the TZD class of anti-diabetic agents. RNA was isolated, and cDNA generated with reverse transcriptase using random primers. Quantification of MPO cDNA was by real-time PCR. MPO cDNA levels were normalized to GAPDH as endogenous control, co-amplified in a dual label multiplex reaction.
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Fig. 1. PPARγ and ERα bind the AluRE in the MPO promoter. A, schematic of the four hexamer half-sites in the MPO AluRE with binding sites for estrogen receptor and PPARγ indicated, and sequence of the oligonucleotide used in the gel shift in B and E. B, double-stranded DNA oligonucleotides encoding the four hexamers of the AluRE with −463A or −463G were incubated with rabbit reticuloocyte lysate programmed with in vitro synthesized mRNA encoding PPARγ and/or RXRα as indicated. The arrow indicates the position of a retarded complex for both A and G allele sequences. C, an oligonucleotide encoding only hexamers 3/4 were incubated with lysates programmed with PPARγ expression vector or control vector. Arrows indicate PPARγ-specific complexes. D, antibodies against PPARγ block complex formation with PPARγ/RXRα on hexamers 3/4. Non-immune serum (nis) does not block. E, estrogen receptor α generates a complex on a DNA oligonucleotide encoding the four hexamers with either −463A or −463G. ERE is a canonical estrogen response element with palindromic repeat AGGTCA with 3-bp spacing. F, ERα forms a complex preferentially with −463A when using a shorter oligonucleotide consisting of hexamers 1/2 with 7 bp of flanking nucleotides. G, ERα forms a complex exclusively with −463A when using an oligonucleotide with hexamers 1/2 with one or two flanking nucleotides. Nucleotides in parentheses are not present in the MPO Alu sequence.

Surprisingly, rosiglitazone had strong yet opposite effects on MPO expression in various donor PBMC, increasing expression by 25–70-fold in some cases, and decreasing expression by 35–70-fold in other cases (Fig. 2A). There was no correlation of MPO genotype or gender with directionality of this response (data not shown). The proximity of ERα and PPARγ binding sites in the AluRE raised the possibility that ERα may be competing with PPARγ for binding. To test this hypothesis, estrogen (17β-estradiol, 10−7 M) was added to the culture medium. In the absence of its ligand, estrogen receptor is restricted to the cytoplasm. Ligand-bound ER is transported to the nucleus where it binds estrogen response elements. The addition of 17β-estradiol was found to block the effects of RS, both positive and negative, in most cases.

PPARγ Ligands Down-regulate MPO in Cells Treated with GMCSF, and Up-regulate MPO in Cells Treated with MCSF—To investigate the reason for the variable response to RS, PBMC were incubated for 24 h with either GMCSF or MCSF, growth factors that promote monocyte survival and differentiation to macrophages. Five different PPARγ ligands were then added for an additional 24 h, in the continued presence of the growth factors. The ligands included the natural prostaglandin metabolite, 15d-PGJ2, and four representatives of the TZD class, rosiglitazone, troglitazone, cigitazone, and MCC-555. RNA was isolated from adherent and nonadherent cells, and cDNA prepared for real time quantitation. In GMCSF-treated PBMC, all five PPARγ ligands down-regulated MPO strongly, by 25–250-fold (Fig. 2B). Conversely, in MCSF-treated PBMC, all five ligands up-regulated MPO by 20–80-fold. This suggested that GMCSF induces conditions, such as co-repressors, which allow PPARγ ligands to down-regulate MPO, whereas MCSF induces conditions, such as co-activators, allowing PPARγ ligands to up-regulate MPO.

PPARγ expression increases significantly as monocytes mature to macrophages (51); thus, we next examined the effects of PPARγ ligands on monocyte-derived macrophages. PBMC were cultured with GMCSF or MCSF for 7 days to generate macrophages. The adherent macrophages were then treated for 24 h with optimal concentrations of PPARγ ligands, 15d-PGJ2, rosiglitazone, troglitazone, cigitazone, and MCC-555 (Fig. 2C). The results were similar to findings with PBMC treated for 48 h; MPO expression was uniformly up-regulated in MC-Mφ, and uniformly down-regulated in GM-Mφ.

Estrogen Blocks Some Effects of PPARγ Ligands by a Mechanism Involving the −463GA Polymorphism—In untreated PBMC, estrogen appeared to block effects of PPARγ ligands in most cases (Fig. 2A). The proximity of the −463GA polymorphism to the PPARγ binding site, and the favored binding by ERα to −463A (Fig. 1), suggested that ERα might preferentially interfere with PPARγ binding on the MPO A allele. To
FIG. 2. PPARγ ligands down-regulate MPO in GMCSF and up-regulate in MCSF. A, varied effects of RS on PBMC in the absence of GMCSF or MCSF. Human PBMC from six random donors were placed in culture medium for 24 h with 0, 2, 5, or 7 μM RS (four points in graphs), in the absence (dark circles) or presence (open squares) of 17β-estradiol (10^{-7} M). The expression level in the absence of RS is designated 0, with -fold induction or repression because of RS treatment indicated. B, PBMC were cultured for 24 h in the presence of GMCSF or MCSF, and then the indicated PPARγ ligands were added for an additional 24 h. The TZD class were represented by MCC-555 (0, 1, and 10 μM), ciglitazone (0, 1, 10, and 50 μM), troglitazone (0, 5, 10, and 50 μM), rosiglitazone (0, 0.1, 1, and 10 μM), along with the natural ligand 15d-PGJ₂ (0, 1, 3, and 6 μM). C, macrophages were obtained by culture of PBMC for 7 days in the presence of GMCSF or MCSF, followed by 24 h with optimal concentrations of MCC-555 (10 μM), ciglitazone (50 μM), troglitazone (50 μM), rosiglitazone (10 μM), and 15d-PGJ₂ (8 μM).
investigate this possibility, we assayed the effects of PPARγ ligands on PBMC and macrophages for genotypes GG, GA, and AA, in the presence or absence of supplemental 17β-estradiol. The representative experiments shown in Fig. 3 assayed varying concentrations of RS. PBMC were initially incubated for 24 h with GMCSF or MCSF, followed by 24 h with RS. The effects were similar for the GG and GA genotypes; RS and PG markedly decreased MPO expression in GM-PBMC (30–60-fold) and increased expression in MC-PBMC (30–35-fold) (Fig. 3A). AA genotype differed, being down-regulated 40-fold in GMCSF, but unaffected in MCSF. Estrogen blocked all PPARγ ligand effects except the down-regulation of GG in GMCSF. The identical experiments performed with the natural ligand 15d-PGJ2 produced the same results, with MPO expression increasing 30–45-fold in MCSF, and decreasing 25–65-fold in GMCSF, with estrogen blocking all effects except the down-regulation of GG genotype in GMCSF (data not shown).

Similar findings were obtained with GM-Mφ and MC-Mφ (Fig. 3B). GG and GA genotypes were markedly up-regulated (18–30-fold) in MC-Mφ and down-regulated (25 to 60-fold) in GM-Mφ. The AA genotype was mildly down-regulated in GMCSF Mφ (7-fold), and did not respond to RS in MCSF-Mφ. Estrogen again blocked all effects except the down-regulation of GG in GMCSF. Equivalent results were obtained using 15d-PGJ2 (data not shown).

The regulation of the homozygous GG and AA genotypes suggested an explanation for the expression pattern of het erozygote GA genotype, which resembled GG genotype in MCSF, and AA genotype in GMCSF. The A allele was not up-regulated by RS in MCSF, suggesting the up-regulation of GA genotype was the result of up-regulation of G allele alone. Estrogen blocks the down-regulation of AA genotype in GMCSF, suggesting the sustained basal expression of A allele could be masking the down-regulation of G allele in the heterozygote GA (Fig. 3, A and B).

Consistent with the data on mRNA levels, MPO enzyme activity increased in MC-Mφ treated with RS, and decreased in GM-Mφ treated with RS (Fig. 3C). The maximal level of MPO activity in macrophages was observed in MCSF-cultured cells treated with RS, and this maximal level was still severalfold lower than that observed in adherent PBMC (Fig. 3C). The activity measured in GMCSF- or MCSF-cultured macrophages may reflect not only newly synthesized enzyme, but could also include some enzyme retained from monocytes, based on a prior study showing that GMCSF prevents the loss of MPO protein during the differentiation of monocytes to macrophages (21).

Table I summarizes the effects of rosiglitazone on PBMC and macrophages from multiple donors. In all cases, MPO expression was strongly up-regulated by RS in MCSF-treated cells, and down-regulated in GMCSF. In GM-PBMC, RS down-regulated GG genotype by an average of 32-fold (p = 0.0001), GA by 45-fold (p = 0.0001), and AA by 32-fold (panel I: GG, GA, AA, rows D, column 2). In MC-PBMC, RS up-regulated GG genotype by 20-fold (p = 0.0001), GA by 39-fold (p = 0.0001), but had no affect on AA expression levels (column 6). Estrogen blocked up-regulation of GG and GA in MCSF (column 8). In GMCSF, estrogen blocked the down-regulation of GA and AA, but reduced GG down-regulation by less than 2-fold (column 4).

In GMCSF macrophages, RS down-regulated GG genotype by 34-fold (p = 0.0001), GA genotype by 32-fold (p = 0.0001), and AA by 7-fold (panel II: GG, GA, AA, rows D, column 2). Thus, the ability of A allele to respond to PPARγ ligands diminishes during differentiation of PBMC to GMCSF-macro- phages. Estrogen blocked down-regulation of GA and AA, but not GG genotype in GM-Mφ (column 4). In MCSF macro- phages, GG genotype was up-regulated by 19-fold (p = 0.0001), and GA genotype by 26-fold (p = 0.0001), and both effects were completely blocked by estrogen (columns 6 and 8). AA genotype did not respond to RS in MCSF.

Table I lists not only the -fold changes in MPO expression with RS and estrogen (D), but also the average Δ threshold cycle (ΔCT) (rows A and B), which is the difference between MPO CT and the CT of the endogenous control gene. In realtime PCR, fluorescence values are recorded at each cycle and represent the amount of product at that point in the amplification process. The threshold cycle (CT) refers to the number of cycles required to reach a fluorescence level significantly above background. If more template is present at the beginning of the reaction, fewer cycles will be required to reach CT. CT is always attained during the exponential phase of amplification, such that each cycle represents a doubling of reaction product. The ΔCT is the difference between the cycles required to attain threshold levels for MPO cDNA versus the control GAPDH cDNA. A difference of 1 CT between target and control cDNA represents a 2-fold difference in RNA levels, whereas a difference of 3 CT indicates an 8-fold difference, and so on. For example, in MCSF-PBMC, GA genotypes had an average ΔCT of 7.5 (Table I, panel I, GA, row B, column 5), indicating MPO cDNA was 27.5- or 181-fold below the level of GAPDH. After rosiglitazone treatment, the ΔCT was reduced to 2.2 (column 6), indicating MPO levels increased to a level only 22.2, or 4.6-fold below GAPDH. The ΔCT values in Table I allow direct comparison of relative MPO mRNA levels in PBMC, macrophages, and in GG, GA, and AA genotypes, all normalized to GAPDH.

The GA Genotype Is Higher Expressing than GG Genotype in the Presence of GMCSF, RS, and Estrogen—The GG genotype was 4.6-fold higher expressing than GA in freshly isolated, untreated PBMC (Table I, panel I, rows A, ΔCT, 7.3 versus 9.5, 4.6-fold higher expressing in GM-Mφ (panel II, row E, column 1), and 7.2-fold higher in MC-Mφ (column 5). Conversely, GA is significantly higher expressing than GG genotype in GMCSF-treated cells in the presence of rosiglitazone and 17β-estradiol. This is the result of the down-regulation of GG genotype by RS, whereas estrogen blocks this effect on the A allele, which then maintains basal expression. In GM-PBMC treated with RS and 17β-estradiol, GA was 52-fold higher expressing than GG (panel I, row E, column 4), and in GM-Mφ with RS and 17β-estradiol, GA was 5.6-fold higher expressing than GG (panel II, row E, column 4). Interestingly, the GA genotype was higher expressing than GG in most circumstances in 48-h treated GMCSF- or MCSF-PBMC (panel I, row E), but not in untreated PBMC or GM- or MC-Mφ (panel II, row E).

The Selective PPARγ Antagonist GW9662 Blocks the Effects of PPARγ Ligands on MPO Expression—TZDs and 15d-PGJ2 function as PPARγ ligands but have also been demonstrated to inhibit gene expression by means independent of the receptor PPARγ (24, 52). For example, 15d-PGJ2 interferes with NFKB-mediated gene regulation by generating adducts with cysteine residues in NFKB and IkB kinase (53). One means to test whether a given effect is mediated by the receptor PPARγ is to use the PPARγ antagonist, GW9662, which covalently modifies cyssteine 286 in the ligand binding domain (54). Although GW9662 reacts with all three PPAR subtypes α, δ, and γ, the antagonist activity is at least 100–1000-fold more potent for PPARγ than α or δ (54); therefore, it is considered a selective PPARγ antagonist.

In Fig. 4A, PBMC were treated with GMCSF or MCSF for 24 h, followed by 24 h with RS (5 or 7 μM) in the presence or absence of GW9662 (5 μM). GW9662 completely blocked the ability of RS to down-regulate MPO in GMCSF or up-regulate MPO in MCSF. Similarly, in Fig. 4B, macrophages were
treated with 9 μM 15d-PGJ$_2$ in the presence or absence of increasing amounts of GW9662. A concentration of 5 μM GW9662 completely blocked the 15d-PGJ$_2$-dependent increase in expression in MCSF, and the decrease in GMCSF. These findings provide evidence that the receptor PPAR$_\gamma$ mediates both the positive and negative effects of PPAR$_\gamma$ ligands on MPO gene expression.

Unlike MPO, Expression of CD36 and Liver X Receptor (LXR) Is Up-regulated by PPAR$_\gamma$ Ligands in Both GMCSF and MCSF Macrophages—We investigated the possibility that other genes regulated through PPAR$_\gamma$ might be subject to down-regulation in GMCSF and up-regulation in MCSF. Scavenger receptor CD36 and LXR are known to be up-regulated by PPAR$_\gamma$ in macrophages (24, 52, 55). We therefore assayed the effects of RS on the expression of these genes in GM-M$_\phi$ and MC-M$_\phi$. Consistent with prior reports, rosiglitazone (10 μM) was found to up-regulate CD36 expression by 8-fold in both GMCSF and MCSF macrophages, and LXR was up-regulated by 2-fold in both cases (data not shown). Thus, the opposing regulation observed for MPO appears to be promoter-specific, and may be AluRRE-specific.

One possible explanation for the differences in RS response for the MPO gene in GMCSF- versus MCSF-treated cells is that key transcription factors are expressed at different levels in these conditions. To investigate this possibility we assayed the expression levels of ER$\alpha$, PPAR$_\gamma$, and SP1 in GMCSF- versus MCSF-treated cells. The differences in expression levels for PPAR$_\gamma$ and SP1 were less than 2-fold in MC-M$_\phi$ and GM-M$_\phi$. However, ER$\alpha$ expression levels were 4-fold higher in MC-M$_\phi$ than GM-M$_\phi$ (data not shown). This may contribute to the greater ability of estrogen to block PPAR$_\gamma$ effects on the A allele in MCSF cultured cells (discussed below).

The Regulation by PPAR$_\gamma$ Ligands and Estrogen Is Repeated in Transgenic Mice Expressing the Human G and A Alleles—Transgenic mice expressing the human MPO G and A alleles allow independent analysis of the two alleles. In human cells, this requires comparison of homozygous GG genotype to the rare AA genotype (3–5% of population). Analysis of allelic expression in heterozygote cells is not possible because of the apparent absence of sequence differences in the mRNA, including exons or 5'- and 3'-noncoding transcribed regions, based on GenBank entries and our sequencing efforts. To facilitate the analysis of the −463GA polymorphism, we generated transgenic mice expressing the G and A alleles under control of extensive native human promoter sequences. The transgenics were created by microinjection of C57BL6/J eggs with a 32-kb restriction fragment including the MPO gene. It is worth noting that the injected alleles did not differ at another reported MPO promoter polymorphism, −129GA (56). PCR analysis confirmed the presence of at least 6 kb of upstream and 4 kb of downstream sequences for both the G and A transgenes. The human MPO gene is appropriately expressed in bone marrow cells, with MPO protein restricted to bone marrow, circulating leukocytes, and subsets of reactive macrophages. Human MPO is not detected in quiescent tissue macrophages, such as brain microglia or liver Kupffer cells (data not shown).

To confirm that the human MPO transgenes were functional, reverse transcription PCR was carried out with cDNA prepared from bone marrow cells. The MPO G transgene was expressed at a high level, equivalent to β$_2$-microglobulin used as endogenous control (Table I, panel III, G, row A). Expression of G allele was 7-fold higher than A allele (Fig. 5A and Table I, panel III, rows A), and the mouse MPO gene was expressed at an intermediate level (Fig. 5A). To demonstrate that the transgene mRNAs coded for functional MPO protein, enzyme activity was monitored by guaiacol oxidation assay. To subtract the contri-

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**Fig. 3.** Estrogen blocks some effects of PPAR$_\gamma$ ligands. A. PBMC of GG, GA, or AA genotype were treated with GMCSF or MCSF for 24 h, in the absence (dark circles) or presence (open squares) of 17β-estradiol (E2), followed by the addition of RS at 0, 1, 3, or 7 μM for an additional 24 h. AA genotype was treated with 0 and 7 μM RS. Basal expression (no RS) for each set is designated as 0. Fold induction/repression indicates the fold change from basal levels because of RS treatment. Set 1 is GG PBMC with GMCSF, no 17β-estradiol, and 0, 1, 3, and 7 μM RS. Set 2 is the same with 17β-estradiol treatment. Set 3 is same as 1, but with MCSF. Set 4 is same as 2, but with MCSF. Sets 5–8 are like 1–4 but with GA genotype. Sets 10–12 are like 1–4 but with AA genotype, and only one concentration of RS. B, macrophages were generated by culturing PBMC in GMCSF or MCSF for 7 days, in the absence (dark circles) or presence (open squares) of 17β-estradiol, followed by addition of RS at 0, 1, 2, 5, or 7 μM for an additional 24 h. AA genotype was treated with 0 and 7 μM RS. Sets are as in A. C, MPO enzyme activity was measured by guaiacol assay in GM-M$_\phi$ and MC-M$_\phi$ treated for 24 h in presence or absence of RS, and in untreated PBMC. The change in optical density at 470 nm was measured, and the rate in milliunits/ml/min was calculated from the slope for the initial 2 min.


**Table I**

**Effect of rosiglitazone on MPO expression**

Asterisks, PBMC or MΦ were treated with GMCSF or MCSF, with or without 17β-estradiol (E2) or rosiglitazone (RS) (10 μM) for 24 h. A and B, mean ΔC\textsubscript{T} is the difference in PCR threshold cycle (ΔC\textsubscript{T}) between MPO and the endogenous control, either human GAPDH or mouse β2-microglobulin. A shows the threshold cycle for untreated cells. B shows the threshold cycle for cells treated with GMCSF, MCSF, with or without estrogen. C, ΔC\textsubscript{T} RS is the change in threshold cycle as a result of RS treatment (difference between ΔC\textsubscript{T} minus or plus RS). D, 2 (ΔC\textsubscript{T} RS) is the fold change in MPO cDNA levels because of RS. Boxes indicate conditions where RS had significant effect on MPO expression. P value is for the RS-induced change in threshold cycle (C\textsubscript{T}) values. E, -fold transcriptional advantage for GG or GA genotypes, or G or A genotype in transgenic mouse macrophages.

### I Human PBMC

|          | CSF | GMCSF | MCSF | GMCSF | GMCSF | MCSF | MCSF | GMCSF |
|----------|-----|-------|------|-------|-------|------|------|-------|
| Estradiol| -   | -     | 17E2 | -     | -     | 17E2 | -    | -     |
| RS       | -   | -RS   | -RS  | -RS   | -RS   | -RS  | -RS  | -RS   |

|          | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| A        | G   | G   | G   | G   | G   | G   | G   | G   |
| B        | 8.5 | 13.5| 10  | 14.3| 6.8 | 4.5 | 10  | 8.1 |
| C        | 8.5 | 13.5| 10  | 14.3| 6.8 | 4.5 | 10  | 8.1 |
| D        | -32 | -20 | 20  | 4   | -32 | -20 | 20  | 4   |

|          | G   | G   | G   | G   | G   | G   | G   | G   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| A        | G   | G   | G   | G   | G   | G   | G   | G   |
| B        | 7.8 | 13.3| 7.8 | 8.0 | 7.5 | 2.2 | 7.4 | 6.5 |
| C        | 7.8 | 13.3| 7.8 | 8.0 | 7.5 | 2.2 | 7.4 | 6.5 |
| D        | -45 | -17 | 39  | 1.5 | -45 | -17 | 39  | 1.5 |

### II Human macrophages

|          | CSF | GMCSF | MCSF | GMCSF | GMCSF | MCSF | MCSF | GMCSF |
|----------|-----|-------|------|-------|-------|------|------|-------|
| Estradiol| -   | -     | 17E2 | -     | -     | 17E2 | -    | -     |
| RS       | -   | -RS   | -RS  | -RS   | -RS   | -RS  | -RS  | -RS   |

|          | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| A        | G   | G   | G   | G   | G   | G   | G   | G   |
| B        | 8.5 | 13.5| 10  | 14.3| 6.8 | 4.5 | 10  | 8.1 |
| C        | 8.5 | 13.5| 10  | 14.3| 6.8 | 4.5 | 10  | 8.1 |
| D        | -32 | -20 | 20  | 4   | -32 | -20 | 20  | 4   |

|          | G   | G   | G   | G   | G   | G   | G   | G   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| A        | G   | G   | G   | G   | G   | G   | G   | G   |
| B        | 7.8 | 13.3| 7.8 | 8.0 | 7.5 | 2.2 | 7.4 | 6.5 |
| C        | 7.8 | 13.3| 7.8 | 8.0 | 7.5 | 2.2 | 7.4 | 6.5 |
| D        | -45 | -17 | 39  | 1.5 | -45 | -17 | 39  | 1.5 |

### III MPO G and A transgenic macrophages

|          | CSF | GMCSF | MCSF | GMCSF | GMCSF | MCSF | MCSF | GMCSF |
|----------|-----|-------|------|-------|-------|------|------|-------|
| Estradiol| -   | -     | 17E2 | -     | -     | 17E2 | -    | -     |
| RS       | -   | -RS   | -RS  | -RS   | -RS   | -RS  | -RS  | -RS   |

|          | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| A        | G   | G   | G   | G   | G   | G   | G   | G   |
| B        | 8.5 | 13.5| 10  | 14.3| 6.8 | 4.5 | 10  | 8.1 |
| C        | 8.5 | 13.5| 10  | 14.3| 6.8 | 4.5 | 10  | 8.1 |
| D        | -32 | -20 | 20  | 4   | -32 | -20 | 20  | 4   |

|          | G   | G   | G   | G   | G   | G   | G   | G   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| A        | G   | G   | G   | G   | G   | G   | G   | G   |
| B        | 7.8 | 13.3| 7.8 | 8.0 | 7.5 | 2.2 | 7.4 | 6.5 |
| C        | 7.8 | 13.3| 7.8 | 8.0 | 7.5 | 2.2 | 7.4 | 6.5 |
| D        | -45 | -17 | 39  | 1.5 | -45 | -17 | 39  | 1.5 |

**P glimpse Regulations MPO Gene Expression**

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In human peripheral blood mononuclear cells untreated with growth factors, the response of the MPO gene to rosiglitazone was variable, in some cases positive and in other cases negative (Fig. 2A), possibly the result of variation in cytokine or hormone levels in the donors. The MPO transgenics provide the advantage of less genetic and environmental variation than in human subjects. We tested the effects of PPARγ/H9253 ligands on untreated bone marrow cells from the MPO G and A transgenics, along with the parental C57BL6/J strain. The cells were incubated with rosiglitazone for 24 h in medium with 10% FCS, prior to isolation of mRNA. MPO G expression was up-regulated by RS, suggesting that mouse bone marrow cells resemble MCSF-treated cells in their response to PPARγ ligands. RS had no effect on MPO A expression (Fig. 6A), again similar to findings for AA genotype in human PMN (8308).
MCSF treated human cells (Fig. 3). The mouse MPO gene, which lacks the primate-specific Alu with PPARγ binding site, did not respond to RS.

The G and A Transgenes Are Down-regulated by PPARγ Ligands in GMCSF-treated Bone Marrow Cells, Whereas G, but Not A, Is Up-regulated in MCSF—Bone marrow cells were first incubated with GMCSF or MCSF for 24 h, followed by addition of RS at 0, 1, 2, 5, or 7 μM or PG at 0, 2, 5, or 7 μM, in the absence (black circles) or presence (open boxes) of 17β-estradiol (10⁻⁷ M) for 24 h. Set 1 shows MPO G transgenic bone marrow treated with GMCSF and RS. Set 2 was the same with 17β-estradiol. Set 3 had GMCSF and PG. Set 4 was same with 17β-estradiol. Sets 5–8 were like 1–4 but with MCSF. Sets 9–16 were like 1–8 but with MPO A transgene. Sets 17 and 18 were C57BL/6J bone marrow treated with RS in GMCSF or MCSF. Macrophages were obtained by culture of bone marrow cells for 7 days in the presence of GMCSF or MCSF, followed by 24 h with RS or PG at 0, 2, 5, or 7 μM. Sets are as in B.

Table I, part III, shows the mean ΔC₇₀ for transgenic MPO G and A macrophages, treated with rosiglitazone, in the presence or absence of estrogen. In GM-Mφ, the 48-fold suppression of G allele (p = 0.0001) was reduced to 19.7-fold by estrogen (columns 2 and 4). In MCSF-Mφ, the 38-fold up-regulation of G allele (p = 0.0001) was completely blocked by estrogen (columns 6 and 8). The A allele failed to respond to RS in either GM-Mφ or MC-Mφ (row D).

In most circumstances, the transgenic G allele was higher expressing than A allele. The G allele was 7.5-fold higher expressing in untreated bone marrow cells (ΔC₇₀ 0.2 versus 3.1) (panel III, rows A), and 3.7–9-fold higher expressing in GM-Mφ and MC-Mφ, respectively (III, row E, column 1 and 5). However, as in human cells, in GM-Mφ treated with RS and estrogen, the A allele was 37-fold higher expressing than G (column 4), because down-regulation of G allele, but not A allele, by RS.

**The Transgenic A Allele Is Unresponsive to PPARγ Ligands in Macrophages**—To obtain transgenic macrophages, bone marrow cells were cultured in GMCSF or MCSF for 7 days. With RS, MPO G was down-regulated 50–60-fold in GM-Mφ and up-regulated 12–55-fold in MC-Mφ (Fig. 6C). 17β-Estradiol reduced (0–2-fold) but did not block the down-regulation of G allele in GMCSF, yet completely blocked the up-regulation in MCSF. In macrophages, the MPO A allele was no longer down-regulated by RS or PG in GMCSF, and remained unresponsive in MCSF. Thus, the MPO A allele lost ability to respond to PPARγ ligands during differentiation to GM-macrophages. The mouse MPO gene remained unresponsive to PPARγ ligands in either GM- or MC-macrophages.

In most circumstances, the transgenic G allele was higher expressing than A allele. The G allele was 7.5-fold higher expressing in untreated bone marrow cells (ΔC₇₀ 0.2 versus 3.1) (panel III, rows A), and 3.7–9-fold higher expressing in GM-Mφ and MC-Mφ, respectively (III, row E, column 1 and 5). However, as in human cells, in GM-Mφ treated with RS and estrogen, the A allele was 37-fold higher expressing than G (column 4), because down-regulation of G allele, but not A allele, by RS.
Conversely, in MC-M\(\phi\), G allele was 294-fold higher expressing than A allele (column 6), because of strong up-regulation of G allele, but not A allele.

Charcoal Stripping of Fetal Calf Serum Permits PPAR\(\gamma\) to Regulate MPO A Allele; Repletion with 17\(\beta\)-Estradiol Reinstates the Block—We hypothesized that the inability of A allele to respond to PPAR\(\gamma\) ligands in MCSF macrophages was because of trace amounts of estrogen present in 10% FCS, thus enabling transport of ER to the nucleus, where it can bind the preferred site at \(463A\). To test this possibility, fetal calf serum was treated with activated charcoal to deplete estrogen and other agents. The use of charcoal-filtered FCS (CF-FCS) allowed RS to strongly up-regulate MPO A in MCSF-treated bone marrow cells (20-fold), whereas unextracted 10% FCS blocked RS-mediated induction (Fig. 7A). To confirm that this rescue was the result of removal of trace amounts of estrogen, we added back 17\(\beta\)-estradiol (10\(^{-7}\) M) to the charcoal-filtered FCS, and thereby reinstated the complete block of RS-induced up-regulation.

Similar findings were obtained in transgenic macrophages. In 10% FCS, the A allele was resistant to RS in GM-M\(\phi\) as well as MC-M\(\phi\). Charcoal stripping of the FCS restored the ability of RS to up-regulate MPO A 20-fold in MCSF, and down-regulate 20-fold in GMCSF (Fig. 7A). Replenishing with 17\(\beta\)-estradiol reinstated the block to PPAR\(\gamma\) ligand effects.

Charcoal-filtered FCS enhanced RS effects on the MPO G allele; down-regulation by RS in GMCSF bone marrow cells increased from 15- to 30-fold, whereas the up-regulation in MCSF bone marrow increased from 30- to 50-fold (Fig. 7B). In GM-M\(\phi\), down-regulation similarly increased from 30- to 50-fold, and there was no change in GMCSF macrophages. This indicates that trace estrogen levels in FCS blunts 2-fold RS effects on the G allele, consistent with weaker binding by ER to \(463G\).

Charcoal filtration of FCS had similar effects in human macrophages (data not shown). In CF-FCS with 10 \(\mu\)M RS, AA genotype was down-regulated 18-fold in GM-M\(\phi\), and up-regulated 34-fold in MC-M\(\phi\). In contrast, in regular FCS, AA genotype had not been affected by RS in MCSF-treated cells (Fig. 3). GA genotype was down-regulated 14-fold by 10 \(\mu\)M RS in regular FCS, and 32-fold in CF-FCS. In MCSF macrophages, GA was up-regulated 12-fold in regular FCS, and 28-fold in

**Fig. 7.** Charcoal-extracted FCS allows regulation of A allele by PPAR\(\gamma\) ligands, and readdition of 17\(\beta\)-estradiol reinstates the block of PPAR\(\gamma\) effects. Bone marrow cells (BM) or macrophages (M\(\phi\)) were obtained from mice transgenic for MPO A (panel A) or G (panel B). Bone marrow cells (BM) were cultured in media containing 10% FCS or 10% CF-FCS, along with GMCSF or MCSF for 24 h prior to addition of RS (0, 5, or 10 \(\mu\)M) for an additional 24 h. Where indicated (+E2, open boxes), estrogen (17\(\beta\)-estradiol, 10\(^{-7}\) M) was added 3 h prior to addition of RS. MPO mRNA levels were quantified by real-time PCR. The eight experimental conditions are indicated at top. Each experimental set includes cells treated with 0, 5, or 10 \(\mu\)M RS. Set 1 was treated with FCS and GMCSF. Set 2 was the same as 1 but with 17\(\beta\)-estradiol. Set 3 had FCS and MCSF. Set 4 was the same as 3 but with 17\(\beta\)-estradiol. Sets 5–8 are the same as 1–4 but with CF-FCS. Bold lines indicate RS-induced changes in expression in CF-FCS (panel A, sets 5 (M\(\phi\)) and 7 (BM and M\(\phi\)). To obtain macrophages, bone marrow cells were cultured in GMCSF or MCSF (10 ng/ml) with 10% FCS for 4 days, then with either FCS or CF-FCS for 3 additional days, prior to addition of RS (0, 5, or 10 \(\mu\)M) for 24 h. Where indicated, estrogen (E2) was added 3 h prior to addition of RS.

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CF-FCS. The readdition of 17β-estradiol completely blocked both effects. It is worth noting that readdition of estradiol to CF-FCS had no significant effect on MPO expression, except to block effects of PPARγ.

Immunodetection of MPO in Macrophages Treated with Rosiglitazone—Rosiglitazone significantly increases MPO mRNA levels in MC-MΦ. To determine whether this results in detectable increases in MPO protein in RS-treated macrophages, we carried out immunocytochemical analysis. Human GMCSF and M-CSF macrophages were incubated with polyclonal antibodies to human MPO (Biosdesign, 1:1000), and monoclonal antibodies to the macrophage marker CD68 (R&D), followed by fluorescein isothiocyanate (FITC)–conjugated secondary antibodies, and analyzed by confocal microscopy. The cells were uniformly of macrophage morphology, and 97% were immunopositive for the macrophage marker CD68 (Fig. 8, C, F, I, and L). MPO was detected in M-CSF macrophages (B), with a peripheral staining pattern resembling that of CD68 (A). Rosiglitazone treatment increased the levels of MPO in apparent vesicles in more central regions (E, D).

In GMCSF macrophages, MPO immunostaining was abundant in the cytoplasm and perinuclear regions (H) and decreased in rosiglitazone treated macrophages (K). MPO was also detected by nonfluorescent means in MC-MΦ treated with RS, using diaminobenzidine (DAB) as substrate for peroxidase conjugated secondary antibodies (N). MPO appears to be in vesicles at higher magnification (O).

The presence of immunodetectable MPO in macrophages untreated with RS is consistent with a previous study showing that culture of monocytes in GM-CSF for 7 days prevents the loss of stored vesicular MPO protein (21). Findings here suggest that RS treatment during the final 24 h increases MPO immunostaining in MC-MΦ, and decreases MPO immunostaining in GM-MΦ, consistent with the observed changes in mRNA levels.

Immunodetection of Human MPO in Early Atherosclerotic Lesions in the LDL Receptor Knockout Model—These findings indicate that the human MPO transgenes are regulated by PPARγ ligands in primary macrophages in culture. To obtain evidence that these transgenes are expressed in vivo in tissue macrophages, we immunostained a section of the aortic valve from MPO G transgenics onto the LDL receptor null background, and fed a high fat diet. These LDL null mice are a model for atherosclerosis and develop fatty streak lesions containing foam cell macrophages, analogous to early human atherosclerotic lesions. A previous study found no mouse MPO in aortic valve lesions in the LDL receptor null model (57), suggesting that this mouse model was unlike human atherosclerosis as regards MPO expression. Findings here suggest that the absence of mouse MPO in vascular lesions may be the result of the lack of response of the mouse MPO gene to PPARγ. Immunostaining of the MPO G/LDL receptor null mice showed that human MPO is present in lesions in aortic valve lesions (Fig. 9A), colocalizing with PPARγ (Fig. 9B). This observation provides evidence that the human MPO G transgene is appropriately expressed in vivo in tissue macrophages at sites containing PPARγ.

DISCUSSION

Results reported here show that PPARγ ligands strongly regulate MPO gene expression. An unusual aspect of this regulation is the opposite effects of PPARγ ligands in macrophages cultured in GM-CSF versus M-CSF. MPO expression is markedly up-regulated in M-CSF macrophages, and down-regulated in GM-CSF macrophages. As evidence that these effects are me-
Fig. 10. Proposed model of competitive binding by estrogen receptor and PPARγ to AluRRE. A, MPO G promoter. Findings here are consistent with a model in which the G promoter is bound by ligand PPARγ in GMCSF-treated cells, down-regulating MPO expression (arrow down). ERα is unable to bind the low avidity −463A site; thus, the addition of estrogen (plus 17β-estradiol) does not block PPARγ binding or MPO down-regulation. In MCSF-treated cells, PPARγ binds and up-regulates MPO G (arrow up). With added 17β-estradiol, ERα is able to enter the nucleus and bind the G promoter, blocking PPARγ binding and MPO up-regulation, such that basal level expression (horizontal arrow) is maintained. The ability of 17β-estradiol to block PPARγ effects in MCSF, but not in GMCSF, may have to do with the severalfold higher levels of ERα expression in MCSF cells. B, MPO A promoter. In GMCSF-treated human PBMC or mouse bone marrow cells, PPARγ binds and down-regulates the A allele. With added 17β-estradiol, ERα is able to enter the nucleus, bind and block PPARγ binding, thereby blocking MPO down-regulation, maintaining basal MPO expression levels. In MCSF-treated cells, supplemental 17β-estradiol was not required to block PPARγ. The low levels of estrogen in 10% FCS were sufficient, suggesting the higher levels of ERα in MCSF, coupled with the higher avidity −463A site, allows sufficient ERα to transport to the nucleus, blocking PPARγ binding. C, MPO A promoter in macrophages. The ability of 17β-estradiol to block PPARγ effects was enhanced in M6. The low levels of estrogen in 10% FCS were sufficient to enable ERα to block PPARγ in GMCSF as well as MCSF. Supplemental estrogen was not required. D, CF-FCS provided evidence that estrogen is responsible for the block of PPARγ effects on the A allele. Charcoal stripping of serum restored the ability of PPARγ ligands to down-regulate the A allele in GMCSF and up-regulate in MCSF, such that the MPO A response is like that of MPO G. The readdition of estrogen reinstated the complete block of PPARγ effects on the A allele.

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mediated by the receptor PPARγ, the selective PPARγ antagonist, GW9662 blocks both the positive and negative effects on MPO expression. This regulation is not specific to particular ligands, having been obtained with four distinct PPARγ ligands of the TZD class, as well as the natural prostanoid 15d-PGJ2.

A second intriguing aspect to this regulation is the influence of estrogen and the −463GA polymorphism. This polymorphism is within an estrogen receptor binding site in hexamer 1/2 of the AluRRE. Estrogen receptor α binds to hexamer 1/2 in both the G and A promoters, binding more effectively to −463A in more stringent conditions. This ERα site is immediately adjacent to the PPARγ binding site in hexamers 3/4. Addition of 17β-estradiol has strong impact on regulation by PPARγ, blocking up-regulation of both alleles in MC-Mφ, and blocking down-regulation of A allele, but not G allele, in GM-Mφ. The A allele is down-regulated by PPARγ in GM-PBMC, and this is blocked by estrogen/ER (Fig. 10B). In MC-PBMC, supplemental estradiol is not required; trace estrogen levels in 10% FCS appear to be sufficient for ER translocation and blockage of PPARγ binding. In macrophages, trace estrogen levels in 10% FCS appear sufficient to block PPARγ binding to the A allele in both GM-Mφ and MC-Mφ (Fig. 10C). Charcoal filtering of FCS depletes estrogen, preventing ER translocation and binding, such that PPARγ is able to down-regulate the A allele in GMCSF and up-regulate in MCSF (Fig. 10D). Both effects are blocked by the readdition of 17β-estradiol. Accordingly, charcoal stripping of serum resulted in comparable regulation of the G and A alleles by PPARγ; the only difference remaining was that the down-regulation of G allele was not blocked by estrogen/ER in GM-Mφ (Fig. 10A). The differential expression of the G and A alleles in physiologically low levels of estrogen (10% FCS) is likely to be biologically significant, as suggested by the gender differences in −463GA genotype association with disease risk in some studies. Indeed, the mutation at −463A may have been evolutionarily selected because of its ability to dampen effects of PPARγ on MPO expression.

Three lines of evidence suggest the AluRRE mediates the effects of PPARγ ligands on MPO gene expression. First, there is the PPARγ binding site in the AluRRE (Fig. 1). Second, the −463GA polymorphism in the AluRRE strongly affects PPARγ regulation, probably through competitive binding by estrogen receptor (Figs. 3, 6, and 7). Third, the mouse MPO gene lacks
the primate-specific AluRRE, and is completely unresponsive to PPARγ ligands (Fig. 6).

The mechanisms underlying the opposite effects of PPARγ ligands in GMCSF versus MCSF macrophages are unknown. Both factors have been used in various studies to induce cultured monocytes to differentiate to macrophages (59, 60, 61). Interestingly, catalase is expressed at high levels in GM-Mφ and is induced by H2O2 whereas in MC-Mφ, catalase is expressed at low levels and is not inducible (62). Catalase consumes H2O2, thereby removing the substrate for MPO. It seems consistent that, in GM-Mφ, MPO is down-regulated whereas catalase is up-regulated, consistent with an antioxidant phenotype. Conversely, MPO is up-regulated and catalase down-regulated in MC-Mφ, consistent with a pro-oxidant phenotype.

The mechanism by which MPO is up-regulated by PPARγ in MCSF and down-regulated in GMCSF is likely to involve the coactivators and corepressors that modulate nuclear receptor activities (63). Ligand binding to PPARγ or other nuclear receptors induces conformational changes resulting in the reorientation of a helical motif termed the AF2 domain, reorienting this motif to the ligand binding pocket (64). This conformational rearrangement promotes the association of coactivators and the dissociation of corepressors. A number of receptor coactivators have been identified including the p160/SRC family, and the dissociation of corepressors. This conformation that allows interaction with corepressors, such as histone acetyltransferase activity important for chromatin remodeling and interactions with the basal transcriptional machinery. In the absence of ligands, nuclear receptors assume a conformation that allows interaction with corepressors, such as nuclear receptor corepressor (N-CoR), silencing mediator for retinoid and thyroid receptor (SMRT), or SHARP (65). These corepressors recruit a histone deacetylase complex that modifies chromatin structure to suppress transcription. The opposing effects of PPARγ ligands may reflect the dominance of coactivators in MCSF and the dominance of corepressors in GMCSF macrophages.

The observation that PPARγ ligands increase MPO expression in MC-Mφ is consistent with established mechanisms in which ligand binds PPARγ, causing dissociation of corepressor complexes, and recruitment of coactivator complexes, enabling transcription. However, the repression of MPO in GM-Mφ is not consistent with this model because this repression is PPARγ ligand dependent. Another means by which PPARγ can repress gene expression is termed transrepression, in which ligand-bound PPARγ competitively sequesters limiting amounts of coactivators SRC-1 and CBP (25), or transcription factors NFκB, AP1, and STAT (28). One observation seems inconsistent with transrepression; estrogen blocked the down-regulation by PPARγ suggesting that ER binding physically blocks PPARγ binding, and if so, PPARγ binding to the MPO AluRRE is necessary for repression. This may represent a novel mechanism for down-regulation through PPARγ, perhaps using a corepressor that associates with PPARγ in the specific context of the AluRRE. It is important to note that the down-regulation of MPO by PPARγ in GM-Mφ was promoter specific; CD36 and LXR were up-regulated by PPARγ in both GM-Mφ and MC-Mφ.

For disease states in which MPO is implicated, such as CAD and AD, the relative presence of GMCSF or MCSF could significantly influence expression levels. Findings here suggest that MPO expression will be higher in monocye/macrophages exposed to MCSF, and lower in GMCSF. Both MCSF (66) and GMCSF (67) are elevated in cerebrospinal fluid in Alzheimer’s patients. MCSF receptor is elevated in microglia surrounding amyloid deposits in AD (68) and in mouse models for AD expressing human amyloid precursor protein (69). Both GMCSF and MCSF are present in human atheromata (21, 70, 71). Atherosclerotic vessels are associated with higher levels of MCSF mRNA than nonatherosclerotic vessels (71), whereas MPO-positive macrophages have been detected in GMCSF-positive atheroma (21). Considering the use of TZDs in diabetic patients with high risk for CAD, our findings suggest that TZDs should reduce MPO expression if GMCSF conditions predominate, and increase MPO expression in MCSF conditions.

Induction of MPO by PPARγ could generate a positive feedback loop promoting accumulation of oxidized lipids in atherosclerotic plaques. Release of MPO by foam cell macrophages results in oxidation of LDL, producing ligands for PPARγ, such as hexadecyl azelaoyl phosphatidylcholine (37). Ligand-bound PPARγ is able to induce MPO expression as well as CD36 scavenger receptor that would promote further uptake of LDL for oxidation by MPO, creating lipid-laden foam cell macrophages at the core of plaques.

An earlier report found that, in serum-free medium, GMCSF (but not MCSF) maintains levels of MPO protein, but not MPO mRNA, in monocyte-derived macrophages (21). Our culture protocol differs significantly in that we use 10% FCS in addition to 10% autologous human serum. Consistent with the earlier report, we find that, in the absence of FCS, MPO mRNA levels drop precipitously after 24 h in culture. In the absence of FCS, MPO mRNA levels were 120-fold lower in GM-Mφ and 1000-fold lower in MC-Mφ (data not shown).

Findings here confirm that the human MPO gene can be expressed in macrophages, with expression increasing markedly in response to MCSF and PPARγ ligands. Nonetheless, levels of MPO mRNA expression are very low in macrophages in the absence of PPARγ ligands, and thus these findings are consistent with the general consensus that MPO expression is low to undetectable in macrophages (19, 21). MPO expression was ~400-fold lower in macrophages than in HL60 cells; therefore, methods less sensitive than real-time PCR are unlikely to detect MPO mRNA in quiescent macrophages. Expression in macrophages increases 20–40-fold in the presence of MCSF and PPARγ ligands. This amount of MPO expression is considerably lower than in bone marrow precursors, yet is likely to be biologically significant, especially at chronic inflammatory sites such as atherosclerotic lesions or Alzheimer’s plaques.

Consistent with our earlier findings (35, 36), the −463G allele is in most circumstances higher expressing than the A allele. The GG genotype was 5–7-fold higher expressing than GA genotypes in macrophages and 5-fold higher expressing than GA in untreated PBMC (Table I). However, in PBMC treated for 48 h with GMCSF or MCSF, expression of A allele increases relative to G allele, such that GA genotype was lower expressing than GG genotype. This raises the possibility that GA/AA genotypes may be higher expressing than GG in circulating monocytes, depending on individual variation in serum levels of GMCSF or MCSF.

The A allele was significantly higher expressed than G allele in the presence of GMCSF, PPARγ ligands, and estrogen. These conditions markedly down-regulate G allele expression, whereas the A allele maintains basal expression levels. GA expression was 4.6-fold higher than GG in GM-Mφ, and 52-fold higher in GMCSF PBMC (Table I). Conversely, the GG genotype had strong transcriptional advantage in MCSF, PPARγ ligands, and estrogen. These conditions markedly induce G allele expression, but do not affect the A allele, which again maintains basal expression. Thus, in disease states in which MPO is implicated, estrogen replacement therapy could be
deleterious for A allele carriers in GMCSF conditions, by preventing the suppression of MPO A by PPARγ. Conversely, estrogen replacement therapy may be beneficial in MCSF conditions, by suppressing the PPARγ-mediated upsurge in MPO expression for both alleles.

The ability of estrogen to block PPARγ effects, especially on the A allele, may underlie gender differences in association of –463GA genotype with disease risk (10, 11, 39, 40, 44, 46). The relative levels of estrogen, GMCSF, or MCSF may vary with age, gender, or genetic subpopulation, and could explain why the G allele is associated with AD in some studies (20, 39) and the A allele in others (38, 40). Because of conversion of testosterone to estrogen by aromatase, aged males may have higher estrogen levels than postmenopausal females. An earlier study found the A allele to be a male risk factor for AD in an aged Finnish population (38). Moreover, AA males were depleted from Finnish aged controls, suggesting selective mortality because of a common disease such as atherosclerosis.

A more recent study of Finnish males found the A allele (GA/AA genotypes) to be associated with increased severity of atherosclerosis, correlating with larger aortic lesions than GG genotypes (16). Conversely, an ultrasonic study of Finnish postmenopausal females (15) found that hormone replacement therapy decreased progression of atherosclerosis in abdominal aorta in GG, but not GA/AA genotypes.

The MPO G and A transgenic mice provided key evidence in this study, confirming observations obtained with the relatively rare AA genotype. The complex regulation of the G and A alleles by GMCSF, MCSF, estrogen, and PPARγ ligands in human cells was reproduced in the transgenic macrophages. The mouse MPO gene was found to be unresponsive to PPARγ ligands, potentially explaining the previously reported absence of MPO in lesions in the LDL receptor-deficient murine model for atherosclerosis. The inability of mouse MPO to be regulated by PPARγ raises questions as to the validity of mouse models for this and other diseases involving MPO. Crossing the human MPO transgenics onto established murine models for atherosclerosis and Alzheimer’s disease should help to humanize these models as regards MPO expression.

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**PPARγ Regulates MPO Gene Expression**

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