Reduced cardiac expression of plasminogen activator inhibitor 1 and transforming growth factor β₁ in obese Zucker rats by perindopril

J E Toblli, G Cao, G DeRosa, P Forcada

Objective: To determine whether angiotensin converting enzyme inhibition by perindopril can reduce cardiac transforming growth factor β₁ (TGFβ₁) and plasminogen activator inhibitor 1 (PAI-1) and therefore control collagen accumulation in an animal model with the metabolic syndrome such as the obese Zucker rat (OZR).

Animals: Male OZR (group 1, n = 10); OZR treated with perindopril (group 2, n = 10); and lean Zucker rats (group 3, n = 10).

Methods: During six months, group 2 received 3 mg/kg/day of perindopril orally and group 1 and group 3 were given a vehicle. Hearts were processed for pathology studies including immunohistochemical analysis with antibodies to PAI-1, TGFβ₁, collagen type I, and collagen type III.

Results: Group 2 had lower blood pressure (126.7 (2) v 148.6 (2.7) mm Hg, p < 0.01) than untreated OZR and had decreased cardiac PAI-1 (3.6 (0.4) v 13.5 (1.7)% of positive area/field, p < 0.01), TGFβ₁ in myocytes (0.13 (0.1) v 9.14 (4.7)%/area, p < 0.01) and in interstitium (19.8 (6.8) v 178.9 (27.4) positive cells/area, p < 0.01), collagen I (3 (0.8) v 13.3 (1)%/area, p < 0.01), collagen III (5 (0.6) v 9.5 (0.9)%/area, p < 0.01), and collagen I to collagen III ratio (0.59 (0.13) v 1.40 (0.15) p < 0.01) compared with untreated OZR.

Conclusion: These results suggest that perindopril reduces cardiac PAI-1 and TGFβ₁ and ameliorates cardiac fibrosis in a rat model with multiple cardiovascular risk factors.

The association of obesity, non-insulin dependent diabetes mellitus, and arterial hypertension constitutes the well known metabolic syndrome X, which is deeply involved in the development of cardiovascular disease with deleterious consequences. In addition, cardiac fibrosis, one of the major factors in the development of heart failure, is highly prevalent in all the aforementioned diseases. Accumulated information supports the notion that angiotensin II has, independently of its hypertensive effect, an essential role in cardiac fibrogenesis by itself and through its action on transforming growth factor β₁ (TGFβ₁) and plasminogen activator inhibitor 1 (PAI-1). Angiotensin II induces TGFβ₁, a multifunctional profibrogenic cytokine that regulates cell differentiation of fibroblast into myofibroblast. Both fibroblast and, especially, myofibroblast regulate fibrogenesis in the heart by producing collagen in the cardiac interstitium. Interestingly, cardiac myofibroblasts express requisite components of the renin–angiotensin system, including angiotensinogen, renin, and angiotensin converting enzyme (ACE), and can generate angiotensin II de novo. Moreover, angiotensin II can also induce PAI-1, which inhibits activation not only of plasminogen but also of plasmin matrix metalloproteinases (collagenases), which are involved in collagen degradation. Consequently, increased PAI-1 concentration either in plasma or in tissue can reduce extracellular matrix degradation, contributes to the accumulation of interstitial collagen, and leads to impaired function of the fibrinolytic system. Therefore, thrombotic events, such as myocardial infarction or stroke, are induced. Collagen type I and collagen type III are the major fibrillar collagens present in the adult heart; as in other organs, collagen turnover in the normal adult heart results from the equilibrium between the synthesis and degradation of collagen I and collagen III. Increased interstitial collagen in the heart enhances organ stiffness and results in diastolic dysfunction, which finally leads to heart failure. Since collagen III is the initial collagen to appear in the tissue repair process, followed by collagen I, evaluating the ratio of collagen I to collagen III gives a picture of the evolution of fibrogenesis.

The antifibrotic effect of ACE inhibition by perindopril has been reported in experimental rat models of left ventricular infarction and cardiac hypertrophy, but never before in a rat model with obesity, high blood pressure, and hyperglycaemia. Since fibrosis is an important cause of cardiac dysfunction in obesity, diabetes, and arterial hypertension, in all of which both TGFβ₁ and PAI-1 are increased, and taking into account that these two factors are stimulated by angiotensin II, intervention against angiotensin II formation and its actions is potentially important in controlling cardiac collagen overproduction. Considering that TGFβ₁ and PAI-1 have an essential role in fibrosis, TGFβ₁ by promoting extracellular matrix expansion and PAI-1 by inhibiting collagenases, pharmacological modulation of these molecules should be the target in the treatment approach against cardiac fibrosis. Accordingly, the purpose of the present study was to explore whether perindopril, an ACE inhibitor with recognised high affinity for tissue ACE, can reduce TGFβ₁ and PAI-1 and hence control fibrogenesis in an animal model with multiple risk factors. We decided to use the obese Zucker rat (OZR) strain in our experiments because its obesity, hypertension, insulin resistance state, mild diabetes mellitus, and hyperlipidaemia make it an attractive model.
to investigate cardiovascular and metabolic abnormalities related to the corresponding human metabolic syndrome.

**METHODS**

All the experiments were approved by the Hospital Aleman ethics committee and the teaching and research committee, and followed the Guide for the care and use of laboratory animals (National Institutes of Health). Ten week old obese (fa/fa) and lean (Fa/fa) (LZR) male Zucker rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were housed in individual cages at 21 (±2°C and a 12 hour light–12 hour dark cycle (7 am to 7 pm). Rats were divided into three groups: OZR group (group 1, n = 10), OZR treated with perindopril (group 2, n = 10), and LZR group (group 3, n = 10). All the animals were allowed to drink tap water and were fed standard rat chow (16–18% protein, Cargill-Argentina, Buenos Aires, Argentina) ad libitum. During six months, group 2 received a daily dose of 3 mg/kg of perindopril by intubation with a stomach tube between 11 and 11.30 am. Group 1 and group 3 received an equal volume of vehicle (normal saline solution) throughout the experiment. The perindopril dose was chosen on the basis of previous studies. The dose was adjusted each week based on calculation of the body weight for each animal. After six months of treatment, all rats were killed by subtotal exsanguination under anaesthesia (sodium thiopental 40 mg/kg intraperitoneally), according to institutional guidelines for animal care and use. The heart was rapidly excised and weighed, and portions of the left ventricle were harvested for light microscopy and immunohistochemical studies.

### Blood pressure measurement

At baseline and throughout the experiment, systolic blood pressure (SBP) was measured monthly by tail cuff plethysmography. Measurements were obtained with the rats restrained in a plastic chamber without anaesthesia. A pneumatic pulse transducer positioned on the ventral surface of the tail distal to the occlusion cuff detected the return of the pulse after a slow deflation of the cuff. Cuff pressure was determined by a programmed electrosphygmomanometer (PE-300, Narco Bio-Systems, Austin, Texas, USA) and pulses were recorded on a Physiograph MK-IIIS (Narco Bio-Systems). A minimum of three readings were taken at each session and the SBP registered was the average of the three readings.

### Biochemical procedures

After the rats had fasted for 14 hours, blood samples were collected from the tail vein in capillary tubes at baseline, as well as at the end of the experiment from the inferior cava vein before the rats were killed. Plasma glucose concentrations were measured by the glucose oxidase method with an automatic analyser (911, Hitachi, Tokyo, Japan). Aliquots of sera were assayed for cholesterol and triglyceride concentrations according to standard methods. Serum insulin was determined by a solid phase, two site immunoassay with monoclonal antibodies (DRG Instruments GmbH, Marburg, Germany). Serum samples were stored before testing. The test detection limit was 0.07 µg/l and the total percentage coefficient of variation of the assay was 4%.

### Heart processing and examination

Hearts were excised from rats and perfused with saline solution through the aorta. The hearts were then cut and pieces were removed from the left ventricle. Short axis fragments from the left ventricle and interventricular septum were fixed in phosphate buffered 10% formaldehyde (pH 7.2) for light microscopy and immunohistochemical studies.

### Table 1 Rat characteristics and biochemical parameters at the beginning and end of the experiment

<table>
<thead>
<tr>
<th></th>
<th>Group 1: OZR (n = 10)</th>
<th>Group 2: OZR + P (n = 10)</th>
<th>Group 3: LZR (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>304.3 (12.4)*</td>
<td>305.6 (15.2)*</td>
<td>220.3 (8.3)*</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>5.6 (0.5)</td>
<td>5.6 (0.5)</td>
<td>5.3 (0.4)</td>
</tr>
<tr>
<td>Insulin (µmol/l)</td>
<td>163.5 (38.8)*</td>
<td>161.8 (31.3)*</td>
<td>41.3 (11.2)*</td>
</tr>
<tr>
<td>Insulin to glucose ratio</td>
<td>28.9 (7.3)*</td>
<td>29.0 (7.6)*</td>
<td>7.7 (2.1)*</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.8 (0.1)*</td>
<td>0.8 (0.1)*</td>
<td>0.8 (0.1)*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.3 (0.1)*</td>
<td>1.3 (0.1)*</td>
<td>0.9 (0.1)*</td>
</tr>
<tr>
<td>End of experiment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>602.9 (52)*</td>
<td>581.7 (28.9)*</td>
<td>375.2 (31.3)*</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>12.8 (0.5)*</td>
<td>12.3 (0.4)*</td>
<td>4.7 (0.4)</td>
</tr>
<tr>
<td>Insulin (µmol/l)</td>
<td>763.7 (103.4)*</td>
<td>500.7 (79.8)*</td>
<td>45.6 (17.8)*</td>
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<tr>
<td>Insulin to glucose ratio</td>
<td>59.1 (8.0)*</td>
<td>40.6 (6.1)*</td>
<td>9.7 (4.0)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>10.9 (1.8)*</td>
<td>7.9 (1.9)*</td>
<td>0.3 (0.1)*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.2 (0.5)*</td>
<td>3.8 (0.7)*</td>
<td>1.5 (0.2)</td>
</tr>
</tbody>
</table>

Data are mean (SD). *p<0.01 v group 3; †p<0.01 v group 2; LZR, lean Zucker rat; OZR, obese Zucker rat; P, perindopril.
and embedded in paraffin for the light microscopy study (Nikon E400, Nikon Instrument Group, Melville, New York, USA). Three micrometre sections were cut and stained with haematoxylin and eosin, periodic acid Schiff reagent, and Masson’s trichrome.

**Immunohistochemical staining**

Paraffin sections were cut in 3 μm widths, deparaffinised, and dehydrated. Endogenous peroxidase activity was blocked by treatment with 0.5% H₂O₂ in methanol for 30 minutes. To evaluate PAI-1 in myocardium, a polyclonal rabbit IgG anti-rat PAI-1 (American Diagnostica, Greenwich, Connecticut, USA) at a dilution 1:100 was used. Monoclonal antibodies to TGFβ1 (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA) at a dilution of 1:100 and to collagen types I and III (Biogenex, San Roman, California, USA) at a dilution 1:100 were used to evaluate the fibrogenic process. Sections were immunostained with a commercial modified avidin-biotin-peroxidase complex technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, California, USA) and counterstained with haematoxylin. The samples were handled as previously described.²¹

**Morphological analysis**

All images were obtained through a light microscope (Nikon E400) with a digital camera (Nikon Coolpix 4500, Nikon Corporation, Tokyo, Japan). All the tissue samples were evaluated independently by two investigators without prior knowledge of the group to which the rats belonged. The first observer obtained the images throughout 20 consecutive microscopic fields at a magnification of 400 (each field measured 1.13 mm² and the total cardiac surface explored was 22.6 mm²) from each sample in each group. All images were then transferred to a personal computer and, with an image analyser (Image-Pro Plus version 4.5 for Windows, MediaCybernetics, Silver Spring, Maryland, USA), a second observer quantified and measured the respective immunostained samples. Lastly, data were averaged to obtain the final result for each group. For every heart, positive immunostaining for PAI-1, TGFβ1, collagen I, and collagen III was assessed as described above. TGFβ1 in the interstitium was expressed as the number of cells that stained positive for TGFβ1/22.6 mm².

**Statistical methods**

Values were expressed as mean (SD). All statistical analyses were based on absolute values and processed by GraphPad Prism version 2.0 (GraphPad Software, Inc, San Diego, California, USA). Gaussian distribution was assessed by the Kolmogorov and Smirnov method with an assumption test. For parameters with a Gaussian distribution, the groups were compared by analysis of variance. The difference of mean values between groups was assessed by the Tukey-Kramer multiple comparisons test. Parameters such as histological data with non-Gaussian distribution were statistically analysed by the Kruskal-Wallis test (non-parametric analysis of variance) and Dunn’s multiple comparison test. Finally, Spearman rank correlation was assessed when appropriate. A value of p < 0.05 was considered to be significant. The estimated statistical power for this study was 80%.

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**Figure 2** (A, B) Cardiac section from OZR (group 1). (A) Representative section showing positive immunostaining for plasminogen activator inhibitor 1 (PAI-1) in the vascular wall and myocytes (arrowheads) (original magnification ×400). (B) Large area with positive immunostaining for transforming growth factor β1 (TGFβ1) in interstitial cells and in myocytes (arrowheads) (original magnification ×400). (C, D) Cardiac section from OZR with perindopril (group 2). (C) Small amount of tissue PAI-1 expression (original magnification ×400). (D) Small area with positive staining for TGFβ1 (original magnification ×400).
RESULTS
At the beginning, at the end, and throughout the study, OZR were significantly heavier than LZR (table 1). In addition, OZR had significantly higher insulinemia, insulin to glucose ratio, cholesterol, and triglycerides (table 1). Concerning the plasma glucose concentration, although obese and lean rats did not differ at baseline (table 1), at the end of the experiment the OZR groups had significant hyperglycaemia \( (p < 0.05) \) compared with the LZR group (table 1). However, at the end of the experiment, the two OZR groups did not differ with respect to the hyperglycaemic state or in cholesterol and triglyceride concentrations (table 1). The insulin to glucose ratio increased in both group 1 and group 2: it was twice the baseline value in group 1 (increasing from 28.9 to 59.3) but only 1.4 times higher in group 2 (from 29.0 at baseline to 40.6 at the end), suggesting some improvement in the insulin resistance state.

Blood pressure changes
At baseline, SBP was similar in all three groups. However, whereas SBP increased significantly in untreated OZR \( (p < 0.05) \) during the study, SBP was significantly lower in OZR treated with perindopril and was similar to the SBP in the LZR group. Figure 1 illustrates the monthly evolution of SBP in all groups throughout the experiment.

Morphological and immunohistochemical findings
At the end of the study, heart weight was significantly higher in the untreated OZR group than in the group of OZR treated with perindopril (table 2). Additionally, when heart weight was related to total body weight in all groups, untreated OZR had a higher heart to body weight ratio than the other groups, which had very similar ratios (table 2).

There was no evidence of positive immunostaining for PAI-1 in the cardiac interstitium in any group. The percentage of cardiac immunostaining for PAI-1 in both vascular wall and in myocytes was significantly increased in animals from group 1 in comparison with the other groups (table 2, fig 2A). At the same time, group 1 had a substantial amount of TGF\(_{\beta 1}\) in the myocytes and a relevant number of interstitial cells with positive immunostaining for TGF\(_{\beta 1}\) (table 2, fig 2B). In addition, collagen types I and III in the cardiac interstitium were both significantly increased in these animals (group 1), with a high collagen I to collagen III ratio (table 2, fig 3A, B). In contrast, all these pathological manifestations were significantly reduced in OZR with perindopril, which was similar to LZR (table 2, fig 2C, D, fig 3C, D). Interestingly, a significant positive correlation between TGF\(_{\beta 1}\) and PAI-1 \( (r = 0.97, p < 0.01) \) was observed in untreated OZR. Conversely, these parameters were not correlated in LZR and OZR with perindopril (TGF\(_{\beta 1}, r = 0.09, \text{not significant} \) and PAI-1, \( r = 0.48, \text{not significant} \)). Moreover, it is worth mentioning that in group 1 serum glucose was positively and significantly correlated with the percentage of PAI-1 and the number of cardiac interstitial cells with positive immunostaining for TGF\(_{\beta 1}\) (fig 4A, B). In contrast, there was no correlation between these variables in groups 2 or 3 (fig 4A, B). Lastly, both the percentage of PAI-1 and the number of cardiac interstitial cells with positive immunostaining for TGF\(_{\beta 1}\) were highly and significantly correlated with the percentages of cardiac collagens I and III in group 1 but not in group 2 (fig 5A–D).
heart disease ACE inhibition reduced cardiac fibrosis in comparison with β blockers; both treatments resulted in a similar reduction in blood pressure. In addition, in patients with hypertensive heart disease, myocardial fibrosis regressed at six months with ACE inhibition by lisinopril compared with hydrochlorothiazide at an equivalent blood pressure. In concordance with this, in genetically obese diabetic mice, which are normotensive, ACE inhibition attenuated fibrinolysis and reduced cardiac perivascular fibrosis, indicating that this effect is independent of the blood pressure lowering outcome. Interestingly, perindopril seems to affect cardiac collagen reduction only when collagen has accumulated in cardiac interstitium. Although we did not include an LZR group treated with perindopril in this study, previous experiments from our laboratory have shown that perindopril administration in LZR does not substantially modify the percentage of interstitial cardiac collagen types I and III.

High glucose concentration is clearly associated with an increase in the expression of both PAI-1 and TGFβ. In addition, glucose tolerance status and insulin resistance states are closely related with PAI-1 concentration in overweight or obese patients. Furthermore, experimental evidence suggests that the degree of hyperinsulinaemia contributes directly to increased PAI-1 in obesity and non-insulin dependent diabetes mellitus. In agreement with this evidence, in the present study serum glucose concentration was remarkably correlated with the tissue expression of both PAI-1 and TGFβ1 in untreated OZR. Since these two factors are unquestionably recognised as essential in the development of the fibrotic process, it is not surprising that in this study collagen accumulation in cardiac interstitium of untreated OZR was considerable. However, although their blood glucose was similar, in OZR treated with perindopril these variables were not correlated. These findings may suggest that perindopril to a certain extent controls both PAI-1 and TGFβ1 in a high insulin and glucose environment.

The renin–angiotensin–aldosterone system (RAAS) and the insulin resistance state have been both identified as essential modulators of PAI-1 in hypertensive patients. In the rat, angiotensin II induces PAI-1 directly through the angiotensin II type 1 receptor independently of its effects on blood pressure in a variety of tissues including heart. By increasing PAI-1 expression, angiotensin II also reduces fibrinolytic activity and stimulates TGFβ1. Since TGFβ1 is also a powerful agonist of PAI-1 expression in adipose tissue, treatments that can ameliorate tissue concentration of TGFβ1 acquire especial importance in the setting of the metabolic syndrome. Notably, in the present study TGFβ1 and PAI-1 were significantly positively correlated in untreated OZR but not in LZR and OZR treated with perindopril. Both ACE inhibition and angiotensin II type 1 receptor antagonism have been shown to decrease PAI-1 in several tissues including the vascular wall. In the present study untreated OZR cardiac PAI-1 increased in both vascular wall and myocytes in conjunction with a positive correlation with the amount of interstitial collagen. Moreover, the amount of cardiac TGFβ1 and cardiac PAI-1 correlated highly in this group. In contrast, in OZR that had received ACE inhibition by perindopril immunostaining of PAI-1 was substantially reduced and the amount of PAI-1 in the interstitial collagen was likewise decreased.

Leptin, an adipocyte secreted hormone that regulates appetite and energy expenditure, has been shown to have profibrotic effects by acting synergistically with angiotensin II through stimulating TGFβ1. Moreover, convincing evidence indicates that leptin and TGFβ1 together exert an additive effect on collagen production. Consequently, since local RAAS is expressed in the heart, it appears congruent that in OZR, which have a recognised hyperleptinaemia, leptin and TGFβ1 acquire especial importance in the setting of the metabolic syndrome. Notably, in the present study TGFβ1 and PAI-1 were significantly positively correlated in untreated OZR but not in LZR and OZR treated with perindopril. Both ACE inhibition and angiotensin II type 1 receptor antagonism have been shown to decrease PAI-1 in several tissues including the vascular wall. In the present study untreated OZR cardiac PAI-1 increased in both vascular wall and myocytes in conjunction with a positive correlation with the amount of interstitial collagen. Moreover, the amount of cardiac TGFβ1 and cardiac PAI-1 correlated highly in this group. In contrast, in OZR that had received ACE inhibition by perindopril immunostaining of PAI-1 was substantially reduced and the amount of PAI-1 in the interstitial collagen was likewise decreased.

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DISCUSSION

In the present study, whereas untreated OZR had high blood pressure and substantially increased cardiac mass, together with a remarkable collagen accumulation in the cardiac interstitium, in group 2 treatment with perindopril reversed this condition in the heart besides controlling blood pressure. Moreover, these modifications were correlated with a significant reduction in both cardiac PAI-1 and TGFβ1 in cardiac interstitium and myocytes.

A possible limitation to bear in mind when interpreting the results of the present study is the lack of an additional antihypertensive treatment group with the same blood pressure as that recorded for OZR treated with perindopril. Therefore, we cannot rule out the possibility that lowering of the blood pressure may participate in the reductions in PAI-1 and TGFβ1. However, we can speculate that the effect on PAI-1 and TGFβ1 in the heart may not be due merely to a decrease in blood pressure. This speculation may be supported by findings in some recent studies that ACE inhibitors, even in a low dose, seem to protect against myocardial fibrosis in OZR. Furthermore, Brilla reported that in rats with renovascular hypertension and hypertensive
interstitial collagen I and collagen III are considerably increased and these two types of collagen are highly significantly correlated with the number of interstitial cells expressing TGFβ1 in the heart, as shown in our experiment. In opposition to this scenario, interaction between the RAAS and perindopril seems to produce an actual benefit by decreasing TGFβ1 and, therefore, preventing fibrosis in these animals. These results agree with our previous studies of the streptozotocin induced diabetic rat model, in which we observed a substantial reduction in fibrosis by an ACE inhibitor in several tissues including heart.37

Selective resistance to insulin signalling in the vasculature and abnormalities of glucose transporter 4 protein have been well documented not only in skeletal muscle but also in myocardium of OZR.38–41 In the current experiment, OZR treated with perindopril had a lower insulin to glucose ratio than untreated OZR. Previous studies have shown that administration of ACE inhibitors is associated with the enhancement of whole body insulin sensitivity in a number of insulin resistant animal models or in insulin resistant humans with high blood pressure.42–46 In the present study, insulin resistance was moderately reduced in perindopril treated OZR compared with untreated OZR, even though the insulin to glucose ratio was significantly higher in perindopril treated OZR than in control LZR. Consequently, part of the effect observed in our study is probably mediated through some improvement in glucose metabolism.

In conclusion, results of the present study suggest that ACE inhibitors with a recognised high affinity for tissue ACE, such as perindopril, have a beneficial effect on the reduction of cardiac PAI-1 and TGFβ1 and hence the modulation of cardiac fibrosis, thus preventing chronic heart disease in a rat model with multiple cardiovascular risk factors. However, even though OZR is a good model of the metabolic syndrome, results obtained by examining this animal model should be confirmed in humans with the metabolic syndrome.

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