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AT1R antagonism improves metabolic and vascular changes of obesity-induced gestational diabetes mellitus

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ABSTRACT

Obesity can overload glucose homeostasis and physiological insulin resistance during gestation which increases the risk of complications like diabetes mellitus or preeclampsia. Angiotensin II /AT_1 receptors are involved in the pathogenesis of vascular effects of obesity/insulin resistance but its role during gestation is not as clear. We sought to determine angiotensin II- AT_1R participation on a diet-induced gestational diabetes mellitus (GDM) experimental model. Female Wistar rats were fed with a standard or hypercaloric diet for 7 weeks. Half of the animals were mated and became pregnant from week 4–7. Animals were treated with saline, irbesartan (30 mg/kg) or metformin (320 mg/kg) for the last two weeks of the protocol. Weight gain, systolic blood pressure (BP), oral glucose tolerance test and vascular contractility were measured at the last day of the protocol (day 19–20 of pregnancy). Hypercaloric diet increased blood glucose, impaired glucose tolerance test, and increased BP in pregnant rats, fulfilling criteria for GDM. Both drugs decreased impaired GTT and relative hyperglycemia. Metformin had no effect on BP but prevented weight increase. In isolated aortas, irbesartan and metformin decreased vasoconstriction only of non-pregnant hypercaloric diet fed animals.

Results support angiotensin II/ AT_1R involvement in BP and glucose homeostasis disturbances observed in present GDM model. Also, provide evidence that a hypercaloric diet can mask pregnancyś physiological hypoglycemia and hypotension without surpassing non-pregnant values. Then, we conclude overweight during pregnancy causes subtle but significant vascular and metabolic damage that might be dismissed in clinical practice.

1. Introduction

One of the most important homeostatic metabolic adaptations of pregnancy is the development of physiologic insulin resistance [1] beginning from midterm and increasing until term [2]. Metabolic homeostasis of pregnancy particularly insulin resistance can be challenged by overweight, triggering gestational diabetes mellitus (GDM). Indeed, GDM is defined as glucose intolerance with varying degrees of severity, recognized for the first time during pregnancy [3] with an estimated worldwide prevalence of 7% [4]. GDM is considered a risk factor for preeclampsia-eclampsia, obstetrical trauma, cesarean section and perinatal mortality [5]. Besides, women who develop GDM, have an increased incidence of insulin resistance and type 2 diabetes later in life [6] a condition that enhances endothelial dysfunction through oxidative stress [7].

On the other hand, renin-angiotensin system (RAS) imbalance clearly contributes to the physiopathology of metabolic/cardiovascular disturbances. This is supported by the well known benefits of angiotensin-converting enzyme inhibitors (ACEI) and AT_1R antagonists on vascular complications of DM [8,9]. Besides, RAS is significantly involved in cardiovascular

Abbreviations: GDM, gestational diabetes mellitus; BP, blood pressure; GTT, glucose tolerance test; RAS, renin angiotensin system; ACE, angiotensin converting enzyme; DM, diabetes mellitus; SD, standard diet; HD, hypercaloric diet; P, pregnant; NP, non pregnant; Irbe, Irbesartan; Met, metformin; PPAR, peroxisome proliferator activator receptor; HOMA, homeostatic model assessment; GLUT, 4 glucose transporter 4.

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adaptations of gestation [10] and its imbalance has been proposed as a triggering factor for the hypertensive disorders of pregnancy. Particularly, an increased angiotensin II concentration has been associated with decreased insulin sensitivity in the offspring of mothers with GDM [11]. Then angiotensin II AT1R stimulation may be responsible for both metabolic and vascular damage produced by GDM.

GDM can have different degrees of severity. Then, hypercaloric diet may cause subtle damage associated with a mild type of GDM. Present work sought to determine AT_1R relative importance on metabolic and vascular damage produced by a hypercaloric diet-induced model of GDM. With the limitations of an experimental model, the objective was to compare the effects of AT_1R antagonist versus an insulin sensitizer on 1) blood pressure 2) fasting plasma glucose 3) glucose tolerance test and 4) vascular reactivity on pregnant and nonpregnant rats fed with standard or hypercaloric diet.

2. Materials and methods

2.1. Animals

Female wistar 12 weeks old rats (n = 60) weighing 250 ± 30 g were kept under standard conditions of light (12 h light/dark cycle) and humidity with free access to food and water. Male wistar rats 12/15 weeks old (n = 15) kept under the same conditions were used for mating with female rats. All procedures were approved by the Official Mexican Norm (NOM-062-ZOO-1969) for the animal handling and were approved by the local Ethical and Research Committee of the ESM Instituto Politecnico Nacional.

2.2. Experimental groups

Animals were fed with standard (SD n = 30) or hypercaloric (HD n = 30) diet for 7 weeks or 48 days of the protocol. Half of the rats were mated at day 28 (day 0 of pregnancy) from which 60–80 % got pregnant. Considering rat gestation average length is 21 days, pregnancy developed from day 28–48 of the protocol. Rats were treated with irbesartan Sigma-Aldrich® (30 mg/kg) [12] (n = 4 NPSD; 5PSD; 4NPHD; 4PHD), metformin Sigma-Aldrich® (320 mg/kg) (n = 5NPSD ;4PSD; 4NPHD; 5PHD) [13] or saline solution 0.9% (≈0.8 ml each) (n = 5 NPSD;4PSD and 4NPHD; 4PHD) for the last 14 days (Fig. 1A).

Drugs were dissolved in saline solution and administered p.o. through a steel device. Standard diet (SD) consisted of rat chow (3.1 kcal/g) and high-caloric diet (HD) (6.3 kcal/g) was prepared by mixing 33% ground commercial rat chow, 33% full fat sweetened condensed milk (Nestle ®), 7% sucrose and 27% water [14]. Diets and tap water were provided *ad libitum.*

2.3. Records in the whole animal

Systolic blood pressure was measured by an indirect tail-cuff plethysmography method (Letica 5007; PanLab, Barcelona) inside a warm (35 °C), softlight and noiseless room. Previously trained animals were placed into appropriate traps and blood pressure was determined as the mean of 3 consecutive successful measurements. Measurements were obtained at the beginning of protocol (day 1), at day 28 (pregnancy day 0) and at day 48 (pregnancy day 20). Fasting blood glucose was measured with reactive strips using a drop of blood obtained from the tail tip using a glucose meter Accu-Chek Advantage glucometer (Roche Diagnostics®, Basel, Switzerland) (reaction is based on glucose dehydrogenase through the ferricyanide/ferrocyanide reaction).

Weight was measured with a triple beam balance and registered at the end of each week of the study.

2.4. Fasting plasma glucose

After a 12 h fasting period, a drop of blood was obtained from the tip of the tail and blood glucose was measured using a Roche® glucometer and reactive strips, at the beginning of protocol, at day 28 and at the end of protocol.

2.5. Glucose tolerance test

At day 47 (day 18–19 of pregnancy), fasting blood glucose was measured before and after a bolus of 0.5 g/kg glucose administered through a cannula that delivered glucose intragastrically. Measurements were taken at 5, 10, 15, 30, 45, 60, 90, and 120 min. Glucose concentrations were graphed vs time and glucose tolerance was determined by calculating the area under the curve from min 5–120 and given in arbitrary units (AU) GraphPad Prism® 5.0 software.

2.6. Studies in isolated vessels

Animals were sacrificed by decapitation and thoracic aorta was excised and cleaned from surrounding connective tissue. Arteries were cut into rings (3–4 mm long) that were placed in tissue bath chambers filled with 10 mL Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5 $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.7, EDTA 0.026. Tissue baths were maintained at 37 °C, pH 7.4 and bubbled with a gas mixture of 95% O_2 and 5% CO_2. Vessel rings were placed between two nickrom hooks in order to fix them to the bottom of the chamber, and to a 50G-TSD125C force transducer connected to a general purpose amplifier DA100C. The amplifier was connected to a data acquisition system MP100 (Biopac System Inc. Santa Barbara, CA, USA®) in order to record isometric tension.

2.7. Analysis and statistics

Data are expressed as the mean of 4–5 animals ± SEM. pD_2 (-Log EC50) and Emax values were obtained by nonlinear regression analysis from concentration-response curves. Statistical evaluation of the data was carried out by one or two-way (ANOVA), with Bonferroni test for comparison of means using GraphPad Prism® 5.0 software. In all comparisons, values of $p < 0.05$ were considered significant between the means.

3. Results

3.1. Treatment effect on body weight

Weight increased in both SD and HD groups during protocol, particularly pregnant rats (data not shown). Neither metformin nor irbesartan significantly changed weight increase of any group along the protocol (Fig. 1B and C). In contrast, weight increase from pregnant animals (both SD and HD) was reduced by treatment with metformin (Fig. 1D and E) while no effect in weight was observed with irbesartan administration.

3.2. Fasting plasma glucose and glucose tolerance

No significant difference in fasting plasma glucose was observed amongst HD and SD groups during the first 28 days of protocol (Fig. 2A). At the end of protocol (day 48) pregnant rats showed the characteristic hypoglycemia described for the last three days of rat pregnancy (Fig. 2A). Indeed, PSD rats showed lower fasting plasma glucose (FPG) values respect to NPSD (51.7 ± 2.8vs 79 ± 3.1 mg/dL P vs NP respectively $p < 0.05$) at day 19–20 of pregnancy (Fig. 2A). FPG values were not significantly increased in

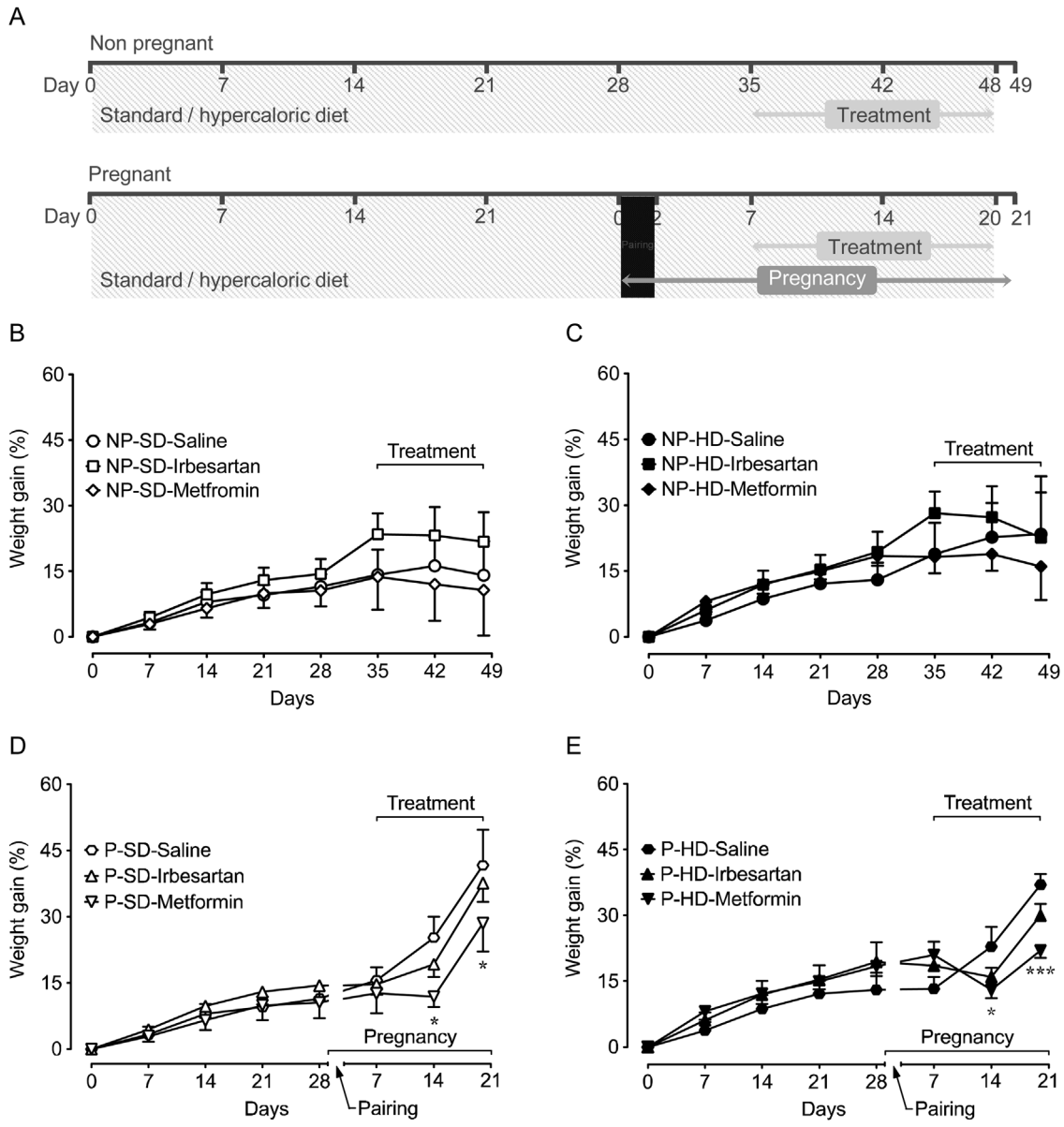

Fig. 1. Weight gain induced by diet and pregnant is sensitive to metformin treatment. A, experimental protocol of 48 days. Timeline that represents the period in which the animals received diet and treatment. Rats were randomly divided in non-pregnant and pregnant groups, each group received standard (SD) or hypercaloric (HD) diet. Animals were treated [saline solution (vehicle), irbesartan or metformin] from day 7 to 19–20 of pregnancy, corresponding from day 35–48 in non-pregnant animals. B–E, the weight increase was monitored weekly using a triple beam mechanical balance from day 0 (basal) to day 48 that corresponds to day 19–20 of pregnancy. Effect of treatment with irbesartan (30 mg/Kg/day p.o.) or metformin (320 mg/Kg/day p.o.) in body weight increase (%) of non-pregnant (NP; B and C) or pregnant (P; D and E) rats, received standard (B and D) or hypercaloric (C and E) diet. Each point represents the media ± s.e.m. of 4–5 independent experiments. *, $p < 0.05$; ***, $p < 0.001$ vs saline solution treatment.

NPHD group (79 ± 3.1 vs 82 ± 2.1 mg/dL NPSD vs NPHD respectively n.s.d.). In contrast, FPG increased in the PHD group respect PSD (51.7 ± 2.8 vs 68 ± 3.7 mg/dL PSD vs PHD respectively $p < 0.05$) all values were measured at the end of protocol (day 48) and of pregnancy (day 19–20) (Fig. 2A).

Met nor Irb treatment for the last 14 days of protocol, significantly changed FPG of nonpregnant or pregnant SD animals (data not shown), but Met diminished FPG of NPHD rats (82 ± 2.1 vs 70 ± 3.2 mg/dL saline vs Met respectively $p < 0.05$) (Fig. 2B). Interestingly Irb, decreased FPG of PHD (GDM) animals (68 ± 2.1 vs 52 ± 1.9 mg/dL no treatment vs Irb treatment respectively $p < 0.05$) (Fig. 2B).

GTT was conducted as an indicator of insulin resistance. Interestingly, pregnancy GTT area under the curve was significantly smaller compared with nonpregnant animals (Fig. 3A). Also, PHD rats (GDM) showed an impaired GTT compared to PSD (81.88 ± 2.01 vs 102.43 ± 2.1 AU $p < 0.05$ PSD and PHD respectively). GTT of PHD (GDM) rats showed increased PG values respect to PSD animals (Fig. 3A), and besides, PHD glycaemia did not reach basal values at the end of the 120 min of the study, supporting the criteria for GDM diagnosis (Fig. 3A). Even when GTT of NPHD rats was also increased, glycaemia reached NPSD values at the end of the study (Fig. 3A). Interestingly, none of the groups showed hyperglycemia at minute 0 and values are considerably low due to the 12 h fasting period.

Treatments with Met and Irbe did not significantly change the GTT of NP SD or HD rats or even from PSD rats (data not shown). Interestingly both Met and Irbe significantly reduced GTT of GDM animals (Fig. 3B).

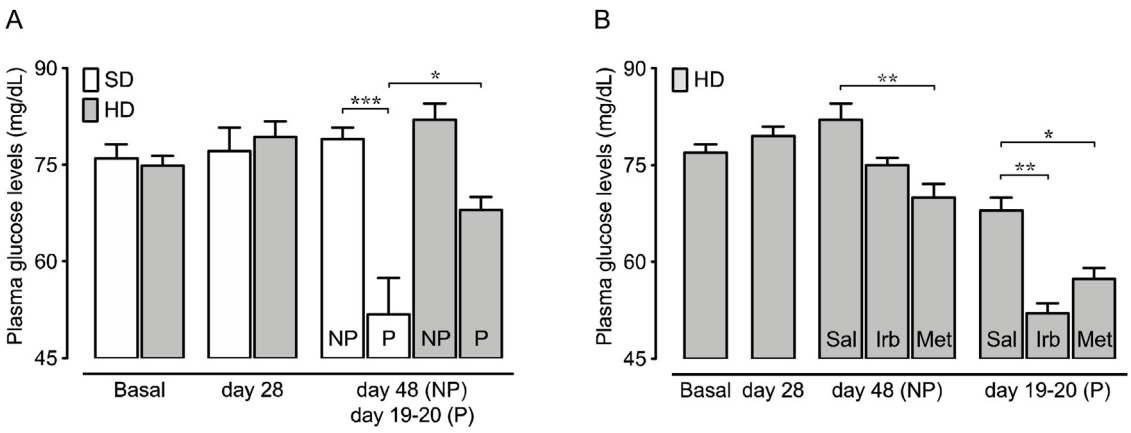

Fig. 2. The relative hyperglycemia characteristic of hypercaloric diet induced GDM, is dependent on the action of angiotensin II. **A**, fasting plasma glucose levels of standard (SD) or hypercaloric (HD) diet fed rats at day 0 (basal), day 28 (day 0 of pregnancy) and day 48 for non-pregnant (NP), corresponding to day 19–20 for pregnant (P). **B**, effect of treatment with vehicle (saline), irbesartan (Irb, 30 mg/Kg/day p.o.) or metformin (Met, 320 mg/Kg/day p.o.) for the last 2 weeks of protocol of hypercaloric diet fed rats (HD) under control non-pregnant and non-treated conditions (basal and day 28) and at day 48 for non-pregnant (NP) or day 19–20 for pregnant (P). Each bar represents the media ± s.e.m. of 4–5 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs control condition.

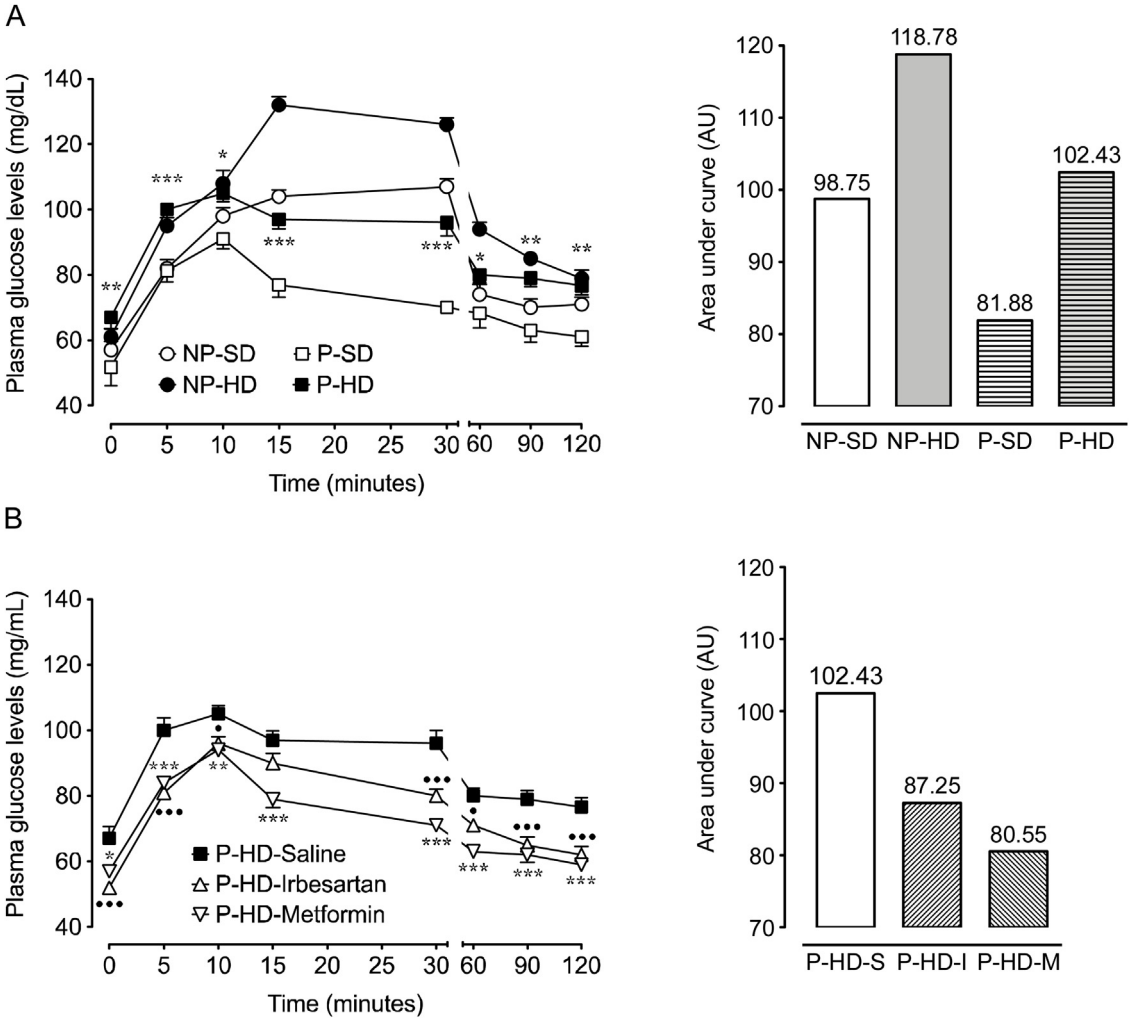

Fig. 3. The attenuation of angiotensin II action improves glucose tolerance in hypercaloric diet induced GDM. Oral glucose tolerance test (GTT). Plasma glucose concentration was determined before (0 min) and after to give an oral glucose load. **A**, the effect of pregnancy and diet on glucose tolerance was measured in the following groups: non-pregnant with standard diet (NP-SD), pregnant with standard diet (P-SD), and pregnant women with hypercaloric diet (P-HD). **B**, effect of treatment with saline, irbesartan (30 mg/Kg/day p.o.) or metformin (320 mg/Kg/day p.o.) of pregnant rats fed hypercaloric diet. Area under the curve in arbitrary units (AU) from 0 to 120 min corresponding to the graph A and graph B, is represented in the right panel of each graph. Each point represents the media ± s.e.m. of 4–5 independent experiments. */·, $p < 0.05$; **, $p < 0.01$; ***/. . ., $p < 0.001$ vs control condition (P-SD or P-HD-Saline).

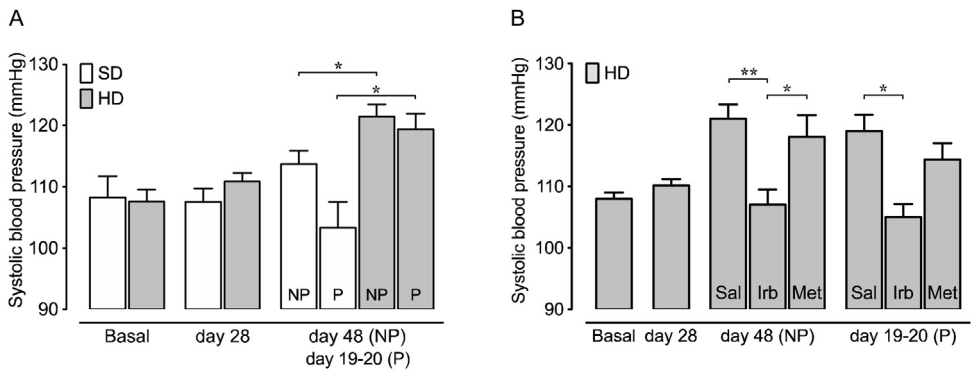

Fig. 4. AT-1 receptor antagonism in hypercaloric diet induced GDM promotes regression of systolic blood pressure to healthy pregnancy levels. Systolic blood pressure was registered at the beginning and at the end of week 4 and 7 of protocol by no-invasive tail cuff method. **A**, rats of the different experimental groups: non-pregnant and pregnant that received standard diet (NP-SD, P-SD) or hypercaloric diet (NP-HD, P-HD). **B**, effect of treatment with saline, irbesartan (30 mg/Kg/day p.o.) or metformin (320 mg/Kg/day p.o.) on NPHD and PHD groups. Each bar represents the media ± s.e.m. of 4–5 independent experiments. *, $p < 0.05$; **, $p < 0.01$ vs control condition.

3.3. Systolic blood pressure

No significant change in BP was observed in NPSD along protocol but it increased in NPHD animals (113.75 ± 2.1 vs 121 ± 1.9 NPSD vs NPHD $p < 0.05$) (Fig. 4A) BP was significantly decreased in PSD animals at the last day of protocol, that corresponds to day 19–20 of pregnancy (113.75 ± 3.05 vs 106 ± 2.8 mm Hg NP vs P $p < 0.05$) (Fig. 4A). PHD blood pressure was increased respect to PSD (Fig. 4A) reverting pregnancy physiological hypotension, even when BP values did not increase enough to reach "hypertension "values (106 ± 2.81 vs 119.3 ± 3.8 mm Hg PSD vs PHD $p < 0.05$) (Fig. 4A).

Treatments did not change BP values of NPSD or PSD animal (data not shown), but Irbe reduced BP of NPHD animals (121 ± 1.9 vs 107.3 ± 3.6 mm Hg NPHD saline vs NPHD Irbe $p < 0.05$) (Fig. 4B). Also, Irbe diminished BP of PHD rats (119.3 ± 3.81 vs 105 ± 2.6 mm Hg PHD saline vs PHD Irbe $p < 0.05$) while Met produced no change (119.3 ± 3.81 vs 118 ± 2.9 mm Hg PHD saline vs PHD Met n.s.d.)(Fig. 4B).

3.4. Isolated organ studies

In order to evaluate the smooth muscle conditions of aorta rings, the force developed by aortic rings in response to KCl 80 mM was measured. No differences were found between groups, indicating that HD diet or GDM did not affect the contractile machinery of the vessel (data not shown).

3.5. Response to phenylephrine

Changes induced by Met or Irbe treatment on phenylephrine-induced aorta vasoconstriction were evaluated. Treatments did not change NPSD nor PSD or PHD Phe-induced aorta contraction (Fig. 5B–D). Interestingly both Met and Irbe decreased the response of NPHD vessels (Emax 2.63 ± 0.3 vs 2.082 ± 0.11 g and 1.75 ± 0.22 g control, Met and Irbe respectively $p < 0.05$) (Fig. 5B).HD produced no change in contractility between experimental groups (data not shown).

4. Discussion

The aim of our work was to support the role of angiotensin II-AT_1R in the initiation of metabolic and vascular damage associated with gestational diabetes mellitus. To achieve this goal irbesartan [9] an AT_1 receptor antagonist and metformin an oral hypoglycemic agent [15] were used as experimental tools. Experimental GDM was achieved through a hypercaloric diet that yields to an älteration of glucose tolerance test that appears for the first time during pregnancyẗhe main feature for the diagnosis of this disease [3].

On the other hand, ACE inhibitors and AT_1R antagonists ability to improve insulin sensitivity and glycemic control in diabetic patients and to reduce the incidence of new-onset type 2 diabetes [16], has supported angiotensin II role as a common mediator of metabolic syndrome from which hypertension and hyperglycemia are the main components. Indeed, Ang II through AT_1R stimulation can disturb insulin signaling associated with an increased Ser instead of Tyr phosphorylation of IRS^{-1} and downriver inhibition of insulin signaling molecules including IRS^{-1} in association with p85 subunit of PI3K, phospho-Ser473-Akt,and phospho-Ser1177-eNOS [17,18]. Besides, it has been demonstrated that pro-inflammatory cytokines, oxidative stress, and free fatty acids induce Ser phosphorylation of IRS-1, inhibiting insulin signaling [18]. Indeed there is evidence that cafeteria-style induced DM, can cause insulin resistance mediated by angiotensin II and oxidative stress [19–21]. Also, high glucose has |been associated with a decrease of TET2 [22] which in turn can promote angiotensin II-mediated cardiac dysfunction [23].

In this work, irbesartan treatment did not change weight increase in any group (Fig. 1,C,D,E) while metformin significantly prevented weight increase of pregnant animals (both SD or HD) (Fig. 1C,D,E). These results can be explained by increased participation of gluconeogenesis for weight gain during gestation. Physiological hypoglycemia (Fig. 2A) and a diminished GTT area under the curve during the last days (19–20) of rat pregnancy that was thrown back by gestational diabetes mellitus were observed in this work (Fig. 3A). Neither irbesartan nor metformin change glucose or GTT values from nonpregnant SD groups (data not shown). Even when FPG was not significantly increased in nonpregnant HD rats, metformin reduced BG values (but not of SD groups), proving glucose homeostasis of rodents can be disturbed by HD for a relative short period of time (48 days) (Fig. 2B). Interestingly, relative hyperglycemia of GDM rats respect pregnant animals fed standard diet, was more significantly reduced by irbesartan that with metformin (Fig. 2B) and both treatments returned GTT to SD pregnancy values (Fig. 3B). Findings provide further evidence that AT_1R has an important role on the triggering of metabolic disturbances of GDM probably through the established cross-talk between insulin and angiotensin II signaling systems [17] in which angiotensin II, through ERK and JNK, induces phosphorylation at serine residues IRS-1 that inhibits activation of Akt, producing alterations in glucose uptake [17]. Agonistic effects of irbesartan on PPARα receptors on adiponectin have also to be considered. Indeed irbesartan has shown to decrease insulin resistance (HOMA) and plasma triglycerides while increasing adiponectin levels and the expression of the

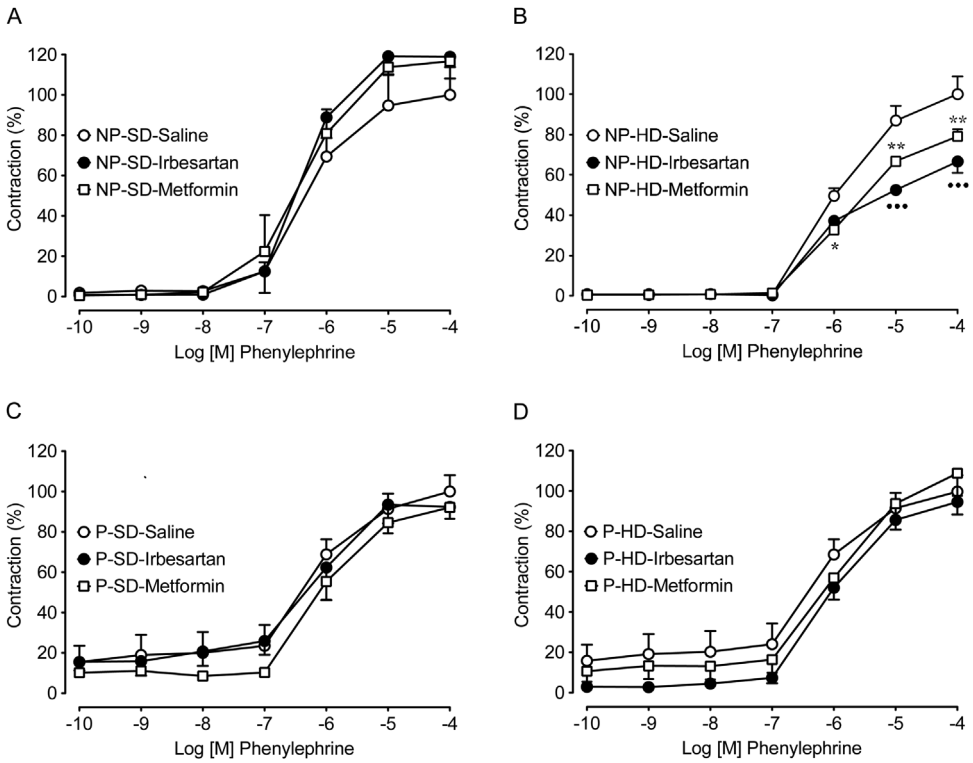

Fig. 5. Effect of AT-1 receptor antagonist (irbesartan) treatment compared to metformin on phenylephrine cumulative concentration-response curve. Contraction (g) induced with phenylephrine (Phe) in isolated thoracic aortic rings (with endothelium) from non-pregnant rats fed standar diet (NP-SD, A); non-pregnant rats fed hypercaloric diet (NP-HD, B) and pregnant rats fed standard diet (P-SD, C) or hypercaloric diet (P-HD, D) on treatment with saline, irbesartan (30 mg/Kg/day p.o.) or metformin (320 mg/Kg/day p.o.). Each point represent the media ± s.e.m. of 4–5 independent experiments. *, $p < 0.05$; **, $p < 0.01$; . . ., $p < 0.001$ vs saline solution treatment.

transporter GLUT 4 in adipocytes of hypertensive rats [12]. Nevertheless, this effect of irbesartan was not supported by present results since treated rats showed no change in weight gain.

Systolic BP is considered the most accurate value for the plethysmographic method in rats so was the value used for comparisons. A decrease in blood pressure was observed in late gestation, that interestingly, was reverted by GDM (Fig. 4A). GDM BP increase was mild with respect to control but significant respect to the pregnant SD group (Fig. 4A). These findings support the link between metabolic changes and hypertension and relates slight BP increases during pregnancy with significant metabolic disturbances that can be underestimated in clinical practice. BP of NP or P SD animals was not changed by irbesartan or metformin, but was increased in NPHD (respect to NPSD) and PHD (respect to PSD) groups (Fig. 4A). Irbesartan decreased BP from NPHD and GDM groups (Fig. 4B) further supporting angiotensin II/AT_1R participation in blood pressure increase as a consequence of hypercaloric diet. Besides, results support irbesartan effectiveness in the treatment of metabolic alterations.

Finally, phenylephrine induced vascular contraction was explored, considering there is evidence of vascular alpha1/AT_1R cross talk generated by diet-induced obesity [24]. GDM showed a tendency to increase contraction even when no significant differences were found between four groups (data not shown). Treatments did not significantly change GDM, PSD or NPSD aorta contraction (Fig. 5A,C,D) but both irbesartan and metformin decreased the contraction of NPHD group (Fig. 5B). These results suggest angiotensin II/AT_1R participation increases with hypercaloric diet but these changes may be ameliorated by pregnancy. Indeed, damage produced by metabolic disturbances can change adrenergic or Ang II receptors functionality, increasing the cross-talk between α1 and AT_1 receptors [25–28]. An increased α1/AT_1 cross-talk has been proposed as an early damage indicator of metabolic disturbances [29]. Both Irbe and Met treatments by different pathways improved plasma glucose levels probably decreasing vasoconstrictors participation on phenylephrine-induced contraction.

Hypoglycemic effects of metformin are attributed to a reduction of hepatic glucose production (mainly through inhibition of gluconeogenesis and to a lesser degree of glycogenolysis) and to an increased insulin-dependent glucose uptake of striated muscle tissue [15] and adipocytes. Metformin increases the activity of tyrosine kinase (TK) insulin receptor (IR) in vascular smooth muscle cells independently of the action of insulin [30] and its primary site of action appears to be the mitochondria where decouples oxidation respiratory chain of substrates of complex I [31]. In various tissues, including adipocytes and skeletal muscle, metformin promotes translocation of glucose transporter 4 and 1 (GLUT4, GLUT1) [32] to the plasma membrane and by stimulating AMPK, metformin can increase TET2 [22]. Secretion of proinflammatory adipokines [33,34], such as leptin can also be considered in the effects observed [35], Leptin can be contributing to the effects observed since it can induce sympathetic vasoconstriction [36] and a selective requirement of the AT1A receptor in leptin-mediated control of resting metabolic rate has been demonstrated [37].

Irbesartan and other AT_1R antagonists, as well as ACEI drugs, are contraindicated during pregnancy because reported increased risk of fetopathy [38]. In the present work, irbesartan was used as a tool to dissect AT_1R role in the pathophysiology of gestational diabetes mellitus. The experimental model used for this work was first validated by Holemans et al. [14] and is consistent with subtle and early metabolic adaptations to obesity during pregnancy.

In conclusion, findings suggest increased blood glucose levels, altered GTT and increased SBP observed in this GDM model are triggered by angiotensin II/ AT_1R. Also, we provide evidence that overweight during pregnancy can trigger mild metabolic dis-

orders that can lead to hypertension. Pregnancy physiologicaĺs drop in BG and BP can be erased by metabolic changes of hypercaloric diet-induced GDM. These increased values may not surpass normal nonpregnant values, increasing the risk of being unnoticed in clinical practice. Attention to these subtle changes can help to improve current standards of care and justify therapeutic nutritional advice to prevent and achieve early identification of pregnancy complications.

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