Apoptosis and proliferation of cultured mesangial cells isolated from kidneys of rosiglitazone-treated pregnant diabetic rats

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Abstract

Background. The peroxisome proliferator activating nuclear receptors (PPAR) are activated in the context of inflammation, diabetes or normal pregnancy. Renal mesangial cells express PPAR-γ which upon activation are capable of exerting anti-inflammatory effects. We investigated the effect of in vivo treatment by rosiglitazone on angiotensin II (A-II) stimulated manifestations of inflammation in cultured renal mesangial cells, such as proliferation, apoptosis, TGF-β1 production and nuclear factor κB (NF-κB) activation, in the situation of pregnancies, complicated or not with diabetes.

Methods. Mesangial cells were isolated from the following groups, receiving or not 5 mg/kg rosiglitazone for 20 days: normal controls, normal pregnant rats, those with streptozotocine induced diabetes and pregnant diabetic rats. Proliferation was assessed by ³H-thymidine incorporation. Apoptosis was evaluated by TUNEL assay. AT-1/AT-2 receptor density was assessed by ¹²⁵I-AT-2 labelling, TGF-β and NF-κB by specific ELISAs.

Results. Rosiglitazone pretreatment resulted in significantly decreased proliferation, apoptosis and reduced responsiveness to A-II stimulation in cultures from controls, pregnant rats and non-pregnant diabetic animals. In the pregnant diabetic group which received rosiglitazone prior to sacrifice, responsiveness to A-II was completely blunted. Moderate attenuation of TGF-β synthesis and significant decrease in the levels of NF-κB in mesangial cell nuclei were observed in all rosiglitazone treated groups.

Conclusions. PPAR-γ activation by rosiglitazone resulted in decreased manifestation of inflammatory hallmarks, including inhibition of mesangial cell proliferation, downregulation of apoptosis and blunted responsiveness to A-II. These anti-inflammatory renoprotective effects were maximally expressed in cultures from pregnant diabetic animals. The therapeutic relevance of these observations is a matter of further investigations.

Keywords: angiotensin II; apoptosis; diabetes; PPAR; pregnancy; proliferation

Introduction

The family of peroxisome proliferator activating nuclear receptors (PPAR) has been shown to play, among others, a significant role in enhancement of cellular glucose uptake and blunting of inflammation [1]. With respect to the kidney, the presence of PPAR, both message and protein, has been recently demonstrated in glomeruli as well as in various tubular segments [2]. In a recently published study, Baylis et al. demonstrated that in a rat model of diabetic nephropathy rosiglitazone, a pharmacologic agonist of PPAR affords a better renal protection compared with ACE inhibitors by a yet unknown mechanism [3].

Thus far, only the PPAR-γ subtype has been identified in renal mesangium [2,4]. Following in vitro activation by physiological or pharmacological ligands, PPAR-γ exert a variety of anti-inflammatory effects on renal tissue, such as blunting of spontaneous or growth factor-induced proliferation and extracellular matrix deposition or activation of apoptosis in immunostimulatory cells infiltrating the inflamed kidney [2,4,5]. In yet a completely different situation, i.e. pregnancy, alterations in the expression of PPAR have also been demonstrated. Thus, PPAR have been shown to be activated in trophoblasts and lutein cells during normal pregnancy [6], while serum of pregnant women was shown to induce or enhance PPAR expression in
Rosiglitazone ameliorates mesangial cell proliferation and apoptosis

cultured human placental cells and gynaecological cancer cell lines [7].

The thiazolidinedione group of drugs, the pharmacological PPAR ligands, is being increasingly used as insulin sensitizers in type 2 diabetic patients. Although theoretically attractive, their effects on the course of renal inflammatory diseases have not been hitherto convincingly established. The present investigation was undertaken to assess the effect of in vivo treatment by rosiglitazone, a PPAR-γ ligand, on A-II stimulated proliferation and apoptosis of mesangial cells in the context of pregnancy, complicated or not by streptozotocin induced diabetes. TGF-β synthesis, as well as the presence of nuclear factor κB (NF-κB) in cytosolic and nuclear fractions of mesangial cells were concomitantly assessed.

Materials and methods

Animals

Thirty-two female (16 pregnant and 16 non-pregnant), 2-months-old Sprague–Dawley rats were included in the study. They were maintained according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the local ethics committee for animal experimentation.

The rats were divided in four main experimental groups (n = 8 in each).
1. Control animals.
2. Pregnant rats.
3. Rats made diabetic by i.p. injection of streptozotocin, 5 mg/kg body weight, in a 0.5 ml bolus.

Each group was divided in two subgroups, a and b, four rats in each:
1a through 4a – not treated with rosiglitazone.
1b through 4b – receiving 5 mg/kg body weight rosiglitazone daily in their drinking water. Blood glucose was measured once before starting the rosiglitazone treatment and once following 3 weeks, prior to sacrifice. In addition, different, similarly treated animals (two from each group) were held in metabolic cages, their 24 h urine collected and used for assessment of total 24 h protein excretion. Following 20 days, i.e. 1 day before the expected deliveries of the pregnant participants, all the animals were sacrificed under fluothane anaesthesia. Their kidneys were removed under sterile conditions.

Mesangial cell culture

Glomerular mesangium was isolated and cultured in a selective mesangial cell culture medium RPMI 1640 supplemented with δ-valine instead of L-valine and with 20% fetal calf serum, as described elsewhere [8]. Unlike cells which can be maintained, partly or solely, in suspension, renal mesangial cells only grow firmly attached to the culture vessel bottom. When detached with trypsin—EDTA solution in order to be equally divided into the wells of the experimental tissue plate, they change their appearance, form small clusters, making cell count under light microscope unreliable. Therefore, when seeded into the wells of the experimental plates and later at each appropriate stage of experiment, cell numbers of the experimental variables were adjusted by total protein measurement, in order to subsequently present the results per milligram cell protein. The cells were seeded in equal protein aliquots on round glass coverslips in 12-well tissue culture plates in the following experimental variations.

1. Untreated control cultures.
2. Cultures stimulated with 10⁻⁸ MAT-2.
3. Cultures pretreated with 10⁻⁶ M losartan (AT-1 receptor blocker) 2 h prior to AT-2 addition.
4. Cultures pretreated with 10⁻⁶ M PD123319 (AT-2 receptor blocker) 2 h prior to AT-2 addition.

Evaluation of proliferation and apoptosis

In cultures assigned for proliferative rate assays, 1 μCi/ml 3H-thymidine was added to the wells. These cultures were terminated 24 h following 3H-thymidine addition, the coverslips removed with forceps, washed three times in PBS and radioactivity counted in a β-counter. The parallel cultures were used for apoptosis rate evaluation. The latter was performed using two independent methods, TUNEL assay and microscopic evaluation following Mayer’s Haematoxylin staining, as described elsewhere [8].

For each experimental variation, a total of 10 slides was examined (n = 10). For each slide, a total count of 1000 cells, combined with a differential count of only the stained apoptotic cells was performed under light microscope. Subsequently, percent apoptosis was calculated as follows:

\[
\%\text{APOPTOSIS} = \frac{\text{ACC} \times \text{TCC}}{100}
\]

where ACC is apoptotic cell count, TCC is total cell count and ACC/TCC is percent apoptotic cells out of total cell count, expressed as %.

Assessment of TGF-β1

Production of TGF-β1 was assessed in cell culture medium by a specific ELISA (Quantikine, R&D Systems, USA). In brief, the tested samples were placed into the wells of a 96-well microplate precoated with a specific rat anti-TGFβ1 antibody. Subsequently, an enzyme-linked polyclonal specific antibody was added, to form a ‘sandwich’ with the immobilized TGF-β1. Following the TMB substrate addition and colour development, the intensity of the colour was measured and translated into the concentration units using ELISA reader.

NF-κB p50/p65 transcription factor assay

Cytosolic and nuclear extracts of mesangial cells were obtained using a Cheimon Nuclear Extraction kit (Chemicon International, USA) according to the manufacturer’s protocol. The prepared extracts were stored at −80°C until used for the assay.

The NF-κB p50/p65 transcription factor assay was performed using a kit combining the principle of the electrophoretic mobility shift assay (EMSA) with the
96-well based ELISA, which enables significantly greater sensitivity compared with conventional EMSA assays (Chemicon International, USA). Whole cell extract from HeLa cell sample was provided by the manufacturer, to serve as a positive control probe. In brief, the prepared cell extracts were mixed with biotinylated oligonucleotide containing the flanked DNA binding sequence for NF-κB (5'-GGGACTTTCC-3'). Following 2h incubation allowing the active (i.e. not bound to Ik-B chain) form of NF-κB to bind to its concensus sequence, the mixed samples were transferred to the streptavidin-coated 96-well plate. Rabbit anti-NF-κB p50/p65 antibodies were used as specific primary antibodies. Goat anti-rabbit, horseradish peroxydase-conjugated antibody was used as a secondary antibody. TMB substrate was added to the wells for the final colour development. The intensity of the colour was read and translated into concentration units in the ELISA reader.

**AT-1/AT-2 receptor density**

Total as well as specific AT-1/AT-2 receptor density was assessed using 125I-Angiotensin II radioactive labelling of cultures pretreated or not with AT-1 or AT-2 receptor blockers (losartan or PD123319, respectively). Following 7h labelling, the glass coverslips were removed, repeatedly washed in PBS and radioactivity counted in a γ-counter.

**Statistical analysis**

The results were evaluated using the non-parametric Mann Whitney test. The differences between the variables yielding P-values <0.05 were considered statistically significant.

**Results**

All the experimental animals subjected to streptozotocin injection developed diabetes within 48h, as evidenced by their elevated blood glucose (Table 1). At the end of the experiment, diabetic animals not receiving rosiglitazone demonstrated significantly higher blood glucose and significantly elevated 24h protein excretion. The latter could be also observed in pregnant non-diabetic rats. All these parameters were significantly attenuated in the parallel, rosiglitazone treated animal subgroups (Table 1).

**Mesangial cell proliferation**

A-II induced significant 3H-thymidine incorporation in mesangial cell cultures of all four experimental groups not receiving rosiglitazone prior to sacrifice (Figure 1, black bars). Blockade of AT-1/AT-2 receptor by losartan 2h before 3H-thymidine addition abolished the enhanced cell proliferation, while blocking of AT-2 receptor by PD123319 had no statistically significant effect.

In cultures from normal controls as well as pregnant normal and non-pregnant diabetic animals which received rosiglitazone for 3 weeks before sacrifice, a significant reduction in A-II stimulated 3H-thymidine incorporation was observed. In mesangial cultures from pregnant diabetic rosiglitazone-treated rats, proliferative response to A-II was statistically not different from the baseline levels (Figure 1, dotted bars).

**Apoptosis**

Basal apoptotic rates of mesangial cells from normal, non-pregnant and non-diabetic animals were significantly lower compared with any other group baseline (Figure 2, P<0.03 in each comparison).

A-II stimulation significantly augmented apoptosis rates in normal control mesangial cells as well as in experimental cultures of rats not receiving rosiglitazone before sacrifice as compared with their respective baselines. By contrast, apoptotic rates of cultures from rosiglitazone-receiving animals were significantly inhibited compared with their rosiglitazone-untreated counterparts (Figure 2, P<0.005 in each comparison). In cultures from pregnant diabetic rosiglitazone-treated animals percent apoptosis was statistically not different from that of control A-II stimulated baseline.

**AT-1/AT-2 receptor density**

Total Angiotensin II receptor density, as well as specific AT-1/AT-2 receptor distribution, were not statistically

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**Table 1. Biochemical data on the experimental animals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline Blood glucose (mg/dl)</th>
<th>Baseline Urine protein (mg/24h)</th>
<th>Day 20 Blood glucose (mg/dl)</th>
<th>Day 20 Urine protein (mg/24h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>115 ± 3</td>
<td>10.5 ± 2.3</td>
<td>112 ± 2.5</td>
<td>11.1 ± 2.18</td>
</tr>
<tr>
<td>Control + rosiglitazone</td>
<td>113 ± 3</td>
<td>11.1 ± 2.4</td>
<td>106 ± 2.1</td>
<td>9.3 ± 1.8</td>
</tr>
<tr>
<td>Pregnant</td>
<td>111 ± 2</td>
<td>10.9 ± 1.9</td>
<td>101 ± 5</td>
<td>14.4 ± 1.75</td>
</tr>
<tr>
<td>Pregnant + rosiglitazone</td>
<td>114 ± 2.5</td>
<td>12.1 ± 1.5</td>
<td>102 ± 1.5</td>
<td>8.3 ± 1.71</td>
</tr>
<tr>
<td>Diabetes</td>
<td>152 ± 4a</td>
<td>12.7 ± 2.4</td>
<td>218 ± 3.4a</td>
<td>17.9 ± 2.3a</td>
</tr>
<tr>
<td>Diabetes + rosiglitazone</td>
<td>154 ± 6a</td>
<td>11.9 ± 3.1</td>
<td>136 ± 2.1</td>
<td>11.2 ± 2.6</td>
</tr>
<tr>
<td>Diabetes + pregnancy</td>
<td>149 ± 5a</td>
<td>12.8 ± 3.4</td>
<td>224 ± 1.9a</td>
<td>18.7 ± 2.4a</td>
</tr>
<tr>
<td>Diabetes + pregnancy + rosiglitazone</td>
<td>151 ± 3a</td>
<td>12.6 ± 4.1</td>
<td>132 ± 3.5</td>
<td>11.3 ± 1.81</td>
</tr>
</tbody>
</table>

*a* significantly different from normal control values.
different in mesangial cell cultures from the four experimental groups, and rosiglitazone treatment did not significantly change these parameters (Figure 3). In all experimental variables, maximal binding of the radioactive tracer, representing the total density, was not statistically different from that in PD123319 (AT-2 receptor blocker) pretreated cultures, indicating that the presence of AT-2 receptors on the cell membranes was unappreciable. In contrast, maximal binding in all the losartan pretreated cultures was significantly lower compared with that of the untreated cultures, signifying that a majority of the receptors capable of binding the radioactive Angiotensin II were of AT-1 type (Figure 3).

TGF-β1 production

TGF-β1 concentrations, presented in Figure 4, varied from basal 247.5±12.2 pg/mg cell protein to rosiglitazone treated 244.4±36.8 pg/mg cell protein in normal controls. In other experimental groups, the results varied from basal 225.3±4.66 to rosiglitazone treated 206.3±27.0 (pregnant rats), from 250.7±24.9 to 203.7±31.5 (diabetic rats) and from 239.1±32.0 to 217.5±18.2 pg/mg cell protein (pregnant diabetic rats). The differences did not reach statistical significance (Figure 4, P > 0.05 in each comparison).

NF-κB studies

In all four experimental groups, i.e. normal controls, pregnant rats, diabetic rats and pregnant diabetic rats, the optical densities of active NF-κB fractions in cytosolic extracts were low and no statistically significant difference between the groups was demonstrable. In contrast, in nuclear extracts the active NF-κB fractions of cells originating from rosiglitazone-treated animals were significantly lower
compared with their respective non-treated counterparts (Figure 5, \( P < 0.005 \) in each comparison).

**Discussion**

The main goal of the present study was to investigate some of the inflammatory responses to A-II of cultured mesangial cells isolated from kidneys of rats receiving rosiglitazone before sacrifice. The study included four experimental groups: normal controls, normal pregnant rats, those with streptozotocin induced diabetes and pregnant diabetic animals.

As expected, mesangial cells from all experimental rats not receiving rosiglitazone before sacrifice responded to A-II stimulation in culture by exaggerated proliferative rate and, on the other hand, by augmentation of apoptotic death. Stimulation of mesangial cell proliferation by A-II was mediated via AT-1 receptor, since blockade of the latter by losartan abrogated this effect while A-II receptor blocker PD123319 proved ineffective. The nature and magnitude of proliferative responses to A-II were similar in all experimental cultures from animals not pretreated with rosiglitazone.

In contrast, mesangial cells isolated from kidneys of rosiglitazone-receiving normal controls exhibited reduced proliferative and apoptotic responsiveness to A-II. Similarly, blunted responses to A-II stimulation were observed in mesangial cells isolated from rosiglitazone-pretreated normal pregnant rats and diabetic non-pregnant animals. Furthermore, in mesangial cells from kidneys of pregnant diabetic rats receiving rosiglitazone prior to sacrifice, both proliferative and proapoptotic responses to A-II were completely abolished.

The altered responsiveness to A-II, a known promoter of mesangial cell apoptosis and proliferation [9], was not due to changes in AT-1/AT-2 receptor density, since this parameter was found similar in all experimental variations, irrespective of prior in vivo
Rosiglitazone ameliorates mesangial cell proliferation and apoptosis

Rosiglitazone, a widely used insulin sensitizer, is a pharmacological agonist of PPAR. PPAR are nuclear receptors which, following formation of heterodimeric units with retinoic acid X-receptor-α, bind to specific DNA locations. When activated with their pharmacological of physiological agonists, including rosiglitazone, they act as transcription factors regulating expression of a broad variety of genes [2,4,5]. Besides mediating insulin and lipid metabolism, PPAR proved to function as antihypertensive, antiproteinuric and anti-inflammatory agents [10–12]. Interestingly, PPAR have been shown to appear in human trophoblasts and lutein cells during normal pregnancy, probably as a part of a preventive anti-inflammatory defense mechanism, while sera from pregnant women proved to induce PPAR expression in several human cell lines [6,7].

PPAR are amply expressed in renal tissue [2,4] and have been shown to be activated in injured or diseased kidneys, such as glomerular sclerosis or diabetic nephropathy. PPAR consist of three isotypes, α, β/δ and γ [2]. Thus far, only the PPAR-γ type has been demonstrated in renal mesangium [5]. Ligands to PPAR-γ, including rosiglitazone, have been shown to inhibit mesangial cell proliferation [2,4] and to induce apoptosis in several cell lines [5]. The underlying mechanisms have not yet been fully elucidated. However, it has been demonstrated that physiological ligands to PPAR-γ are capable of blocking the activation of NF-κB [13]. NF-κB is a DNA binding protein necessary for transcription and selective activation, or inhibition, of a variety of genes regulating inflammatory processes, including those responsible for augmentation of inflammatory cytokine production, extracellular matrix deposition, exaggerated cell proliferation and apoptosis [14,15]. It was therefore of interest to investigate the influence of rosiglitazone treatment on the presence of active form of NF-κB in the nuclei of mesangial cells. In the present experiment, we separately measured NF-κB in extracts from the cytosol and from the nuclei of the experimental cell samples. NF-κB content of the cytosol was found extremely low, which is probably not surprising, considering that it is present in the cytoplasm in its inactive form, bound to an inhibitory I-κB protein, and is translocated to the nuclei only upon activation via cleavage of the bond with I-κB chain [14,15]. In contrast, in nuclear extracts from the cells of diabetic and pregnant diabetic rats the levels of NF-κB were found significantly elevated compared with their rosiglitazone treated counterparts or to normal controls. This would indicate that a considerable amount of NF-κB, activated in the context of diabetes-induced inflammation, was translocated from cytosol to the nuclei. In turn, lower levels of NF-κB in the nuclei of cells from rosiglitazone-treated animals would indicate that activation of PPAR-γ receptors by rosiglitazone abrogated NF-κB activation, apparently by suppressing its proteolytic dissociation from the inhibitory I-κB protein.

TGF-β1 synthesis showed a tendency to decrease in all cultures originating from rosiglitazone-pretreated animals, however, no statistically significant inhibition was demonstrable for any experimental group. Similarly to our data, no evidence on any direct influence of rosiglitazone on formation of TGF-β1 have been thus far reported in the literature. Nevertheless, rosiglitazone, among other PPAR ligands, has been shown to inhibit various pro-inflammatory responses, which are known to be induced by TGF-β1, in a variety of cells of non-renal origin [16]. The role of rosiglitazone in attenuation of TGF-β-mediated renal inflammation is yet to be elucidated.

Renal mesangium plays an important role in progression of inflammatory diseases. Deleterious mesangial cell proliferation is one of the main features of IgA nephropathy, membranoproliferative glomerulonephritis, lupus nephritis and diabetic nephropathy [17,18]. Renin–angiotensin system is activated in most renal inflammatory diseases, while PPAR-γ agonist treatment proved to reduce glomerular and tubular tissue damage in human and experimental diabetic nephropathy, over and above the hypoglycaemic effect [3]. Mesangial cell proliferation is often accompanied by augmented apoptosis. Both processes are mediated via NF-κB and induced by the same stimuli [19]. In a number of different experimental settings, not involving PPAR-γ ligands, a prolonged systemic infusion of A-II resulted in activation of NF-κB in glomerular and tubular tissues of rat or murine kidneys [12,20]. This, in turn, brought about upregulation of angiotensinogen synthesis, consequent long-term activation of the renin–angiotensin system, eventual downregulation of PPAR and, hence, further suppression of anti-inflammatory defense mechanisms. In this context, ACE inhibition proved to be effective in ameliorating the course of a variety of renal, human and experimental inflammatory processes [12,20]. Furthermore, any treatment aimed at inhibiting the deleterious mesangial cell proliferation improved the course of renal inflammatory diseases [18]. Moreover, in comparing the protective effects of PPAR activation vs ACE inhibition on deterioration of renal function in a diabetic nephropyathy rat model, the former has been found to confer far better renal protection [3]. Our results seem to provide an important tool to support this observation, demonstrating that in vivo treatment with rosiglitazone results in suppressed activation of NF-κB and, consequently, in inhibition of proliferative responsiveness of mesangial cells to A-II. In a diabetic animal model, this would represent a powerful anti-inflammatory renoprotective mechanism. Therefore, it is noteworthy that this suppressive effect was maximal in mesangial cells originating from pregnant diabetic animals. Further studies are required to scrutinize the role of mesangial cell apoptosis in this setting. The therapeutic relevance of these observations in the context of inflammatory renal diseases not involving diabetes remains to be elucidated.
Conflict of interest statement. None declared.

References


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