Effects of peroxisome proliferator-activated receptor-γ agonists on the generation of microparticles by monocytes/macrophages

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Aims

Microparticles are membrane vesicles shed by cells upon activation and/or apoptosis. Microparticles are involved in several processes, including blood coagulation and thrombosis. In addition to their role in the regulation of lipid metabolism, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists exert other effects, both dependent on and independent of PPAR-γ activation. Some PPAR-γ agonists have been linked to an increased risk of thrombotic diseases. We aimed to investigate the potential role of PPAR-γ agonists on the generation of procoagulant microparticles by human monocytes/macrophages.

Methods and results

Monocytes/macrophages were isolated from the buffy coats of normal donors. Cells were incubated with three structurally unrelated PPAR-γ agonists, namely, rosiglitazone, pioglitazone, and 15-deoxy-D¹²,¹⁴-prostaglandin J₂. Microparticle generation was assessed as phosphatidylserine concentration by a prothrombinase assay, after capturing the microparticles onto annexin V-coated wells. Intracellular calcium concentration was assessed by a fluorescent probe. Extracellular signal-regulated kinase (ERK) phosphorylation was assessed by western blot. Tissue factor expression on microparticles was measured with a one-stage clotting assay. Rosiglitazone and 15-deoxy-D¹²,¹⁴-prostaglandin J₂, but not pioglitazone, caused a dose-dependent, significant increase in intracellular calcium mobilization and tissue factor-bearing microparticle generation. EGTA inhibited microparticle generation. The specific PPAR-γ inhibitor, GW9662, also inhibited microparticle generation. Finally, rosiglitazone and 15-deoxy-D¹²,¹⁴-prostaglandin J₂ caused phosphorylation of ERK; inhibition of ERK by PD98059 inhibited microparticle generation.

Conclusion

The PPAR-γ agonists rosiglitazone and 15-deoxy-D¹²,¹⁴-prostaglandin J₂, but not pioglitazone, caused an increase in procoagulant, tissue factor-bearing microparticle generation by human monocytes/macrophages. The effect was dependent on ERK phosphorylation and partly mediated through intracellular calcium mobilization; however, direct activation of the PPAR-γ ligand was also involved.

Keywords

Microparticles • Peroxisome proliferator-activated receptor-γ • Monocyte/macrophage • Thrombosis • Extracellular signal-regulated kinase activation

1. Introduction

Microparticles (MPs), also referred to as ectosomes or microvesicles, are subcellular structures, ranging in size between 50 nm and 1 μm, released by the plasma membrane of activated and apoptotic cells.¹ As well as cytoplasmic content, MPs have complex surface membrane constituents, and it has now become clear that they represent a disseminated storage pool of bioactive effectors and participate both in the maintenance of homeostasis and in the pathogenesis of disease.¹ The processes in which the contribution of MPs has been
demonstrated include inflammation, vascular function, angiogenesis and blood coagulation. This list, however, is incomplete, and is being constantly updated.

The mechanisms whereby MPs promote blood coagulation are at least two-fold. As a result of the loss of activity of the enzyme flipase, which contributes to maintain the membrane asymmetry characteristic of resting cells, MPs expose on their surface the negatively charged phospholipids, such as phosphatidylserine (PS), normally segregated in the inner leaflet. PS is an essential component of the multi-molecular complexes tenase and prothrombinase, which activate factor X and factor II (prothrombin) into factor Xa and factor IIa (thrombin), respectively, as well as of the tissue factor (TF)/factor VII(a) complex. In the absence of primary in vivo data, it is still generally assumed that activated platelets represent the main source of PS in the coagulation process, but the possible contribution of MPs as sources of PS has long been recognized and is now becoming more appreciated. Furthermore, MPs potentially carry TF on their membranes. TF is an integral membrane protein that functions as an essential cofactor for factor VII(a) and is involved in the initiation of the coagulation cascade. Over the last decade, the classical view of TF as a for factor VII(a) and is involved in the initiation of the coagulation pathway, and possibly others, might influence the release of MPs.

2.1 Reagents and kits
RPMI 1640 medium, penicillin, streptomycin, -glutamine, fetal bovine serum, phosphate-buffered saline (PBS), Trypan Blue, Ficol-Hypaque, dextran, sodium citrate, calcium chloride, Pio, EGTA, protease inhibitors (aprotinin, pepstatin A, leupeptin, phenylmethylsulphonyl fluoride), and phorbol-12-myristate-13-acetate were obtained from Sigma (Milan, Italy). Rz, 15-deoxy-
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prothrombin. In the conditions used, the rate of thrombin formation is limited by PS availability and is therefore proportional to MP concentration. A chromogenic substrate was finally added to quantify thrombin concentration with a microplate reader (Titerkit Multiskan MCC ELISA reader; Flow Laboratories, McLean, VA, USA). As the assay depends on the availability of calcium ions, in the experiments in which EGTA was used during MP generation, 10 mM CaCl₂ was added prior to MP analysis.

2.6 Measurement of intracellular calcium concentration

Molecular Probes Fluo-4 NW Calcium Assay kit was used to measure the changes in the [Ca²⁺], of monocytes/macrophages. Briefly, pre-washed monocytes/macrophages on a 96-multiwell plate (1 x 10⁵ per well) were loaded with 100 µL of the dye loading solution containing fluo-4 NW dye and probenecid, according to the manufacturer’s instructions. The 96-well plate was incubated at 37°C for 30–45 min in the dark, and Rz, 15d-PGJ₂, Pio, and the calcium ionophore A23187 (as a positive control) were added to the cells in the presence or in the absence of GV99662. The changes in fluo-4 NW fluorescence were measured by the Wallac 1420 Victor 2 (PerkinElmer, Milan, Italy) at an excitation wavelength of 494 nm and an emission wavelength of 516 nm. Calcium mobilization was recorded over time (up to 3600 s) and analysed using Wallac 1420 Software version 3 (PerkinElmer Life and Analytical Sciences, Wallac, Milan, Italy). The increase in [Ca²⁺], was calculated as the difference between the mean stimulation fluorescence value and the mean baseline fluorescence observed × 100 (percentage increase vs. baseline).

2.7 Assessment of MP-bound TF activity

TF activity was measured in MPs generated from human monocytes/macrophages by a one-stage clotting time assay, as previously described, except that plasma rendered MP free by ultracentrifugation (100 000 × g for 2 h) was used instead of normal plasma. Briefly, normal, MP-free, human plasma (100 µL) was added to MPs (100 µL) in a 37°C bath. Calcium chloride (100 µL; 25 mM) was then added, and time to formation of a visible clot upon recalcification was recorded. The results are expressed in arbitrary units (a.u.) of procoagulant activity by comparison with a standard curve obtained using a human brain thromboplastin standard. This preparation is assigned a value of 1000 a.u. for a clotting time of 30 s. An anti-human TF antibody was used to assess the specificity of the test.

2.8 Western blot analysis for detection of phosphorylated ERK1/2

Monocytes/macrophages (4 x 10⁵) were seeded in 10 cm² culture dishes and, after overnight incubation, washed three times with pre-warmed serum-free RPMI and then treated with Rz,15d-PGJ₂, Pio, or phorbol-12-myristate-13-acetate (as positive a control) for 30 min. At the end of the treatment period, the cells were washed with ice-cold PBS and detached with 35 µL of lysis buffer, a solution containing 20 mM Tris—HCl (pH 7.5), 10% glycerol, 137 mM NaCl, 10% sodium dodecyl sulphate, 1% Triton X-100, 10 mM EDTA, and protease inhibitors (1 µg/mL aprotinin, 1 µg/mL pepstatin A, 1 µg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride). The cell lysate was cleared by centrifugation at 14 000 × g for 30 min at 4°C. The protein content in the supernatant fraction was determined by a commercially available kit based on the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s directions. Equal amounts of sample lysate (20–30 µg) were subjected to 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis according to Laemmli, and the proteins were transferred onto pure nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat dry milk (Bio-Rad Laboratories) in tris buffered saline-Tween containing 0.5% Tween 20, 50 mM Tris—HCl (pH 7.6) and 150 mM NaCl for 24 h at 4°C and were then incubated with a mouse anti-phosphorylated ERK1/2 (anti-p-ERK1/2; 1:300 dilution) overnight at 4°C. The membranes were then incubated with an antibody against α-tubulin (1:3000 dilution) for 1 h at 37°C to confirm equal protein loading. At the end of this period, the membranes were incubated with a horseradish peroxidase-conjugated goat antimouse antibody (1:1000 dilution) for 2 h at 37°C. Finally, the membranes were washed twice with tris buffered saline-Tween and once with tris buffered saline. The immunoreactive bands were visualized using an electrochemiluminescence western blotting detection reagent according to the manufacturer’s instructions.

Band intensity was calculated using image analysis software (Image version 1.42q; NIH, Bethesda, MD, USA).

2.9 Flow cytometric analysis of MPs

To characterize the MPs generated from monocytes/macrophages, samples were analysed by flow cytometry using FACSCalibur (Becton Dickinson, Oxford, UK). Data analysis was performed using WinMDI 2.9 software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA, USA). MP-containing supernatants (12 mL) were submitted to ultracentrifugation (100 000 × g for 2 h, 4°C), and the pellet was resuspended in 2 mL RPMI. One-hundred microlitres of MP were added for 30 min on ice to a phycoerythrin-conjugated antibody against CD14 (DakoCytomation, Glostrup, Denmark). Incubation was performed in the dark, and the expression of the surface marker was analysed. CD14 positivity was defined on phycoerythrin fluorescence vs. forward scatter dot plot representations. Regions corresponding to MPs were defined on forward vs. side-angle light scatter intensity dot plot representations. The forward light scatter setting was E-01, and a total of 10 000 events were analysed.

2.10 Data presentation and statistical analysis

Unless otherwise indicated, data are shown as means ± SEM from n independent, consecutive experiments; comparisons among groups were made by either ANOVA for repeated measures followed by Bonferroni’s analysis or Student’s paired t-test, as appropriate, using Prism Software (GraphPad, San Diego, CA, USA). Values of P < 0.05 were considered statistically significant.

3. Results

3.1 Rz and 15d-PGJ₂, but not Pio, induce the generation of procoagulant MPs by monocytes/macrophages

To investigate whether PPAR-γ agonists induce the release of MPs, monocytes/macrophages were stimulated with different concentrations of Rz, of the naturally occurring PPAR-γ agonist, 15d-PGJ₂, and of Pio for 1 h; the supernatants were collected, and MPs were quantified as nanomolar PS. As shown in Figure 1, monocytes/macrophages generate MPs upon stimulation with both Rz (Figure 1A and D) and 15d-PGJ₂ (Figure 1B and E) in a dose-dependent manner; the effect reached statistical significance at 80 µM for Rz and at 20 µM for 15d-PGJ₂. In contrast, Pio did not exert any effect at the highest concentration (80 µM; Figure 1C).

The cellular origin of these MPs was analysed by flow cytometry. As shown in Figure 1F, approximately 96% of MPs were CD14 positive, confirming their monocytic origin.
3.2 Role of Ca\(^{2+}\) mobilization in Rz- and 15d-PGJ\(_2\)-induced generation of MPs by monocytes/macrophages

The mechanisms that regulate the generation of MPs are not completely defined; however, it is known that MPs are released following activation or cell death, the latter being due to either apoptosis or necrosis.\(^{18}\) Mobilization of Ca\(^{2+}\) is a critical step in the cell activation process. To investigate whether PPAR-\(\gamma\) agonists affect intracellular calcium mobilization, monocytes/macrophages were loaded with Fluo-4 NW probe and stimulated with Rz, 15d-PGJ\(_2\), or Pio. Our data show that Rz (80 \(\mu\)M) and 15d-PGJ\(_2\) (20 \(\mu\)M), but not Pio (80 \(\mu\)M), induce a rapid increase in [Ca\(^{2+}\)], observed over time. The calcium ionophore, A23187 (12 \(\mu\)M), was used as a positive control (Figure 2). To investigate the role of Ca\(^{2+}\) mobilization in generation of MPs further, the calcium chelator, EGTA, was used. The addition of EGTA prior to monocyte/macrophage stimulation with Rz or 15d-PGJ\(_2\) largely inhibited MP generation (Figure 3).

3.3 Effect of PPAR-\(\gamma\) inhibition on shedding of MPs stimulated by PPAR-\(\gamma\)

PPAR-\(\gamma\) agonists have been shown to exert some effects independent of PPAR-\(\gamma\) activation.\(^{29–31}\) To investigate the role of PPAR-\(\gamma\) activation, the specific inhibitor, GW9662, was used. GW9662 did not affect intracellular calcium mobilization induced by either Rz or 15d-PGJ\(_2\) (see Supplementary material online, Figure S1); however, GW9662 caused a significant inhibition of the MP generation induced by Rz and 15d-PGJ\(_2\) (Figure 4).
3.4 Role of ERK in PPAR-γ agonist-induced MP release

PPAR-γ agonists are also known to stimulate MAPK activities in certain cell lines.32 We sought to investigate whether ERK phosphorylation is involved in Rz- and 15d-PGJ₂-induced shedding of MPs.

**Figure 2** Evaluation of intracellular calcium mobilization in monocytes/macrophages treated with Rz (80 μM; upright filled triangles), 15d-PGJ₂ (20 μM; inverted filled triangles), Pio (80 μM; filled diamonds), or the calcium ionophore, A23187 (12 μM; filled circles). The cells were pre-loaded with the fluorescent probe fluo-4 NW for 45 min prior to the addition of the relevant agonist; the changes in [Ca²⁺] were monitored for 3600 s, and the increase in fluo4-NW fluorescence was calculated as described in the Methods; data are from one experiment representative of three.

**Figure 3** Effect of EGTA on MP generation by monocytes/macrophages induced by peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists. The cells were pre-treated for 30 min with EGTA (1 mM) prior to stimulation for 1 h with Rz (80 μM) or 15d-PGJ₂ (20 μM). *P < 0.05 for EGTA-treated vs. untreated cells (ANOVA); n = 4.

**Figure 4** Effect of GW9662 on MP generation by monocytes/macrophages stimulated with PPAR-γ agonists. The cells were pre-treated for 30 min with GW9662 (80 or 20 μM to match the agonist concentration) before stimulation for 1 h with Rz (80 μM) or 15d-PGJ₂ (20 μM). *P < 0.05 for GW9662-treated vs. untreated cells (ANOVA); n = 4.

Figure 5A shows that both Rz and 15d-PGJ₂ induce ERK phosphorylation in monocytes/macrophages. Furthermore, the selective non-competitive inhibitor of the MAPK pathway, PD98059, inhibits MP generation (Figure 5B). In contrast to Rz and 15d-PGJ₂, Pio did not induce ERK phosphorylation (Figure 5C), consistent with the hypothesis that ERK phosphorylation is involved in Rz- and 15d-PGJ₂-mediated MP generation.
3.5 Rz and 15d-PGJ2, but not Pio, induce the expression of MP-bound TF

The procoagulant activity of MPs is due to the exposure of PS on their surface and is also enhanced by the presence of functional TF.15 To evaluate whether Rz and 15d-PGJ2 induce the generation of TF-bearing MPs by monocytes/macrophages, we analysed the procoagulant activity of purified MPs released by treated and untreated cells through a one-stage clotting test. As shown in Figure 6, Rz and 15d-PGJ2 induce a 2- to 2.5-fold increase in procoagulant activity of MPs. A monoclonal antibody to TF (epitope specific for amino acids 1–25; American Diagnostics, Stamford, CT, USA; 30 μg/mL) inhibited most of the procoagulant activity (not shown), confirming the identity of this activity with TF. In contrast, Pio did not exert any effect. The MAPK inhibitor, PD98059, and the specific PPAR-γ inhibitor, GW9662, inhibited the up-regulation of the expression of MP-bound TF.

As platelets respond to numerous different stimuli with the generation of TF-bearing MPs,10 we performed experiments in which a number of platelets corresponding to 10 times the number of mononuclear cells were treated exactly as described for mononuclear cells. In our hands, contaminating platelets are consistently less than two per mononuclear cell, so that the number of platelets used in our experiments is in large excess compared with the number of platelets that can potentially contaminate our preparations. We could not detect any significant generation of MP-bound TF upon incubation with rosiglitazone (not shown).

4. Discussion

The aim of this work was to investigate whether Rz and other PPAR-γ agonists might influence release of procoagulant MPs by human monocytes/macrophages. Our results show that, indeed, the thiazolidinedione, Rz, as well as the naturally occurring agonist, 15d-PGJ2, cause an increased release of MP. It has previously been shown that Rz causes an increase in intracellular calcium in monocytes, and that this property of Rz is responsible for an inhibition in actin polymerization.24 In turn, it is known that destabilization of the actin cytoskeleton, at least in platelets, causes disconnection of the plasma membrane and MP formation.33 Thus, the present data, including the demonstration that Rz and 15d-PGJ2 cause an increase in [Ca2+]i, are consistent with the hypothesis that PPAR-γ activation stimulates MP shedding in monocytes/macrophages through calcium mobilization. The demonstration that EGTA largely inhibits the effect lends further support to this hypothesis. However, we also show that the PPAR-γ inhibitor, GW9662, does not affect calcium mobilization while still inhibiting MP formation, which indicates that PPAR-γ agonist-induced MP shedding via calcium mobilization is independent of ligand–receptor interaction. A recent report by Li et al. has demonstrated the involvement of the ERK pathway in the generation of MPs by human monocytes stimulated with cigarette smoke extract.34 As PPAR-γ agonists induce ERK phosphorylation,32 we speculated that the ERK pathway might be involved in the generation of MPs induced by PPAR-γ agonists. Our data showing that ERK is phosphorylated by PPAR-γ agonists and that ERK inhibition abolishes MP generation are indeed consistent with the hypothesis.

Thus, it appears that Rz and 15d-PGJ2 stimulate MP formation through at least two distinct mechanisms, one dependent on calcium mobilization, largely independent of the PPAR-γ receptor, and possibly mediated through ERK phosphorylation,35 and one mediated through the activation of the PPAR-γ receptor.

In conclusion, we demonstrate that two structurally unrelated PPAR-γ agonists, the member of the thiazolidinedione family, Rz,
and the naturally occurring prostaglandin, 15d-PGJ_{2}, up-regulate the expression of procoagulant, TF-bearing MPs by human monocytes/macrophages through both a PPAR-γ activation-independent increase in [Ca^{2+}], and a PPAR-γ activation-dependent mechanism. Another member of the thiazolidinedione family, namely Pio, failed to show the same effect. Differences among members of this family in terms of biological activity have been reported. Regarding calcium handling, a recent study aimed to investigate the chemical sensing profiles of transient receptor potential (TRP) channels, for example, has led to the observation that Rz causes an increase in [Ca^{2+}], in cells overexpressing TRP canonical 5 (TRPC5) channel. In contrast, Pio, as well as two other members of the same family, troglitazone and ciglitazone, completely failed to evoke TRPC5 activity. The specific requirements for TRPC5 channel activation were not investigated further, but the authors conclude that the thiazolidinedione moiety is insufficient for TRPC5 modulation.

In light of the growing interest in MPs as physiologically relevant effectors in human blood coagulation, these data add to our knowledge on the mechanisms underlying their generation.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

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**References**


35. Franklin RA, Atherfold PA, McCubrey JA. Calcium-induced ERK activation in human T lymphocytes occurs via p56(lck) and CaMK-kinase. Mol Immunol 2003;37:675–683.
