Ciglitazone and 15d-PGJ2 Induced Suicidal Erythrocyte Death

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Key Words

Anemia • Eryptosis • Apoptosis • Calcium • Cell volume

Abstract

Side effects of peroxisome proliferator activated receptor gamma (PPAR γ) agonists such as ciglitazone include anemia, which in theory could be due to decreased formation or premature death of erythrocytes. A form of suicidal erythrocyte death is eryptosis, which is characterized by cell shrinkage and by breakdown of phosphatidylserine asymmetry leading to phosphatidylserine exposure at the cell surface. Phosphatidylserine-exposing erythrocytes are recognized by macrophages, engulfed, degraded and thus cleared from circulating blood. Triggers of eryptosis include increase in intracellular Ca²⁺ concentration. The present study thus explored, whether the PPAR γ agonist ciglitazone or the natural PPAR γ ligand 15deoxy-delta12,14-prostaglandin J2 (15d-PGJ2) are capable to trigger eryptosis. Phosphatidylserine exposure was determined from annexin V binding and cell shrinkage from decrease of forward scatter of human erythrocytes in FACS analysis. Both,

ciglitazone ($\geq 5 \mu\text{M}$) and 15d-PGJ2 ($\geq 3 \mu\text{M}$), within 24 hours increased phosphatidylserine exposure and at concentrations of 10 μM led to a significant loss of the cell volume. Ciglitazone further stimulated hemolysis, which, however, affected only a fraction of erythrocytes undergoing eryptosis. According to Fluo3 fluorescence of human erythrocytes, 10 μM ciglitazone or 15d-PGJ2 increased intracellular Ca²⁺ activity. In conclusion, ciglitazone and 15d-PGJ2 trigger eryptosis at least in part by an increase in the cytosolic Ca²⁺ concentration. The effect most likely contributes to the anemia observed following treatment with PPAR γ agonists.

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Introduction

Peroxisome proliferator activated receptor gamma (PPAR γ) agonists enhance insulin sensitivity and thus lower plasma glucose concentration particularly in patients with insulin resistance [1]. The drugs may, however,

increase the risk of congestive heart failure [2]. Moreover, side effects of PPAR γ agonists include anemia, which may limit the use of those drugs [3]. In theory, anemia could result from decreased formation or premature clearance of circulating erythrocytes. Erythrocytes may be cleared by eryptosis, the apoptosis-like suicidal death of mature defective erythrocytes [4]. PPAR γ agonists such as ciglitazone are known to trigger suicidal death or apoptosis of nucleated cells [5, 6].

Natural PPAR γ ligands such as 15deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15d-PGJ2) have similarly been shown to trigger apoptosis [5]. Thus, the present study explored, whether the PPAR γ agonist ciglitazone or the natural ligand 15d-PGJ2 are capable of triggering eryptosis.

As demonstrated by various laboratories, an increased intracellular Ca²⁺ activity, which may result from Ca²⁺ entry through Ca²⁺-permeable cation channels [7], is one of the key stimulators of eryptosis [8]. Ca²⁺ triggers scrambling of the cell membrane with subsequent phosphatidylserine exposure at the cell surface [4, 8]. Phosphatidylserine - exposing erythrocytes are recognized by phosphatidylserine receptors on macrophages [9] and thus engulfed and degraded [10]. In addition, an increase in the intracellular Ca²⁺ concentration leads to activation of Ca²⁺-sensitive K⁺ channels [11] with subsequent hyperpolarization of the cell membrane, Cl⁻ exit through Cl⁻ channels [12], cellular loss of KCl and osmotically obliged water and thus cell shrinkage [8].

The present study explored the effect of ciglitazone and 15d-PGJ2 on intracellular Ca²⁺ concentration, phosphatidylserine exposure, and cell volume of mature erythrocytes.

Materials and Methods

Solutions

Erythrocytes were drawn from healthy volunteers, and erythrocyte concentrates were obtained as described recently using standard leukocyte depletion filters [13]. The study was approved by the local ethics committee of the University of Tübingen (project#: 184/2003V). Virtually leukocyte-free erythrocyte concentrates were used in this study. Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), 5 glucose, 1 CaCl₂. In Ca²⁺-free Ringer solution, 1 mM CaCl₂ was replaced by 1 mM glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). Ciglitazone and 15d-PGJ2 (both from Calbiochem, Bad Soden, Germany) were dissolved in DMSO. In all control experiments, the vehicle DMSO was applied at the highest concentration used in this series of experiments.

Fig. 1. Effect of ciglitazone on Ca²⁺-dependent Fluo3-fluorescence of erythrocytes: A. Original histograms of Fluo3-fluorescence of human erythrocytes following a 6 hours exposure to 0 μ M (left panel) or 10 μ M ciglitazone (right panel). B. Arithmetic means $\pm$ SEM (n = 8) of the Fluo3-fluorescence following a 6 hours exposure to 0 μ M (control, open bar) or to 10 μ M (closed bar) ciglitazone. * indicates significant (p < 0.05) difference from the absence of ciglitazone (unpaired t-test).

FACS analysis

FACS analysis was performed as described [8]. After incubation in the presence or absence of ciglitazone or 15d-PGJ2, cells were washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES/NaOH (pH 7.4), 5 CaCl₂. Erythrocytes were suspended in a solution composed of annexin V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin buffer (dilution of 1:50). After 10 min of incubation, samples were finally diluted 1:5 in annexin-binding buffer and measured on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analysed by forward scatter and annexin fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca²⁺

Intracellular Ca²⁺ measurements were performed as described [14]. Briefly, erythrocytes were loaded with

Fig. 2. Effect of 15d-PGJ2 on Ca^{2+} -dependent Fluo3-fluorescence of erythrocytes: A. Original histograms of Fluo3-fluorescence of human erythrocytes following a 6 hours exposure to 0 μM (left panel) or 10 μM natural PPAR γ ligand 15deoxy-delta12,14-prostaglandin J2 (15d-PGJ2) (right panel). B. Arithmetic means $\pm$ SEM (n = 4) of the Fluo3-fluorescence following a 6 hours exposure to 0 μM (control, open bar) or to 10 μM 15d-PGJ2 (closed bar). * indicates significant ($p < 0.05$) difference from the absence of 15d-PGJ2 (unpaired t-test).

Fluo-3/AM (Calbiochem; Bad Soden, Germany) by addition of 2 μl of a Fluo-3/AM stock solution (2.0 mM in DMSO) to 1 ml erythrocyte suspension (0.16% packed cell volume in Ringer). The cells were incubated at 37°C for 15 min under protection from light. An additional 2 μl aliquot of the Fluo-3/AM stock solution was added with incubation carried out for 25 min. Fluo-3-loaded erythrocytes were centrifuged at 400 g for 5 min at 22°C and washed two times with Ringer solution containing 0.5% bovine serum albumin (Sigma) and one time with Ringer. For flow cytometry, Fluo-3/AM-loaded erythrocytes were re-suspended in 1 ml Ringer (0.16% packed cell volume) containing ciglitazone (10 μM), 15d-PGJ2 (10 μM), or vehicle alone (DMSO) and incubated for 6 hours at 37°C. Then, Ca^{2+} -dependent fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Fig. 3. Effect of ciglitazone on erythrocyte forward scatter: A. Original histograms of forward scatter of human erythrocytes following a 24 hours exposure to 0 μM (left panel) or 10 μM ciglitazone (right panel). B. Arithmetic means $\pm$ SEM (n = 13-18) of forward scatter following a 24 hours exposure to 0 μM (control, open bar), to 5 μM , or to 10 μM 15d-PGJ2 (closed bars). * indicates significant ($p < 0.05$) difference from absence of 15d-PGJ2 (ANOVA).

Measurement of hemolysis

Erythrocytes were incubated for 24 hours at 37°C in the presence of 5 μM , 10 μM , or 15 μM ciglitazone. The incubation was stopped by centrifugation (2 min at 1000 g), and the supernatants were harvested. Hemolysis of control erythrocytes in distilled water was defined as complete hemolysis. As a measure of hemolysis, the hemoglobin (Hb) concentrations of the supernatants were determined photometrically at 546 nm upon transforming the Hb into cyanometHb as described earlier [15]. Sample values were expressed as the percentage of complete hemolysis.

Statistics

Data are expressed as arithmetic means $\pm$ SEM, and statistical analysis was made by paired or unpaired t-test or ANOVA, as appropriate. $p < 0.05$ was considered as statistically significant.

Fig. 4. Effect of 15d-PGJ2 on erythrocyte forward scatter: A. Original histograms of forward scatter of human erythrocytes following a 24 hours exposure to 0 μM (left panel) or 10 μM 15d-PGJ2 (right panel). B. Arithmetic means ± SEM (n = 13-18) of forward scatter following a 24 hours exposure to 0 μM (control, open bar) or to 10 μM 15d-PGJ2 (closed bar). * indicates significant (p < 0.05) difference from absence of 15d-PGJ2 (t-test).

Results

Exposure of human erythrocytes to the PPAR γ agonist ciglitazone significantly increased Fluo3-fluorescence within 6 hours pointing to increase in cytosolic Ca²⁺ concentration (Fig. 1). Significant effects were observed at a ciglitazone concentration of 10 μM (Fig. 1). The effect was mimicked by exposure of erythrocytes to the natural PPAR γ ligand 15d-PGJ2. Exposure to 10 μM 15d-PGJ2 also significantly increased Fluo3 fluorescence (Fig. 2).

An increase in the intracellular Ca²⁺ activity has previously been shown to activate Ca²⁺-sensitive K⁺ channels [11], thus leading to hyperpolarization of the cell membrane and subsequent extrusion of KCl. The loss of

Fig. 5. Effect of ciglitazone on erythrocyte annexin V-binding: A. Original histograms of annexin V-binding of human erythrocytes following a 24 hours exposure to 0 μM (left panel) or 10 μM ciglitazone (right panel). B. Arithmetic means ± SEM (n = 13-18) of annexin V-binding following a 24 hours exposure to 0 μM ciglitazone (control, open bar) or to concentrations of ciglitazone ranging from 2 μM to 10 μM (closed bars). * indicates significant (p < 0.05) difference from absence of ciglitazone (ANOVA). C. Arithmetic means ± SEM (n = 8) of annexin V-binding following a 24 hours exposure to 0 μM (left bars) or 10 μM ciglitazone (right bars) in the presence (white bars) or absence (black bars) of extracellular Ca²⁺. ### indicates significant (p < 0.001) difference (ANOVA).

Fig. 6. Effect of 15d-PGJ2 on erythrocyte annexin V-binding: A. Original histograms of annexin V-binding of human erythrocytes following a 24 hours exposure to 0 μ M (left panel) or 10 μ M 15d-PGJ2 (right panel). B. Arithmetic means $\pm$ SEM (n = 7) of annexin V-binding following a 24 hours exposure to 0 μ M 15d-PGJ2 (control, open bar) or to concentrations of 15d-PGJ2 ranging from 1 μ M to 10 μ M (closed bars). * indicates significant (p<0.05) difference from absence of 15d-PGJ2 (ANOVA).

KCl and osmotically obliged water shrinks the cells, which is reflected by a decrease of the forward scatter in FACS analysis. Fig. 3 indeed shows that a 24 hours exposure to 10 μ M ciglitazone led to a decrease of the erythrocyte forward scatter. The natural PPAR γ ligand 15d-PGJ2 similarly shrunk the cells (Fig. 4).

Increased cytosolic Ca²⁺ activity has further been shown to trigger cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocyte surface [8, 14]. Phosphatidylserine exposure is disclosed by annexin V binding. As illustrated in Fig. 5A,B, ciglitazone (≥ 5 μ M) indeed enhanced the percentage of annexin V-binding erythrocytes within 24 hours. In the nominal

Fig. 7. Ciglitazone induced hemolysis: Arithmetic means $\pm$ SEM (n = 5) of hemoglobin release following a 24 hours incubation without (open bar) or with (closed bars) ciglitazone at concentrations ranging from 2 to 10 μ M. Hemolysis is expressed as the percentage of complete hemolysis in distilled water. * indicates significant difference from the absence of ciglitazone (ANOVA).

absence of Ca²⁺, the effect of ciglitazone on annexin V-binding was significantly blunted (Fig. 5C). The natural ligand 15d-PGJ2 (≥ 3 μ M) similarly increased erythrocyte annexin V-binding (Fig. 6).

To explore, whether ciglitazone-induced phosphatidylserine exposure is paralleled by hemolysis, hemoglobin release into the supernatant was quantified following treatment of the erythrocytes with ciglitazone. As illustrated in Fig. 7, ciglitazone indeed triggered some hemolysis, which, however, affected only one third of the erythrocytes exposing phosphatidylserine. Thus, ciglitazone-mediated effects on phosphatidylserine exposure cannot be explained by cell lysis.

Discussion

The present study demonstrates that the PPAR γ agonist ciglitazone and the natural PPAR γ ligand 15d-PGJ2 enhance cytosolic Ca²⁺ activity in mature human erythrocytes.

The increased cytosolic Ca^{2+} activity could contribute to or even account for the stimulation of phospholipids scrambling leading to phosphatidylserine exposure at the erythrocyte surface [4].

Whether or not this effect is mediated by activation of PPAR γ , cannot be decided on the basis of the present observations. PPAR γ -independent ciglitazone effects have been reported [16]. On the other hand, the effective concentrations are well in the range of those utilized to stimulate PPAR γ [17-21]. In any case, side effects of ciglitazone may include eryptosis, which could contribute to the anemia of patients treated with PPAR γ agonists [3].

Eryptosis has previously been observed in a wide variety of anemic conditions, including sickle-cell anemia [22], beta-thalassemia [23], glucose-6-phosphate dehydrogenase (G6PD)-deficiency [23], phosphate depletion [24], iron deficiency [25], Hemolytic Uremic Syndrome [26], sepsis [27], malaria [28] and Wilson disease [29]. Moreover, enhanced eryptosis could contribute to the anemia following excessive formation, treatment or intoxication with hemolysin Kanagawa [30], listeriolysin [31], PGE₂ [32], lipoxygenase inhibitor Bay-5884 [33], platelet activating factor [13], paclitaxel [34], methylglyoxal [35], chlorpromazine [36], cyclosporine [37], amantadine [38], curcumin [39], amyloid peptides [40], valinomycin [41], aluminium [42], lead [43], mercury [44], gold [45] and copper ions [29]. Thus, eryptosis participates in the pathophysiology of several diseases and contributes to side effects of a wide variety of drugs.

At least in theory, eryptosis could foster erythrocyte adherence to the vascular wall [46] with subsequent impairment of microcirculation [46]. Trapping of

annexin-V binding erythrocytes has previously been observed in the renal medulla following renal ischemia [47]. Adhesion of erythrocytes to the vascular wall could participate in hemostasis [48] and may favour the development of thrombosis [46]. This effect may, however, be more than outweighed by the endothelium-protecting, blood pressure lowering, anti-inflammatory, vasodilatory and anti-atherogenic effects of at least some PPAR γ agonists [49]. Moreover, the glucose lowering effect of PPAR γ agonists should decrease HbA1c and thus reduce oxygen affinity [50] which would facilitate oxygen delivery to peripheral tissues. Nevertheless, the stimulation of erythrocyte phosphatidylserine exposure with subsequent enhancement of adherence may present a hitherto unrecognized side effect of PPAR γ agonists, which at least in theory may become pathophysiologically significant under unfavourable conditions.

In conclusion, the peroxisome proliferator activated receptor gamma (PPAR γ) agonist ciglitazone and the natural PPAR γ ligand 15d-PGJ2 stimulate eryptosis. The clearance of suicidal erythrocytes from circulating blood could contribute to the development of anemia.

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