LOW DOSE DEXAMETHASONE IN THE RAT: A MODEL TO STUDY INSULIN RESISTANCE

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Running title:
An insulin resistance model

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ABSTRACT

The main aim of this study was to set up a new animal model to study insulin resistance. Wistar rats (6-7 for group) received for 4 weeks in EXPERIMENT 1: A) Vehicle, B) 2µg/day subcutaneous Dexamethasone, C) Metformin 400 mg/Kg/day per os, and D) Dexamethasone + Metformin; while in EXPERIMENT 2: A) Vehicle, B) Dexamethasone, C) Dexamethasone + Arginine 2% (as substrate of the nitric oxide synthase for nitric oxide production) in tap water and D) Dexamethasone + Isosorbide dinitrate 70 mg/Kg (as direct nitric oxide donor) in tap water. Insulin sensitivity was significantly reduced by Dexamethasone already at week 1, before the increase in blood pressure (day 15) without significant changes in body weight as compared to vehicle. Dexamethasone treated rats had significantly higher triglycerides, haematocrit and insulin while serum total nitrates/nitrites were lower as compared to vehicle. The concomitant treatment with metformin minified all the described effects of dexamethasone. In experiment 2 only isosorbide dinitrate was able to prevent the observed dexamethasone induced metabolic, haemodynamic and insulin sensitivity changes. Chronic low dose subcutaneous dexamethasone (2µg/day) is an useful model to studying the relationships between insulin resistance and blood pressure in the rat and dexamethasone might decrease insulin sensitivity and increase blood pressure through an endothelium-mediated mechanism.

Key words: glucocorticoids, metabolic syndrome, metformin, and hypertension

Abbreviations: Dexamethasone = DEX; Metformin= MET; Arginine = ARG; Isosorbide dinitrate =ISN; systolic blood pressure =SBP; nitric oxide synthase = NOS; nitric oxide= NO, Free Fatty Acid= FFA
INTRODUCTION

There is considerable evidence that abnormalities of glucose, insulin and lipoprotein metabolism occur more frequently in untreated hypertensive patients than in normotensive subjects. It could be argued that the relationship between high blood pressure and metabolic disorder are incidental, but, on the other hand, there is evidence that changes in glucose, insulin and lipoprotein metabolism play a role in the etiopathology and/or clinical course of hypertension. In human hypertension subtle changes in adrenal steroid metabolism has been suggested (28,29,36) indicating that glucocorticoids, besides the well known Cushing syndrome, might have an important role in the development of high blood pressure. Moreover glucocorticoids have been shown to reduce cellular glucose uptake affecting the glucose transport system per-se (7), with no direct effects on the insulin receptor (26). Thus glucocorticoids alter glucose metabolism and in turn they have a role in the development of peripheral insulin resistance. Insulin resistance may be an impetus for the development of hypertension, impaired carbohydrate tolerance and lipids alteration, but the underlying mechanisms are still unclear. Similar metabolic abnormalities occur in rodent models of hypertension. For example, endothelial dysfunction precedes hypertension in an experimental model of fructose-induced insulin resistance (9,24), but fructose-induced insulin resistance is not easily comparable to humans. At the best of our knowledge there is no established model of insulin resistance induced by dexamethasone without the dramatic catabolic side effects commonly seen with glucocorticoids. We previously showed that it is possible to increase blood pressure for long term in rats with subcutaneous doses of dexamethasone in the order of micrograms/day (31) without appreciable catabolic effects. This animal model is not associated with sodium retention, but with sodium
shift from the intracellular to the extracellular space (12), and the effects on blood pressure is opposite to those obtained by intracerebroventricular dexamethasone administration (32). The main aim of this study was to establish if our previously described “old model” of glucocorticoids induced hypertension in the rat is indeed an useful “new” model to studying insulin resistance in the metabolic syndrome. To do so we used our previously described animal model (31) evaluating the effects of metformin, a well-established drug able to ameliorate insulin sensibility in rat (25).
RESEARCH DESIGN AND METHODS

EXPERIMENT 1

ANIMALS

Male Wistar rats, 400 grams weight, were used through the experiments in groups of 6-7 animals each. All rats were housed in an automatically light-controlled animal facility (12 hours on, 12 hours off) with constant temperature (22°C) and humidity, with free access to food (Mil mice and rats GLP Diets, Mucedola Srl, Italy) and tap water ad libitum. Two weeks before starting the experiment animals were accustomed to handling, blood pressure and blood glucose measurements (by a vein puncture in the tail). Four groups were thereafter treated for 4 weeks:

A) Vehicle: tap water and daily subcutaneous injection of 0.9% NaCl (75 µl) at 8.00 a.m and 8.00 pm,

B) DEX: tap water and daily subcutaneous injections of Dexamethasone (1 µg in 75 µl 0.9% NaCl) at 8.00 a.m and 8.00 pm,

C) MET: Metformin in tap water (3.5 mg/ml) and daily subcutaneous injection of 0.9% NaCl (75 µl) at 8.00 a.m and 8.00 pm,

D) DEX+MET: metformin dissolved in tap water (3.5 mg/ml ) and daily subcutaneous injection of Dexamethasone (1 µg in 75 µl 0.9% NaCl) at 8.00 a.m and 8.00 pm,

METHODS

Three times a week systolic blood pressure was measured in the morning (tail cuff Method, Letica, Le 5001 Pressure Meter, Spain) in the conscious lightly restrained animal after the animals being pre-warmed at 38°C for 10 minutes as previously described (12,31,32), body weight and tap water consumption were recorded at the same time. At days +8, +14 and +26 after blood pressure measurement, in the
conscious rats blood from a tail vein was obtained for glucose measurement using a reflectometer (Lifescan One Touch Profile, Johnson-Johnson Company, Minneapolis, USA), immediately before and 30 minutes after receiving intraperitoneal fast acting insulin (1.6 U/Kg Actrapid, Novo Nordisk). In this “insulin tolerance test” the 1.6 U/Kg dose and the 30 minutes values were choosen to calculate the maximum decrease in blood glucose. This method was, in our laboratory, repetitive as shown in Figure 1, where the effects of three different doses of intraperitoneal insulin (0, 0.8 and 1.6 U/Kg) on blood glucose, over 80 minutes after insulin injection are given. The reflectometer was validated in our laboratory against an automatic analyser (Hitachi 912, automatic analyser, Boehringer Mannheim, using standard reagents) for values from 30 mg/dl to 200 mg/dl (n=68, \( r^2 = 0.921 \) \( p<0.001 \)).

At day +28 after the last blood pressure measurement, while the rats were fasting from midnight, under pentobarbital anaesthesia a large blood sample from the abdominal aorta was obtained. Serum cholesterol, triglycerides, OH-butyrate and uric acid (Hitachi 912), Na+, K+ (flame photometer, Beckman) were measured. One hundred and fifty µl blood were collected in a micro-haematocrit capillary tube and after spin at 5,000 rpm for 10 minutes, used for the determination of haematocrit. Insulin was measured in duplicate by rat insulin RIA Kit (Diasorin, Stillwater, Minnesota, USA). Total nitrates/nitrites were measured by a validated colorimetric assay kit (Cayman) with high sensibility (1µM) for both NO2- and NO3- in serum to achieve an estimate of \textit{in-vivo} Nitric Oxide (NO) production, being nitrates (NO3-) and nitrites (NO2-) the end product of NO (13). Intraerythrocyte \( \text{Na}^+ \) (\( \text{Na}^+/\text{RBC} \)) was measured following a published method (5) with the only modification of using 0.5 ml full blood. Blood for Free Fatty Acids was obtained at the same time and collected
in iced (+4°C) EDTA tubes, immediately centrifuged at 4°C and the plasma frozen at –80°C until assayed. Free Fatty Acids were measured by a colorimetric Method using a Boeringher Mannheim kit.

Heart, liver and the left kidney were excised and weighted. The measurement was corrected for body weight.

HISTOLOGY
Liver and left kidney tissues after fixation in 10% buffered formalin were dehydrated with ethyl alcohol and then included in paraffin. Section of 5 µm were obtained by a microtome (Top Rotary S-130,pabish). PAS (periodic acid Schiff) coloration for polysaccharides has been used to evaluate glycogen content in the hepatocytes. Two independent observers not aware of the different treatments gave independently comment on the histological material.

STEADY-STATE GLUCOSE CONCENTRATION.
Steady-state glucose concentration (24) was measured to confirm the findings of the “insulin tolerance test” in the period preceding the rise in blood pressure observed in dexamethasone treated rats. Steady-state glucose concentration (SSGC) was measured in additional 4 rats after 8 days Dexamethasone treatment alone (before any significant rise in systolic blood pressure) or with Metformin following the described protocol of Reaven (24). In brief under anaesthesia with Pentothal Sodium (Tiopentale Sodico 0.5 gr, Farmaceutici Gellini) the right femoral vein was cannulated for insulin and glucose infusion at the fixed doses of 2.5 mU/Kg/min and 8 mg/Kg/min, respectively for 3 hours. Tail blood samples for glycemia were taken at 15 minutes intervals during the last hour infusion and the 4 obtained values were used as mean.
EXPERIMENT 2

ANIMALS AND METHODS

In order to better characterize the possible endothelial involvement in the development of insulin resistance in this animal model, four additional groups of 6 animals each were compared in a 28 days experiment. The main reason for this second study was to evaluate if nitric oxide production (measured as said above as total nitrates and nitrites) was restored giving exogenously the endogenous substrate for nitric oxide production, Arginine. The Arginine effects were compared with those obtained giving exogenously directly NO through a NO-donor, isosorbide dinitrate.

A) Vehicle: tap water and daily subcutaneous injection of 0.9% NaCl (75 µl), at 8.00 a.m and 8.00 pm

B) DEX : tap water ab libitum and daily subcutaneous injections of Dexamethasone (1 µg in 75 µl 0.9% NaCl), at 8.00 a.m and 8.00 pm

C) DEX+ARG : daily subcutaneous injections of Dexamethasone (1 µg in 75 µl 0.9% NaCl) at 8.00 a.m and 8.00 pm and 2% Arginine in tap water ab libitum

D) DEX+ISN: daily subcutaneous injections of Dexamethasone (1 µg in 75 µl 0.9% NaCl) at 8.00 a.m and 8.00 pm and: isosorbide dinitrate 70 mg/Kg/die in tap water ab libitum.

Thus in this second experiment, Arginine was given as substrate of NOS for the production of nitric oxide (NO), while isosorbide dinitrate was given as direct NO donor, in order to establish the role of endothelium in this animal model. Animals were studied as in experiment 1 including insulin tolerance test evaluation. After 1 and 4 weeks treatment, immediately after blood pressure measurements, 2 ml blood were obtained from the tail in the conscious rat for biochemistry determination and haematocrit.
Statistical analysis

Data are presented as mean±SEM. After ANOVA measurements, with pair wise Newman-Keul’s test for multiple comparisons, parameters found significantly different were subsequently analysed with the paired (within groups, different times) or unpaired (between groups, same time) two tail Student t-test when appropriate, p<0.05 was taken as significant. Data were analysed using Sigma Stat 3.0 program.

Principles of laboratory animal care (NIH publication n°86-23, 1985) were followed in these experiments.
RESULTS

EXPERIMENT 1

All rats completed the 28 days experiments. Water consumption was equal in the groups (32-38 ml /day/ rat) a part for a slight decrease limited only between day +1 and +2 for rats receiving metformin. Both DEX+MET and MET rats drunk 21-26ml/day/rat during these days, probably due to the rats accustoming to the drug taste, thereafter the daily water intake was similar to that one of the other groups. Thus the average metformin intake in the DEX+MET and MET rats was 183-228 mg/Kg/day for day 1 to 2 and 280-333 mg/Kg for the remaining days. Systolic blood pressure increased significantly only in DEX rats from day + 15, reaching the zenith at day +18 and remaining significantly elevated till day +28 with a net increase of more than 20 mmHg from basal (Figure 2, top). The remaining rats did not show significant changes in SBP through the study although final SBP in DEX+MET rats was somehow 5 mmHg more elevated, but not significantly, than in the other two remaining groups (Vehicle and MET). Heart rate did not show significant difference during the study in the four groups of rats (Table 1). Body weight did not change significantly through the study in any group (Table 1). At the end of the 4 weeks serum insulin was significantly increased in DEX rats and decreased in metformin rats as compared to vehicle while blood glucose was equal in the four groups (Table 1), Dexamethasone treated rats showed also significantly lower (p=0.032) levels of serum total nitrates/nitrites while haematocrit, cholesterol, triglycerides, FFA and insulin were significantly higher as compared to vehicle (Table 1). These effects were completely reversed by the concomitant use of metformin (DEX+MET), but serum cholesterol remained higher than in vehicle. No significant changes for the other considered parameters were evident among the different groups of animals.
included intraerythrocyte Na+ (12.1 ± 1.3, 10.4 ± 0.5, 9.8 ± 0.8, 12.5 ± 1.4, mEq/10^6 RBC for Vehicle, MET, DEX and DEX+MET treated rats respectively).

Insulin sensitivity as estimated by the 30 minutes drop of blood glucose after i.p. fast acting insulin, was significantly reduced in DEX treated rats as compared to the other groups at time +8, +12 and +26 (Figure 2, bottom). Similar results were obtained when insulin sensitivity was estimated by the steady-state plasma glucose during insulin/glucose infusion. Mean steady-state glycemia during the third hour insulin/glucose infusion was significantly higher in DEX rats as compared to DEX+MET: 128±6 vs 84±5 mg/dl, p< 0.02, while serum insulin at the end of the three hours infusion was comparable in the two groups.

At the end of the study no significant difference in heart, liver and kidney weight (corrected for body weight) was evident in the four groups of rats. Glycogen content, determined histological in the hepatocytes was reduced in Dexamethasone treated rats as compared to vehicle and metformin only treated rats. Moreover in dexamethasone treated rats an increased deposit of lipids at the liver level was evident (Figure 3). The addition of metformin in Dexamethasone rats partially restored the content of glycogen at the liver level, dramatically reducing the liver lipids content. No significant differences in the different groups of rats were evident regarding kidney structure although the glomerular apparatus in DEX rats appeared bigger than in the other groups (data not shown).

**EXPERIMENT 2**

All animals completed the 28 days experiment and water consumption was equal in the groups (30-37 ml/day/rat) without significant changes in rats given both ARG or ISN as compared to vehicle or DEX. Rats treated with DEX+ISN did not show any significant change in systolic blood pressure, heart rate and body weight through
the 28 days experiments as compared to vehicle (data for final measurement at day 28 are shown in table 3). ISN treatment in addition to DEX was also able to completely reverse the effects of dexamethasone treatment in terms of triglycerides, haematocrit and delta glycemia changes after intraperitoneal insulin (Table 2). On the other hand, DEX+ARG treated rats did not significantly differed from dexamethasone-only treated rats (DEX, table 3). Thus only the direct NO donor ISN given in association with dexamethasone was able to restore the conditions seen in the vehicle group.
DISCUSSION

In the present paper we describe for the first time, at the best of our knowledge, a new animal model of glucocorticoid-induced insulin resistance possibly due to endothelial dysfunction. We previously showed that it is possible to increase blood pressure for long term in rats with subcutaneous doses of dexamethasone in the order of micrograms/day without appreciable catabolic effects and a complete inhibition of ACTH secretion (31) and without sodium retention (12). The main aim of this study was to establish if this “old model” of glucocorticoids induced hypertension is a useful “new” model to studying insulin resistance. Using our “pure glucocorticoid” model, characterised by the absence of catabolic side effects, we were able to show that dexamethasone-induced insulin resistance (increase in serum insulin concentration, decreased insulin tolerance and higher state-state glucose concentration during exogenous insulin/glucose infusion) precedes hypertension and is accompanied by other features of the metabolic syndrome (increased serum FFA, cholesterol and triglycerides, and decreased NO production as marker of endothelial dysfunction). Insulin resistance in our animal model clearly precedes the development of hypertension and we were able to show that dexamethasone treatment reduces total nitrates/nitrites. Indeed total nitrates/nitrites may be considered a good marker of the Nitric Oxide Synthase (NOS) activity (13), suggesting an endothelial dysfunction as *primum movens* for the development of this dexamethasone-induced insulin resistance. NO undergoes a series of reaction with several molecules present in biological fluids as plasma and urine. The final in vivo products of NO are nitrite (NO$_2^-$) and nitrate (NO$_3^-$). Since the relative proportion of nitrite and nitrate, may vary, as a better index we measured the sum of the two as previously reported (13). The addition of metformin to dexamethasone treatment restores serum total nitrates/nitrites to the levels observed in the vehicle group,
suggesting that metformin might have overcome the dexamethasone induced endothelial dysfunction, the most probable pathogenetic mechanism of insulin resistance in this animal model. Pharmacological blockade of Nitric Oxide Synthase (NOS) activity has suggested an important role of its product, nitric oxide (NO), in regulating insulin sensitivity and carbohydrate metabolism (1, 27). Data obtained in our second experiment confirm this hypothesis. Indeed Arginine did not modify DEX induced increase in systolic blood pressure, and serum triglycerides, it was not able to restore the circulating levels of nitrite/nitrates to the levels observed with vehicle and did not modify the dexamethasone-induced decrease in insulin sensitivity. When isosorbide dinitrate treatment is added to dexamethasone, serum nitrates/nitrites values higher than in the vehicle, although not significantly, were achieved and only isosorbide was able to prevent the rise in systolic blood pressure and in circulating triglycerides and the decrease in insulin sensitivity induced by dexamethasone alone. Mice with gene disruption of endothelial nitric oxide synthase show insulin resistance (21) stressing the importance of this system in the modulation of the peripheral insulin sensitivity at least in animals. Our data suggests that an endothelial disruption caused by dexamethasone treatment, inactivates the NOS that has an important role in this newly described animal model of insulin resistance and metabolic syndrome.

Insulin resistance, before the development of type 2 diabetes and/or hypertension, may cause endothelial dysfunction with a key role in the pathogenesis of vascular complications (8,33). Metformin is able to increase peripheral insulin sensitivity and insulin-mediated glucose uptake in the cells, increasing insulin-induced translation of Glut-4 from an intracellular pool to the plasma membrane and increasing the functional activity of the glucose carrier without altering the de-novo synthesis of the glucose carrier both in-vivo (15) and in-vitro (15). In this way metformin might
reverse the concomitant endothelial dysfunction. Indeed, recently, the UKPDS has shown that metformin treatment in overweight type 2 patients, reduces significantly the occurrence of myocardial infarction (35), as compared to sulphonylureas or insulin in normal weight type 2 patients (34), suggesting additional effects of metformin (2), besides lowering glycaemia. It is well known that glucocorticoids alter insulin sensitivity and they have a role in altering glucose metabolism and blood pressure regulation. Most of the already described “glucocorticoids” animal models do not take into account catabolism, with muscle atrophy (6,17,37) and increased blood pressure that can affect the measurement of insulin sensitivity by altering peripheral blood flow. Moreover no data on the long-term glucocorticoid treatment are available as well regarding a possible endothelial involvement. We choose metformin as a well established (25), both in-vivo and in-vitro, drug therapy to ameliorate peripheral insulin resistance in rats, as well in humans. Its wide use in diabetes is mainly due to its effects on peripheral insulin action increasing glucose uptake and utilization (30) although recently a direct effect of metformin on restoring $\beta$-cell insulin secretion response in vitro has been described. The reduction in blood pressure induced by metformin in insulin resistant rats is apparently through a direct mechanism with a nitric oxide-dependent relaxation (10). Indeed metformin has been shown able to attenuate the development of hypertension in the SHR (19) usually reported to be insulin resistant (4,17). These effects of metformin on blood pressure are not present in other animal models of hypertension not characterised by insulin resistance (38), indicating that metformin is not able per-se to decrease blood pressure.

An additional alternative “non-esterified fatty acid hypothesis” may be added to explain glucocorticoids induced insulin resistance (17). Our data show that dexamethasone treatment increase Free Fatty Acid (FFA) in plasma and this might
have contributed to insulin resistance, while metformin treatment restores free fatty acid (FFA) metabolism (21), as we found in this experiment. Metformin increases the effects of infused L-Arginine on lowering blood pressure decreasing platelet aggregation and blood viscosity in NIDDM patients. We did not measure blood viscosity in our experiment, but metformin+dexamethasone (DEX+MET) rats showed, as compared to dexamethasone only treated rats (DEX), lower haematocrit levels, lower triglycerides, and lower FFA; all features that can explain a decrease in blood viscosity. Feeding rats with the nitric oxide synthase inhibitor, L-N(omega)nitroarginine, elevates serum triglycerides and cholesterol and lowers hepatic fatty acid oxidation, affecting the activity of hepatic carnitine palmitoyltransferase, the rate limiting enzyme of fatty acid oxidation, increasing circulating free fatty acid due to a reduction in fatty acid oxidation (11). Indeed raised FFA concentrations have been associated with the development of hypertension (3), skeletal muscle insulin resistance (23) decreased insulin secretion (20) and fatty liver (14), this last being regarded as the hepatic consequence of the metabolic syndrome due to specific hepatic insulin resistance (14). In our experimental model, dexamethasone, together with the other classic features of metabolic syndrome such as insulin resistance, hypertriglyceridemia etc, was able also to increase the hepatic fatty accumulation, as observed in humans in whom non-alcoholic fatty liver disease is a feature of the metabolic syndrome with insulin resistance (14). Metformin dramatically reversed this fat accumulation together with the other features of the metabolic syndrome. Recently (39), metformin has been shown able to activate AMPK (AMP-activated protein kinase) in hepatocytes leading to reduction in acetyl-CoA carboxylase activity, induction of fatty acid oxidation and suppression of the expression of lipogenic enzymes. These new data may provide a unified explanation for the pleiotropic effects of metformin, in particular regarding
modulation in circulating lipids and reduction in hepatic lipid synthesis and fatty liver as we observed in our experiment. The effects on cholesterol, triglycerides and FFA were observed with dexamethasone treatment and reversed by the concomitant use of metformin. Thus we postulate that dexamethasone, by altering NOS expression might alter lipid metabolism with an increase in FFA and consequently insulin resistance. Since in human hypertension subtle changes in adrenal steroid metabolism has been suggested (28,29,36) indicating that glucocorticoids, besides the well known Cushing syndrome, might have an important role in the development of high blood pressure, this animal model is useful to study this aspect of human hypertension.

In conclusion the two most important finding of this study are: 1) long term – low dose subcutaneous dexamethasone induces insulin resistance that precedes hypertension and both conditions are reversed by the concomitant treatment with metformin, 2) dexamethasone induced insulin resistance might be due to endothelial dysfunction. Our data indicate that low-dose dexamethasone induced hypertension in rats, an “old model” (12,31,32) of hypertension, can be revisited on the light of an endothelial dysfunction causing insulin resistance and consequently some of the commonly observed features of the metabolic syndrome (hypertension, lipid-metabolism alteration, fatty liver etc). In particular the presence also of the hepatic consequences of the insulin resistance in humans (fatty liver) appears to complete the picture of metabolic syndrome due to reduced insulin sensitivity This model would be useful for studying the insulin resistance/blood pressure relationships.
Acknowledgments

We acknowledge the excellent technical work of Mrs Maristella Spissu and Mr Gianluigi Fenu. Dr Cristiana Severino is at present working in the Servizio Diabetologia, Dipartimento Struttura Clinica Medica e Patologia Speciale Medica University of Sassari as a Post-Doc yellow.
Table 1

Body weight, heart rate, biochemical serum parameters and haematocrit at the end of the 4 weeks experiment in the different groups of rats. (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>MET</th>
<th>DEX</th>
<th>DEX+ MET</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(grams)</td>
<td>415 ±18</td>
<td>400 ±21</td>
<td>390 ±17</td>
<td>395 ±22</td>
</tr>
<tr>
<td><strong>Heart rate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(bpm)</td>
<td>330 ±25</td>
<td>340 ±30</td>
<td>345 ±28</td>
<td>365 ±35</td>
</tr>
<tr>
<td><strong>Haematocrit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td>40.2 ±0.5</td>
<td>40.4 ±0.2</td>
<td>42.0 ±0.3  **</td>
<td>41.4 ±0.7</td>
</tr>
<tr>
<td><strong>T-Cholesterol</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>70 ±6</td>
<td>72 ±3</td>
<td>99.6 ±9    **</td>
<td>115 ±10    **</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>142 ±18</td>
<td>121 ±17</td>
<td>249 ±20    **</td>
<td>136 ±18</td>
</tr>
<tr>
<td><strong>Free Fatty Acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/l)</td>
<td>0.29 ±0.14</td>
<td>0.25 ±0.14</td>
<td>0.81 ±0.18 **</td>
<td>0.18 ±0.08</td>
</tr>
<tr>
<td><strong>OH-butyrate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(nmol/l)</td>
<td>0.76 ±0.3</td>
<td>0.72 ±0.4</td>
<td>0.86 ±0.4</td>
<td>0.79 ±0.5</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µU/ml)</td>
<td>22 ±2.1</td>
<td>14 ±0.9 * $</td>
<td>$ 32 ±2.3 *</td>
<td>21 ±1.9</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>77 ±4.5</td>
<td>78 ±2.4</td>
<td>75 ±4.8</td>
<td>73 ±3.2</td>
</tr>
<tr>
<td><strong>Uric acid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg/dl)</td>
<td>1.1 ±0.1</td>
<td>0.7 ±0.1</td>
<td>0.9 ±0.1</td>
<td>1.1 ±0.1</td>
</tr>
<tr>
<td><strong>Nitrites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol/ml)</td>
<td>78 ±9</td>
<td>62 ±9</td>
<td>42 ±10 *</td>
<td>63 ±5</td>
</tr>
</tbody>
</table>

*p<0.05 , ** p<0.01, vs vehicle; $p<0.05, vs Dexamethasone
Table 2

Principal biochemical serum parameters, systolic blood pressure and body weight in the four groups of animals at day 7 and at the end of the 28 days experiment 2.

<table>
<thead>
<tr>
<th>Treatment Days</th>
<th>Vehicle +7</th>
<th>+28</th>
<th>DEX +7</th>
<th>+28</th>
<th>DEX+ARG +7</th>
<th>+28</th>
<th>DEX+ISN +7</th>
<th>+28</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mmHg)</td>
<td>118±2</td>
<td>119±3</td>
<td>120±5</td>
<td>151±6**</td>
<td>121±4</td>
<td>149±5**</td>
<td>120±3</td>
<td>125±4</td>
</tr>
<tr>
<td>Body Weight (grams)</td>
<td>405±25</td>
<td>400±21</td>
<td>386±23</td>
<td>388±30</td>
<td>384±31</td>
<td>371±22</td>
<td>393±24</td>
<td>390±28</td>
</tr>
<tr>
<td>Glycemia (mg/dl)</td>
<td>75±3</td>
<td>78±4</td>
<td>72±4</td>
<td>73±4</td>
<td>77±3</td>
<td>79±4</td>
<td>73±4</td>
<td>72±4</td>
</tr>
<tr>
<td>Insulin µU/ml</td>
<td>23±2.0</td>
<td>21±1.9</td>
<td>38±2.3*</td>
<td>33±2.5*</td>
<td>34±2.2*</td>
<td>29±2.0*</td>
<td>22±1.8</td>
<td>20±2.2</td>
</tr>
<tr>
<td>Δ% G</td>
<td>-44±4</td>
<td>-41±5</td>
<td>-27±5</td>
<td>-24±4</td>
<td>-28±5</td>
<td>-29±7</td>
<td>-39±7</td>
<td>-36±8</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>------</td>
<td>138±19</td>
<td>------</td>
<td>232±21***</td>
<td>------</td>
<td>215±20**</td>
<td>------</td>
<td>135±20</td>
</tr>
<tr>
<td>FFA mmol/l</td>
<td>------</td>
<td>0.24±0.13</td>
<td>------</td>
<td>0.79±0.15**</td>
<td>------</td>
<td>0.68±0.16</td>
<td>------</td>
<td>0.23±0.10</td>
</tr>
<tr>
<td>Nitrates/nitrites (µmol/ml)</td>
<td>64±7</td>
<td>80±8</td>
<td>35±10*</td>
<td>41±10*</td>
<td>42±13*</td>
<td>47±12*</td>
<td>79±8</td>
<td>69±6</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>40.3±0.7</td>
<td>40.0±0.8</td>
<td>41.8±0.5**</td>
<td>43.2±0.4**</td>
<td>43.2±0.6**</td>
<td>42.9±0.5**</td>
<td>40.±0.5</td>
<td>40.2±0.6</td>
</tr>
</tbody>
</table>

SBP= systolic blood pressure, TG = triglycerides, FFA= Free Fatty Acids
Δ% G= blood glucose variation (% from basal) after 1.6 U insulin Actrapid i.p.
*P<0.05, **P<0.01, *** P<0.001 vs vehicle same day
Legends to Figures

Figure 1
Blood glucose changes from basal (time 0) after intraperitoneal fast acting insulin measured every 10 minutes over a total period of observation of 80 minutes. Data were obtained in three rats receiving, in three different days, either vehicle or insulin at 0.8 and 1.6 U. Blood was obtained in the conscious rats from a tail vein and glucose measured by a reflectometer (Lifescan One Touch Profile, Johnson-Johnson Company, Minneapolis, USA).

Figure 2
Top
Systolic Blood Pressure (SBP, mmHg), in the four groups of rats during the 28 days experiment 1. ▲ Dexamethasone, ● Dexamethasone + Metformin, ◆ Metformin, ■ Vehicle

Data are mean ± SEM, p<0.05, ** p<0.001 vs vehicle

Bottom
Delta changes in blood glycemia 30 minutes after fast acting intraperitoneal Actrapid insulin (Novo-Nordisk), after 8 (open bars), 14 (grey bars) and 26 (dotted bars) days treatment. (experiment 1)

Data are mean ± SEM, * p<0.05 vs vehicle
Figure 3

Histological patterns of hepatocytes in the four groups of rats in experiment 1.

Top left = Metformin treated rats (MET), Bottom left = Dexamethasone + Metformin treated rats (DEXA+MET), Top right = Dexamethsone (DEXA) treated rats, Bottom right = Vehicle treated rats.

In DEXA it is evident the reduction in glycogen granules (red) while the empty spaces around the nuclei represent the lipid accumulation area (left empty by ethyl alcohol dehydration and paraffin inclusion) in comparison to Vehicle rats. Treatment with Metformin (DEXA+MET) restored the hepatocytes glycogen content and reduced the lipid content.
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Figure 2
Figure 3