Effects of pioglitazone and metformin on carbohydrate metabolism in experimental models of glucose intolerance

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Abstract
The aim of the study was to compare the effects of pioglitazone and metformin on carbohydrate metabolism in diabetic and insulin resistant rats. Male albino rats were randomized into seven groups. The first, control group received high carbohydrate diet. The second, third and fourth groups were fed high fat diets for 6 weeks and either left untreated, or given pioglitazone (2.7 mg/kg/day) or metformin (180 mg/kg/day) in the last 3 weeks. The 5th, 6th, and 7th groups were made diabetic by single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg) on day 15 of the 6-week high fat regimen. They were either left untreated, or given pioglitazone or metformin in the last 3 weeks. High fat diet induced insulin resistance evidenced by increased serum glucose, associated with increase in liver glucose-6-phosphatase and decrease in liver glucose-6-phosphate dehydrogenase and glucokinase activities. No significant difference was observed between rats treated with either pioglitazone or metformin. Both treatments showed significant increase in glucose-6-phosphate dehydrogenase activity by ~20 %, decrease in glucose-6-phosphatase activity by ~25 %, and decreases in liver and kidney glycogen. In diabetic rats, both pioglitazone and metformin decreased elevated serum glucose by ~30 %. Only metformin caused more than 2-fold increase in hepatic glycogen content, and normalized glucose-6-phosphatase activity. Only pioglitazone normalized elevated renal glycogen content and increased glucose-6-phosphate dehydrogenase activity. In conclusion pioglitazone and metformin had comparable effects on estimates of carbohydrate metabolism and insulin sensitivity in insulin resistant rats, but different effects in diabetic rats.

Key words: Pioglitazone, metformin, carbohydrate metabolism, diabetes, insulin resistance, high fat diet

Introduction
Type 2 diabetes mellitus (T2DM) is the most common endocrine disorder worldwide, affecting more than 171 million subjects. The incidence of T2DM is increasing rapidly, and it is estimated that by the year 2030, this number will almost double. The greatest increase in prevalence is expected to occur in developing countries due to urbanization and lifestyle changes, perhaps most importantly adopting a "Western-style" diet.¹ In Egypt, one study estimated the combined prevalence of diagnosed and undiagnosed diabetes in the Egyptian population ≥ 20 years of age to be 9.3% with a gradient increase from rural (4.9%) to urban areas from lower (13.5%) to higher (20%) socioeconomic standard.²

Insulin resistance, defined as a state of reduced responsiveness to normal circulating levels of insulin, plays a major role in the development of T2DM. While there is a genetic component involved in the onset appears to be triggered by lifestyle. Obesity, along with physical inactivity, can account for approximately 50% of the variability in the insulin-mediated glucose disposal in healthy, non-diabetic, normotensive individuals.³ High saturated fat, high calorie, processed carbohydrate and low fiber diets increase the incidence of insulin resistance.⁴

Pioglitazone and metformin are extensively used in Egypt and worldwide to treat patients with T2DM. Both drugs are considered to be insulin “sensitizers”. However, the detailed mechanism of action of these two drugs is still unraveled and further investigations are needed to compare their clinical efficacy in different models of insulin resistance.

Metformin, the only available biguanide in the market, inhibits glucose production potentially through effects on adenosine monophosphate activated protein kinase (AMPK). Whereas pioglitazone, a thiazolidinedione, activates peroxisome proliferators activated receptor (PPAR) gamma and improves hepatic insulin sensitivity, primarily through indirect effects on lipid metabolism.⁵ The treatment of prediabetic patients with metformin decreased new diagnosis of T2DM by

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Pioglitazone and metformin appear to have additional effects in ameliorating oxidative stress and inflammation thus rendering them as attractive tools for the prevention of insulin resistance and diabetes. To our knowledge, very few studies are available that directly compare the effects of pioglitazone and metformin on different pathways of carbohydrate metabolism in experimental models of insulin resistance and diabetes. Reports about the comparison between the two drugs with regard to efficacy on glycemic control as related to modulation of metabolism are scarce as well. For the objectives of adding information to these unraveled areas, we studied the effects of pioglitazone and metformin monotherapy on key enzymes of HMP (hexose monophosphate) shunt, gluconeogenesis and glycolysis in liver, as well as on hepatic and renal glycogen contents in experimental models of insulin resistance and diabetes. Many studies did not pick major differences between the effects of the two drugs. Some distinctions are displayed in the results.

Materials and Methods

Chemicals

Pioglitazone hydrochloride was provided by the raw materials department of NODCAR, Cairo, Egypt. Metformin hydrochloride was kindly provided by CID Pharmaceuticals, Cairo, Egypt. All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Animals

Male Wistar albino rats weighing 170-190 g, purchased from National Research Centre, Cairo, Egypt, were used in the study. Rats were housed in stainless steel cages and were kept in controlled environment. They had free access to water throughout the study. All procedures were made according to European Community Guidelines for the use of experimental animals and reviewed by the Animal Ethics Committee at NODCAR.

Diets

Rats were randomly assigned to either one of two diet regimens; control rats (n=9) were fed high carbohydrate diet (20% Kcal protein, 10% fat, 70% carbohydrate), all the remaining rats (n=70) were fed high fat diet (20% Kcal protein, 60% fat, 20% carbohydrate).

Diets used in this study as outlined in Table 1, are given ad libitum for six weeks. Food was withdrawn five hours before blood sampling on day 43. Rats were weighed at the beginning of the study and then weekly until the end of the study.

Experimental design

Rats were randomly divided into seven groups. The first group (n=9) received high carbohydrate diet for six weeks (42 days) and then left untreated (control group). All the other six groups received high fat diet (HF) continuously for six weeks. Among the six groups, following three weeks of high fat diet ingestion, three groups (each n=8) were treated with either pioglitazone (2.7 mg/kg/day, given as suspension by oral tube, HF Pio), metformin (180 mg/kg/day, given as solution by oral tube, HF Met), or no drug (HF) for three weeks. The other three groups were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ, 50 mg/kg), freshly prepared in 0.1 M citrate buffer at pH 4.5 on day 15 and allowed to drink oral glucose 5% w/v overnight. The minimal dose of insulin (1 unit/rat) was given for each rat on the 2nd and 3rd day after STZ administration. Induction of diabetes was confirmed on day 21 when the serum glucose level was at least 250 mg/dl. Diabetic rats were then randomly divided into three groups, diabetic untreated (STZ-HF, n=7) and diabetic treated with either pioglitazone (STZ-HF Pio, n=6) or metformin (STZ-HF Met, n=6) with the same protocol as that of high fat fed rats (Table 2).

Table 1: Composition of diets used in this study

<table>
<thead>
<tr>
<th>Diet/ Constituent</th>
<th>High Carbohydrate Diet (HCHO) g/kg Diet</th>
<th>High Fat Diet (HF) g/kg Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>188</td>
<td>254</td>
</tr>
<tr>
<td>Corn starch</td>
<td>438</td>
<td>169</td>
</tr>
<tr>
<td>Sucrose</td>
<td>219</td>
<td>85</td>
</tr>
<tr>
<td>Wheat bran/cellulose</td>
<td>38</td>
<td>51</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>41</td>
<td>339</td>
</tr>
<tr>
<td>Gelatin</td>
<td>14</td>
<td>19</td>
</tr>
<tr>
<td>Salt mix</td>
<td>50</td>
<td>65</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>9.7</td>
<td>13</td>
</tr>
<tr>
<td>Vitamin E acetate</td>
<td>--</td>
<td>0.31</td>
</tr>
<tr>
<td>Vitamin A (1000 IU/ml)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin D (150 IU/ml)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DL-Methionine /L-cystine</td>
<td>2.3</td>
<td>3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>69</td>
<td>20</td>
</tr>
<tr>
<td>Fat</td>
<td>10</td>
<td>59</td>
</tr>
<tr>
<td>Protein</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Caloric value (kcal/g)</td>
<td>3.82</td>
<td>5.20</td>
</tr>
</tbody>
</table>

Diet/ Constituent and their composition are as per Table 1.
The effects of administration of pioglitazone and metformin for 21 days on study parameters in high fat-fed and STZ diabetic rats. Data are presented as mean ± S.E.

**Table 2: Assignments of the study groups to drugs and diets**

<table>
<thead>
<tr>
<th>Days/Groups</th>
<th>Control</th>
<th>HF</th>
<th>HF Pio</th>
<th>HF Met</th>
<th>STZ-HF</th>
<th>STZ-HF Pio</th>
<th>STZ-HF Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 → 14</td>
<td>x</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>x</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>16 → 21</td>
<td>x</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>22 → 43</td>
<td>x</td>
<td>+</td>
<td>Δ</td>
<td>+</td>
<td>•</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

x = High carbohydrate diet, + = High fat diet, Δ = Pioglitazone (2.7 mg/kg BW/day), • = Metformin (180 mg/kg BW/day), S = Streptozotocin (50 mg/kg BW)

**Blood sampling and tissue collection**

On day 43, blood samples were collected in plastic centrifuge tubes and allowed to clot at 4°C for 30 min. Serum was then separated by centrifugation at 3000 rpm for 20 min. Rats were then killed. Livers and kidneys were separated, washed with ice-cold saline, plotted dry with filter paper, weighed and prepared at once for studying enzyme activities and glycogen contents.

**Enzyme activities and glycogen content**

100 mg fresh liver tissue was immediately homogenized in 4 ml of ice cold EDTA/physiological saline solution and centrifuged at 15000 rpm for 20 min at 1.5°C. 0.5 ml of the clear supernatant was used for glucose-6-phosphate dehydrogenase assay as described by Lühr and Waller. One unit of glucose-6-phosphate dehydrogenase is defined as the amount of enzyme needed to convert 1 μmol of glucose-6-phosphate to 6-phosphogluconate min in the presence of β-NADP at pH 7.5 at 25°C. For glucose-6-phosphatase assay, another 100 mg fresh liver tissue was similarly homogenized in four ml ice cooled citrate buffer and centrifuged at 15000 rpm for 30 min at 1.5°C. 0.1 ml of the clear supernatant was used for glucose-6-phosphatase assay as described by Taussky and Short. One unit of glucose-6-phosphatase is defined as the amount of enzyme needed to liberate 1 micro m mole of inorganic phosphate from glucose-6-phosphate per min at pH 6.5 at 37°C. Regarding glucokinase assay, one gram of fresh liver tissue was homogenized with nine ml of Tris-KCl-EDTA buffer and centrifuged at 15000 rpm for one h at 1.5°C. 0.025 ml of the clear supernatant was used for glucokinase assay according to the method of Jamdar and Greengard. One unit of glucokinase is defined as the amount of the enzyme required to phosphorylate 1 micro m mole of glucose.
per min at pH 7.6 at 30°C. For determination of glycogen content, 250 mg fresh liver tissue was digested with 5 ml 30% KOH and 0.5 ml of the digested tissue was used for glycogen precipitation using 3 ml absolute ethanol. Glycogen was then isolated by centrifugation for 15 min at 4000 rpm and determined according to Montgomery method. The same above procedure was used for renal glycogen determination but using 2 ml of tissue digest instead of 0.5 ml.

**Blood glucose, triglycerides and insulin**

Blood glucose and triglycerides levels were determined in the sera using UDI (United Diagnostic Industry) glucose and triglycerides enzymatic kits, respectively, whereas serum insulin was estimated using rat insulin ELISA kit purchased from Shibayagi, Japan.

**Statistical analysis**

All statistical analyses were performed using Statistical Package for Social Science (SPSS), version 10 software. All values were presented as means ± S.E. (standard error). Comparisons among groups were made by application of two-way analysis of variance ANOVA followed by LSD post hoc analysis. Differences were considered statistically significant if p< 0.05.

**Results**

**Experiments on high fat-fed rats**

As shown in table 3, HF rats did not show significant differences in body, liver and kidney weights when compared with control rats. Similarly, serum triglycerides and insulin were not significantly affected. On the other hand, serum glucose levels were significantly elevated in HF rats by 18%. This elevation was associated with 18% increase in glucose-6-phosphatase activity, 31% decrease in glucose-6-phosphate dehydrogenase activity, and 46% decrease in glucokinase activity as compared with control rats. Moreover, liver glycogen contents were reduced by 34%, though not significant.

Oral administration of pioglitazone (2.7 mg/kg/day) for 21 days was able to significantly decrease body weight and glucose-6-phosphatase activity by 12% and 22%, respectively as compared to HF group. Meanwhile, glucose-6-phosphate dehydrogenase activity was increased by 29%. Other parameters were affected by pioglitazone administration but not significantly under the conditions of the experiment. We were not able to detect significant differences between results of the aforementioned pioglitazone group and the results of oral administration of metformin (180 mg/kg/day) for 21 days.

**Experiments on high fat-fed STZ diabetic rats**

As compared to control rats, STZ-HF rats showed significant decreases in body weights, serum insulin levels, and liver glycogen contents by 17%, 51% and 85%, respectively. These were associated with significant increases in kidney weights by 42%, renal glycogen contents by 92% and serum glucose levels by 314%. Serum triglycerides levels were not altered significantly. With regard to liver carbohydrate metabolizing enzymes, significant increase in glucose-6-phosphatase activities by 22% and decreases in glucose-6-phosphate dehydrogenase and glucokinase activities by 47% and 65%, respectively were observed in STZ-HF rats as compared to control animals (Table 3).

Oral administration of pioglitazone (2.7 mg/kg/day) and metformin (180 mg/kg/day) significantly reduced blood glucose level by 32% and 29%, respectively. Pioglitazone therapy was associated with a decrease in renal glycogen contents by 52% and an increase in glucose-6-phosphate dehydrogenase activity by 20%. On the other hand, metformin therapy was associated with an increase in hepatic glycogen contents by 125% and normalization of glucose-6-phosphatase activity.

**Discussion**

The main objective of our study is to compare the effects of pioglitazone and metformin on carbohydrate metabolism and insulin sensitivity in prediabetic and diabetic states. We are unaware of similar study that focuses on the similarities and differences between the two drugs on carbohydrate metabolism. Although it is commonly stated that thiazolidinediones lower glucose concentration primarily by increasing glucose uptake and metformin by decreasing glucose production, the data supporting these statements are scarce and often contradictory. In vitro and animal studies have identified multiple potential targets for these drugs. Moreover, the results have not always been consistent in human.

For these reasons, we tested the drugs in two models of impaired glucose tolerance; one is induced by HF diet only (prediabetic state) and the other is induced by high fat diet associated with impaired insulin secretion (diabetic state).

The model of HF diet-induced glucose intolerance had been extensively used by others and was shown to impair carbohydrate metabolism, increase hepatic glucose production and induce insulin resistance. It is generally accepted that high fat diets can be used to generate a valid rodent model for the metabolic syndrome with insulin resistance and compromised beta cell function. Sunflower oil, which is used in this study to induce insulin resistance, is very similar in composition to safflower (carthame) oil that has been used in previous studies for that purpose. Sunflower oil is very abundant in Egypt and represents the main oil used in daily diets.

It was previously reported that rats fed HF diets for three weeks exhibited moderate hepatic insulin resistance as compared with rats fed HF diets for six weeks. The six weeks model was thus used in this
study. Results demonstrated that the six-week sunflower-rich diet increased hepatic glucose output through several mechanisms; significant reduction in hepatic glycogen deposition, stimulation of the gluconeogenic glucose-6-phosphatase activity and decreased hepatic glucose utilization through reducing the activities of the HMP shunt enzyme glucose-6-phosphate dehydrogenase and glycolytic enzyme glucokinase in liver.

The molecular mechanisms behind HF diet-induced insulin resistance are still not fully revealed. However, several mechanisms were postulated. One study correlated the increased glucose intolerance caused by HF diet to the elevated levels of plasma nonesterified fatty acids. Others referred to the elevated circulating leptins, PPAR-gamma genotype susceptibility, increased fatty acid oxidation in muscles, and deficiency in muscle mitochondria. Özela and associates indicated that neither defects in insulin receptor function nor elevated membrane glycoprotein PC-1 activities are involved in the development of insulin resistance in rats with high fat feeding, and the insulin resistance induced with high fat feeding is likely due to postreceptor defects in skeletal muscle. The door is still open for more investigations to come.

Impairment of insulin production by streptozotocin added to the results of high fat diet several modifications. Compared to the HF group, STZ-HF animals experienced lower body weights, hepatic glycogen contents, serum insulin levels and activities of glucose-6-phosphate dehydrogenase and glucokinase. Meanwhile higher renal weight, renal glycogen content, and serum glucose levels were observed. This pattern well complies with the reported biochemical changes in experimental model of T2DM.

Doses chosen for pioglitazone and metformin in this study are in harmony with their therapeutic doses in human, being equivalent to 30 mg/day for pioglitazone and 2000 mg/day for metformin. Drugs were administered for three weeks to study their effects when given as chronic treatments, and were administered concomitantly with diet to study their effects under uncontrolled diet conditions.

In presence of HF diet, administration of pioglitazone or metformin caused almost similar biochemical changes, but with few exceptions. Both drugs did not show significant effects in this model with regard to liver and kidney weights, serum glucose and insulin levels, and glucokinase activity. On the other hand, they significantly improved glucose-6-phosphate dehydrogenase activity and normalized glucose-6-phosphatase activity. These findings are consistent with the findings of Sugiyama and associates for pioglitazone effect on glucose-6-phosphatase activity and oppose their findings for pioglitazone on glucokinase activity, which showed increased hepatic glucokinase activity by pioglitazone. This may be attributed to the differences in the insulin resistance model used, as Sugiyama et al. used genetically obese Wistar rats. For metformin, these findings are consistent with those of Mithieux and associates. The shift of glucose-6-phosphate flux to HMP shunt, mediated by reduction of glucose-6-phosphatase and increase in glucose-6-phosphate dehydrogenase, is also consistent with the findings of Kletzien and associates for pioglitazone and Mithieux and co-workers for metformin.

However, it was noticeable that the decrease of glucose-6-phosphatase activity was not accompanied by stimulation of hepatic glycogenesis. Rather, a remarkable decrease in liver glycogen was observed for both drugs as compared to normal and untreated HF animals. These data contradict the findings of Mithieux et al, for metformin and that of Sugiyama et al, for pioglitazone. In the former study, liver glycogen content was dramatically increased three- to five times by concomitant administration of 50 mg/kg/day metformin for seven days with HF diet for six weeks. In the second study, 0.3-3 mg/kg/day for seven days pioglitazone enhanced insulin-stimulated glycogen synthesis in genetically-obese hyperglycemic rats. Meanwhile, our results are consistent with the findings of Radziuk and Pye who reported the decrease of both gluconeogenesis and glycogen synthesis by metformin and with those of Otto et al, who reported inhibition of glycogen synthesis by metformin in cultured rat hepatocytes. Contradictory data for the metabolic effects of pioglitazone and metformin are not uncommon in literature. One of the observed differences between pioglitazone and metformin is the reduction in body weight of rats on pioglitazone therapy. These rats showed reduced rate of body weight gain from the beginning of the study.

To compare the effects of pioglitazone and metformin in the diabetic state, rats were fed HF diet before STZ injection and this feeding was continued throughout the study. High fat feeding induced insulin resistance and STZ injection induced partial beta cell destruction and insulin deficiency, giving a diabetic model similar to the pathophysiologic changes in T2DM, being preceded by insulin resistance state before beta cell failure and appearance of overt diabetes.

With regards to the effects of pioglitazone and metformin on diabetic rats, both drugs reduced serum glucose level by ~30 % in STZ-treated rats. This complies with the findings of Pavo and co-workers who reported comparable improvement in glycemic control by pioglitazone and metformin in patients with T2DM. The decrease of glycogen content in liver and the increase in kidneys in STZ animals well complies
with previous reports. Only pioglitazone was able to reduce elevated renal glycogen content in STZ-HF rats. On the other hand, only metformin increased hepatic glycogen content. These observations are similar to the findings of Okine and co-workers who observed an increase in hepatic glycogen content of STZ induced diabetic mice after metformin administration. This contradicts our findings of metformin effect on hepatic glycogen content of HF rats, suggesting that the inhibitory effect of metformin on glycogen synthesis is lost under hyperglycemic conditions. Previous studies have shown that STZ diabetic rats exhibited severe loss of body weight associated with a significant increase in kidney weight as well as kidney weight to body weight ratio. These alterations were also evident in our study. The increase in kidney weight in STZ-HF Met group correlated with the increased renal glycogen.

STZ-HF rats showed increased levels of glucose-6-phosphatase activity associated with reduced activities of both glucose-6-phosphate dehydrogenase and glucokinase activities. This shift is mainly induced by the high glucagon/insulin ratio in STZ-treated animals. Similar to its effect in HF rats, metformin was able to normalize glucose-6-phosphatase activity in STZ-HF rats. This is consistent with the findings of Heishi and co-workers, who found reduction in glucose-6-phosphatase gene expression associated with reduction of glucose-6-phosphatase activity in livers of obese diabetic db/db mice. However, the insignificant effect of metformin on glucose-6-phosphatase dehydrogenase activity contradicts the findings of Ashokkumar and co-workers that demonstrated the ability of metformin to restore glucose-6-phosphate dehydrogenase activity almost to control levels. However, this experiment was done in neonatal STZ-induced diabetic rats, where a single 100 mg/kg STZ injection was given to two days old rats. On the other hand, pioglitazone was able in to increase glucose-6-phosphate dehydrogenase activity in STZ-HF rats, similar to its effect on HF rats.

Finally, our salient conclusions of this study are:

1) High-sunflower oil diet impairs glucose tolerance and disrupts carbohydrate metabolism via decreasing hepatic glycogen content and impairing activities of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and glucokinase.

2) STZ induced diabetes caused marked elevation in serum glucose level associated with marked decrease in serum insulin level and body weight, impairment of carbohydrate metabolism as demonstrated by dramatic decrease in hepatic glycogen content, increase in renal glycogen content and impairment of activities of glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and glucokinase.

3) In high fat diet rats, metformin and pioglitazone had almost similar effects; both activated glucose-6-phosphate flux to HMP shunt, mediated by reduction of glucose-6-phosphatase and increase in glucose-6-phosphate dehydrogenase activities.

4) In diabetic high fat diet rats, metformin and pioglitazone equally depressed elevated serum glucose level by ~30%. Pioglitazone therapy was associated with a decrease in renal glycogen and an increase in glucose-6-phosphate dehydrogenase activity. On the other hand, metformin therapy was associated with an increase in hepatic glycogen and normalization of glucose-6-phosphatase activity.

Further comparative studies between pioglitazone and metformin are recommended on the metabolic, cellular and molecular levels to show whether pioglitazone has any added favorable actions than the cheaper, safer, and early-used metformin.

References
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