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Ph.D. thesis:

CARDIOVASCULAR AND METABOLIC ADAPTATION TO PREGNANCY IN SPONTANEOUSLY HYPERTENSIVE RATS

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A Mamma e Papà,
grazie per gli innumerevoli sforzi e
sacrifici che fate affinché possa realizzare i miei sogni.

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INDEX

1. INTRODUCTION

Hypertensive disorders in pregnancy remain a major cause of maternal, fetal and neonatal morbidity and mortality not only in less developed but, also, in the industrialized countries. Pregnant women with hypertension are at higher risk for severe complications such as abruptio placentae, cerebrovascular accident, organ failure, and disseminated intravascular coagulation. The fetus is at risk for intrauterine growth retardation, prematurity, and intrauterine death. Physiologically, blood pressure falls in the second trimester, reaching a mean of 15 mmHg lower than levels before pregnancy. In the third trimester, it returns to pre-pregnancy levels. This fluctuation occurs in both normotensive and chronically hypertensive women.

The definition of hypertension in pregnancy is not uniform. It used to include an elevation in blood pressure during the second trimester from a baseline reading in the first trimester, or to prepregnancy levels, but a definition based on absolute blood pressure values (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) is now preferred. Hypertension in pregnancy is not a single entity but comprises:

- *Pre-existing hypertension*, which complicates 1-5% of pregnancies and is defined as blood pressure ≥ 140/90 mmHg that either predates pregnancy or develops before 20 weeks of gestation. Hypertension usually persists more than 42 days post partum. It may be associated with proteinuria.

- *Gestational hypertension*, which is pregnancy-induced hypertension with or without proteinuria. Gestational hypertension associated with significant proteinuria (> 300 mg/l or > 500 mg/24 h or dipstick 2+ or more) is known as *pre-eclampsia*.

Hypertension develops after 20 weeks' gestation. In most cases, it resolves within 42 days post partum. Gestational hypertension is characterized by poor organ perfusion.

- *Pre-existing hypertension plus superimposed gestational hypertension with proteinuria*. Pre-existing hypertension is associated with further worsening of blood pressure and protein excretion ≥ 3 g/day in 24-hour urine collection after 20 weeks' gestation; it corresponds to previous terminology "chronic hypertension with superimposed pre-eclampsia".

- *Antenatally unclassifiable hypertension:* hypertension with or without systemic manifestation, if blood pressure was first recorded after 20 weeks' gestation. Re-assessment is necessary at or after 42 days post partum. If hypertension is resolved by then, the condition should be re-classified as gestational hypertension with or without proteinuria. If the hypertension is not resolved by then, the condition should be re-classified as pre-existing hypertension. Edema occurs in up to 60% of normal pregnancies, and is no longer used in the diagnosis of pre-eclampsia.

The spontaneously hypertensive rat (SHR) represents a widely used genetic animal model of hypertension (Okamoto and Aoky, 1963). Several studies have shown that the SHR can serve as suitable model for essential hypertension to study neuroendocrine and metabolic abnormalities that characterize this disease during pregnancy. Aoi et al (1976) showed that blood pressure decreases midway through pregnancy in SHR and reaches level similar to those found in control Wistar Kyoto rats (WKY). Moreover, other authors found a remarkable reduction of pressure at delivery (Zamorano et al., 1980; Lindheimer et al., 1983; Ahokas et al., 1990, Mattace Raso et al., 2007). The pressure decrease near term could be related to an

increase of nitric oxide in pregnancy (Conrad et al., 1993), or the release of hypotensive substances by the placenta (Nakanishi et al. 1980) rather than an alteration of insulin resistance (Tanigawa et al. 1999).

Nevertheless, the molecular mechanisms behind the modification of hypertensive state during pregnancy remain to be elucidated.

1.1 Role of renin-angiotensin system (RAS) in physiopathology of pregnancy

During pregnancy, a number of physiological changes occur in the maternal circulation to accommodate the growing fetus. These changes usually include an increase in cardiac output and a decrease in arterial blood pressure and total peripheral resistance. Among many vasomotor systems regulating the blood pressure, the renin-angiotensin system (RAS) plays an important role. RAS participates in electrolyte homeostasis, in the maintenance of vascular tone, and in cardiovascular remodelling. Angiotensin II (Ang II), the main component of RAS, regulates blood pressure, body fluid volume and electrolyte balance interacting with the type 1 (AT1) and the type 2 (AT2) receptor (Hall et al., 1999; Timmermans et al., 1993). The RAS plays a major role in the physiological regulation of the kidney, including the control of renal microvascular and tubular function. Well-known renal actions of Ang II mediated by the AT1 receptor include increased tubular sodium absorption at low doses, inhibition of reabsorption at higher doses, afferent and efferent arteriolar vasoconstriction, and glomerular mesangial cell contraction and constriction of renal vessels, including the arcuate and interlobular arteries and vasa recta (Arendshorst et al., 1999; Navar et al., 1996). These actions produce integrated physiological actions including decreased renal blood flow, glomerular filtration rate, and sodium excretion. In addition to direct effects of Ang II on vascular smooth muscle and tubule cells, the peptide can stimulate the release of vasoactive factors from endothelial, vascular smooth muscle, mesangial, interstitial, or other cell types within the kidney. Thus the vascular and tubular actions of Ang II can be regulated by cell-to-cell (paracrine or autocrine) mediators produced in response to Ang II, thereby

dampening or amplifying the primary effects. The most frequent integrated response to Ang II is net vasoconstriction, and the best known counteracting vasodilator mechanisms include NO and the vasodilator products of arachidonic acid metabolism, especially prostaglandin E_2 (PGE_2) and prostaglandin I_2 (PGI_2).

The physiological actions of Ang II at AT2 receptor seems to act in opposite to the AT1 receptor. In general, the AT2 receptor inhibits cell growth and proliferation and promotes cell differentiation, counterbalancing the opposite effects of Ang II at the AT1 receptor (Meffert et al., 1996; Stoll et al., 1995). AT2 receptor has been shown to play an important role in Ang II stimulation of a number of renal vasodilator substances, including bradykinin (BK) and NO (Carey et al., 2000). Because BK is also a renal autacoid that stimulates NO production through the B2 receptor, it was possible that the AT2 receptor stimulates a renal BK-NO-cGMP vasodilator cascade (Siragy et al.,1990) (figure1 and 2).

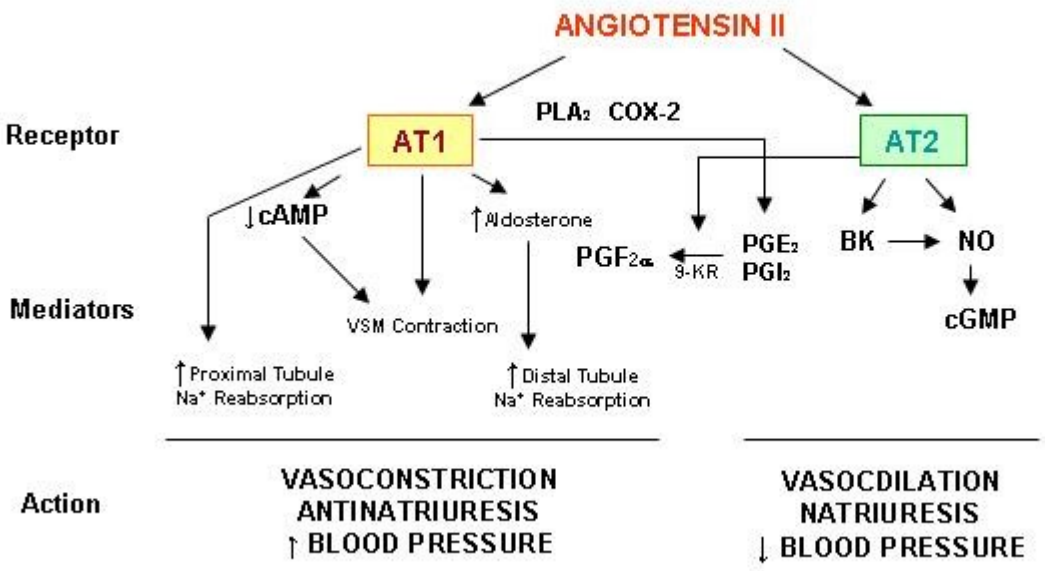

Fig 1. Schematic description of the regulation of blood pressure and renal function by the AT1 and AT2 receptor.

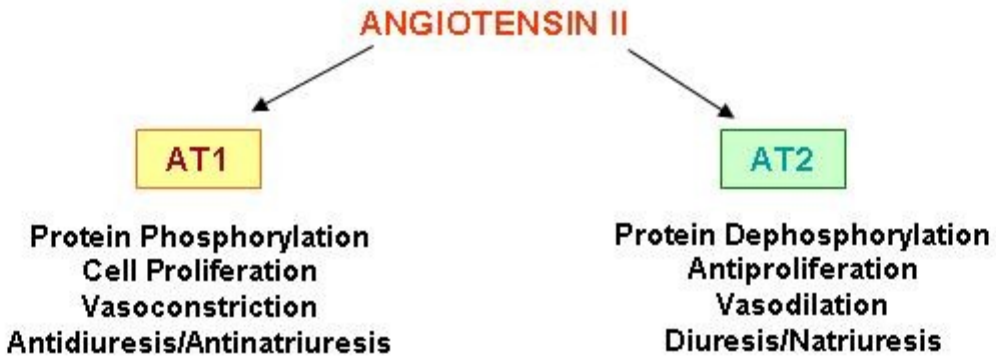

Fig 2. Schematic description of the balance of actions mediated by AT1 and AT2 receptors. AT2 receptor actions counterbalancing actions of AT1 receptors.

There is accumulating evidence to indicate that Ang II is also capable of inducing inflammatory response in the vascular wall. Ang II modifies several steps of inflammatory response, such as increase of vascular permeability, leukocyte infiltration, tissue hypertrophy/proliferation, and fibrosis (Cheng et al., 2005).

Hypertension is associated with an increased risk for tissue injury that may be mediated by endothelium dysfunction, the ongoing of inflammatory process, with overproduction of superoxide (O_2^-) and other reactive oxygen species (ROS) in cardiovascular system and kidney (Zalba et al., 2000; Zhan et al.,2004). It's well documented that Ang II, via the AT1 receptors, enhances the production of ROS through stimulation of NAD(P)H oxidase in the vascular wall. The increased oxidative stress contributes to endothelial dysfunction and to vascular inflammation by stimulating the redox-sensitive transcription factors (NF-kB) and by upregulating adhesion molecules, cytokines, and chemokines (Cheng et al., 2005). As well documented NF-kB regulates expression of proinflammatory enzymes such as cyclooxygenase (COX)-2 and inducible nitric-oxide synthase (iNOS), that may exacerbate tissue damage.

Cyclooxygenase metabolites have been implicated in functional and structural alterations in glomerular and tubulointerstitial inflammatory diseases (Takahashi et al.,1990). Previous studies have suggested that cyclooxygenase inhibitors may acutely decrease hyperfiltration in diabetes and inhibit proteinuria and/or structural injury (Hommel et al., 1987). Hong et al. (2000) focused their experiments on the potential role of excess nitric oxide (NO) production by iNOS in the pathogenesis of hypertension in the SHR. Excessive NO would result in peroxynitrite anion formation, protein tyrosine nitration, hydroxyl radicals generation and thereby oxidative/nitrosative stress and hypertension (Klahr, 1998; Espey et al., 2002; Modlinger et al., 2004). It should be noted that the reduction in NO bioavailability in the SHR is, in part, due to Ang II-mediated increase in superoxide production and

impaired superoxide scavenger activity (Adler and Huang, 2004). Nonetheless, iNOS expression is significantly increased in the SHR (Chou et al., 1998; Vaziri et al., 1998) and its down-regulation by pyrrolidine dithiocarbamate (PDTC), which is a known to inhibit iNOS induction (Liu et al., 1997; Hong et al., 1998) could contributed to the prevention of hypertension in PDTC-treated SHR (Rodriguez-Iturbe et al., 2005).

Other studies have demonstrated that the treatments with either antioxidant or immunosuppressive/anti-inflammatory agents improve hypertensive state in SHR (Rodriguez-Iturbe et al., 2002; 2003; 2005).

1.2 Role of placental proteins in physiopathology of pregnancy.

The rat placenta has been widely used as a model to study placental development. Briefly, the rat placenta is composed of two distinct zones, the junctional zone (invasion and endocrine function) and the labyrinth zone (transport barrier) (Knipp et al., 1999). The junctional zone is adjacent to the maternal compartment and is mainly involved in uterine wall invasion and the production of hormones/cytokines. The labyrinth zone is the main barrier to diffusion and acts to regulate the transfer of nutrients and wastes between the maternal and fetal compartments. Of note is the fact that syncytial trophoblast cells form the transport barriers in both the rat and human placentas (Ogata et al., 1997). Fatty acids are of critical importance in normal development of the foetus, due to the fact that fatty acids serve as obligatory constituents of biological membranes (Uauy et al., 1999), concentrated fuel storage (Uauy and Hoffman, 2000), and precursors of intracellular signalling molecules (Narumiya and Fukushima, 1986). In addition, insufficient fatty acid supply has been demonstrated to result in foetal intrauterine growth retardation (IUGR), fetal facial dysmorphology and severe postnatal growth retardation (Abel, 1984; West, 1994; Denkins et al., 2000). Central to these observations is the role of the placenta in influencing bi-directional transfer of fatty acids between the maternal and fetal circulations. Fatty acids are hydrophobic, and depending on their physicochemical properties, may passively diffuse into cells across the lipid bilayer membrane (Hamilton, 1998; Hamilton and Kamp, 1999). However, the capacity of fatty acid transfer by free diffusion is limited and not sufficient to satisfy the demand of the developing foetus. Therefore, a facilitative, directional transfer of fatty acids from

the maternal circulation to the foetus is required to properly meet foetal demands. Consistent with this observation, Hornstra et al. demonstrated that fatty acid transport is highly directional, with a strong preference in the direction from the mother to the foetus (Hornstra et al., 1995). Dutta-Roy and other investigators have demonstrated that there exists a preference for transporting long-chain polyunsaturated fatty acid (LCPUFAs) over nonessential fatty acids in the human placenta and in BeWo cells, a human choriocarcinoma cell line (Campbell et al., 1997; Campbell et al., 1998). This observation cannot be explained by simple diffusion of fatty acids alone. Recently, several fatty acid transport proteins have been found in the rat and human placentas and in in vitro trophoblast cell culture models (Campbell, et al, 1994; Knipp et al., 2000). These proteins were identified as plasma membrane fatty acid binding protein (FABPpm), fatty acid translocase (FAT), fatty acid transport protein (FATP) and members of cytosolic fatty acid binding proteins (FABPs). All of these proteins are known to function as fatty acid transferring proteins in other tissues and their expression in the both the rat and human placentas have been shown. However, the regulation of fatty acid transport across the placenta remains to be elucidated. In recent years, it has become clear that fatty acids act in an autocrine manner to regulate their metabolism, uptake and transport (McDonald and Lane, 1995; Vamecq and Latruffe, 1999). Further investigations demonstrated that this autocrine effect may be facilitated by nuclear hormone receptors of the peroxisome proliferator-activated receptor (PPAR) family (Lemberger et al., 1994; Schoonjans et al.,1996). There are currently three PPAR isoforms, α, β, and γ, that have been identified in various tissues from several species (Mukherjee et al., 1994; and 1997). A wide

range of structurally different chemicals, including long chain fatty acids, eicossanoids, leukotrienes, hypolipidemic drugs, and antidiabetic agents (Forman et al., 1997; Kliewer et al., 1997; Krey et al., 1997) have been demonstrated to bind and activate PPAR isoforms. It must be noted that for each PPAR isoform, binding has a moderate degree of specificity, and certain compounds may only interact with a particular isoform (Krey et al., 1997). Although each of the three PPAR subtypes is known to be expressed in the rat placenta, in particular PPARα and PPARγ, measured by RT-PCR and immunohistochemical analyses, seem to be expressed at higher levels toward term, the period of maximal fetal and placental growth, in response to the increased fetal demand (Wang et al.,2002). PPARα has been demonstrated to play a role in regulating lipid catabolism (Krey et al., 1993), whereas PPARγ was demonstrated to promote the differentiation of preadipocyte fibroblasts to the mature adipocytes (Peraldi et al., 1997). Recently, PPARγ has also been shown to be an important regulatory factor for placental development (Barak et al., 1999; Waite et al., 2000). PPAR isoforms have been demonstrated to form a heterodimer with another nuclear hormone receptor family, the 9-cis-retinoic acid receptor (RXR) isoforms that are activated by ligand binding of 9-cis-retinoic acid (Levin et al., 1992). Three isoforms of RXR family, α, β, and γ, have been

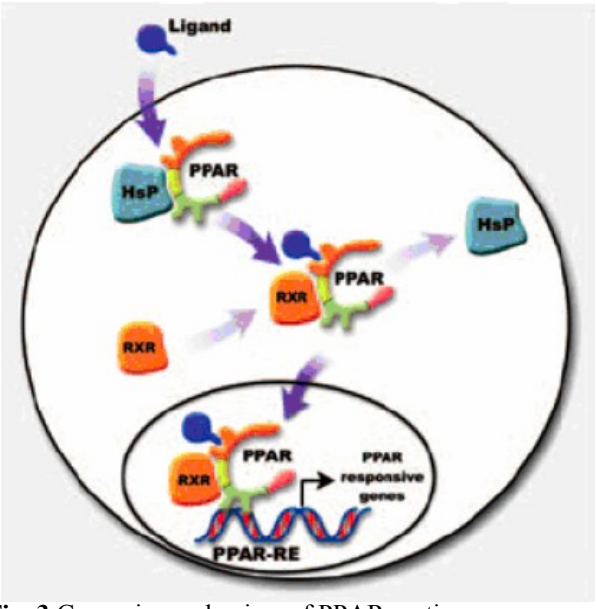

Fig. 3 Genomic mechanism of PPARs action

characterized in different species (Freebern et al., 1999). The individual PPAR/RXR heterodimers bind to the peroxisome proliferator response element (PPRE) in the promoter region of target genes to control gene transcription (Schoonjans et al., 1996; Simoneau et al., 1999) (figure 3). The PPAR/RXR heterodimers have been demonstrated to regulate the transcription of several target genes including FAT, FATP, several FABP subtypes, acyl-CoA oxidase, phosphoenolpyruvate carboxykinase (PEPCK) (Schoonjans et al., 1995; Schoonjans et al., 1996), which collectively exert integrative effects on lipid homeostasis.

It has been previously shown that nitric oxide (NO), a free radical gas, is an important bio-regulator in the cardiovascular, immune, nervous (Moncada et al., 1991) and reproductive systems (Izumi et al., 1993). The maternal circulatory adaptations that accompany pregnancy are also influenced by NO, reducing blood pressure and vascular tone and attenuating response to vasoconstrictors (Molnar M and Hertelendy, 1992). During pregnancy NO synthase (NOS) activity is expressed in the placental villous tree where it may contribute in regulating placental blood flow, and thereby fetal nutrition and growth. It appears that NO contributes to the maternal circulatory adaptation that accompany pregnancy, for example, reduced blood pressure and vascular tone and an attenuated responses to vasoconstrictors (Molner M. and Heertelendy F. 1992). The placental circulation, which is critical for delivery of nutrients and oxygen to the growing fetus in exchange for fetal-derived metabolic waste products, is influenced mainly by local synthesis of NO. In addition to the constitutive endothelial eNOS, some authors claim the presence of inducible iNOS at the end of pregnancy (Casado et al., 1997), as principal sources of NO in

placenta. Beyond a key role in blood pressure decrease and fetal perfusion, placental NO seems to act in a paracrine fashion to modulate uterine function, and its down-regulation is related to initiation of labour (Purcell et al., 1997).

Differently from NO system, the level of COX-2 increases significantly toward the end of gestation and contributes to pregnancy maintenance and labour initiation. Moreover, prostaglandins (PGs) induce myometral contractility, regulate fetal adaptation to labour process, maintain uterine and placental blood flow and contribute to changes in extracellular matrix metabolism associated with cervical ripening during parturition, fetal adaptation to the labor process, and maintenance of uterine and placental blood flow (Challis et al., 2002). Spatial expression of COX-2 intensity shifts from the labyrinthine zone to the maternal-invasive junctional zone with the advance of pregnancy (Xu et al., 2005). This shift suggest that PGs biosynthesis in the decidua at late-gestation regulates parturition, (Gibb, 1996) and thus, a shorter transfer to the myometrium enhances the significance of their biological effects. Interestingly, PGE_2 increased metalloproteinase (MMP)-9 gelatinolytic activity responsible for collagen degradation within the fetal membranes. Alterations in these processes might influence pregnancy outcome.

The fetal membrane rupture and trophoblastic cells invasion process involve the degradation and remodelling of extracellular matrix (ECM) mainly due to MMPs, a family of zinc-dependent proteolytic enzymes. They are synthesized as secreted or transmembrane proenzymes and processed to an active form by the removal of an amino-terminal propeptide.

As known the strength of the amnion and chorion is, in large part, a result of collagen. Collagens I, III, IV, V and VI have been described in various layers of the amniochorion. Degradation of collagen during labour is controlled by MMPs which have specificities for different collagen types as modulated by tissue inhibitors of matrix metalloproteinases (TIMPs). The ratio of the MMP/TIMP for particular collagens thus determines whether collagen is degraded. Degradation, along with the deposition rate of new collagen by fibroblasts, determines the resultant strength of the tissues. MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9 have been described in the amniochorion. Major investigative work in fetal membranes has been done with MMP-2 and MMP-9, and somewhat less with MMP-1 and MMP-3. Most reports suggest that fetal membrane MMP-2 is constitutive, not responding to cytokines, or changing with premature rupture of membranes or labor (term or preterm) (Maymon et al., 2001; Xu et al., 2002; Fortunato et al., 1999). In contrast, MMP-9, in both the latent form and active form, has been shown to increase in amniotic fluid with preterm premature rupture of membranes (Fortunato et al., 1999), in amniotic fluid of rhesus monkey after labor induction with cytokines (Vadillo-Ortega et al., 2002), and in human fetal membrane tissue (Xu et al., 2002). MMP-9 can also be induced in fetal membrane tissue with PGE2, PGF2α , tumor necrosis factor (TNF)-α and ROS (McLaren et al., 200; Ulug et al., 2001; Arechavaleta-Velasco et al., 2002; Buhimschi et al., 2000; Zaga et al., 2004).

The selective timing of MMP-9 expression just before delivery makes it a potential candidate as a molecular marker in the initiation of labour (Uchide et al 2000),

differently from other MMPs, such as MMP-2, that is constitutively expressed during all phases of pregnancy and enhanced in late gestation (Vadillo-Ortega et al., 2005).

1.3 Hormone modifications in physiopathology of pregnancy: role of leptin and ghrelin

In the last decade many data report the hormones influence on pressure control. In particular among these, leptin (Ob), the product of the *ob* gene, is thought to play a critical role in the pathogenesis of hypertensive disorders in pregnancy and actually is considered as a marker of pre-eclampsia, a form of pregnancy induced hypertension. In fact, maternal leptinemia is significantly higher in pregnancies complicated by pre-eclampsia than gestational age matched controls (Teppa et al. 2000). The increase of hormone level predates the development of preeclampsia (Anim-Nyame et al. 2000) and it has been shown that leptin gene expression in the placenta is augmented in severe pre-eclampsia (Laivouri et al. 2006). Moreover, a dysregulation of autocrine and paracrine function of leptin in fetal-maternal interface can be implicated not only in pregnancy-induced hypertension, but also in gestational diabetes and in the intrauterine growth retardation, including disturbance of fetal bone (Bajoria et al., 2002).

Leptin, may directly or indirectly influence reproductive function because regulates mother's metabolism, fetus growth and development via pituitary, hypothalamic and placental receptors. Recent reports have demonstrated that leptin levels are elevated in serum during human and rodent gestation (Henson & Castracane 2006). In particular, during rat pregnancy, the high levels of leptin indicate the existence of a physiological state of central leptin resistance, that might explain the increased food intake observed during gestation (Garcia et al., 2000).

It is well recognised that leptin is produced in several organs and tissues besides

white adipose tissue, such as heart, mammary epithelial cells, and placenta. This hormone inhibits food intake, regulates energy expenditure, is a permissive signal to the reproductive system and is a metabolic hormone affecting insulin secretion, lipolysis, and sugar transport (Trayhurn et al., 1999). Leptin has been proposed as a lipostatic factor that regulates the amount of body fat stores by means of a closed feedback loop involving the hypothalamus (Rohner Jean Reaneaud et al. 1996; Erickson et al., 1996).

The physiological actions of leptin are linked to the interaction of the hormone with specific receptors (Ob-R), which use the JAK/STAT pathway of signal transduction. Different isoforms of the Ob-R exist, including a long isoform (Ob-Rb) with signalling capacity and short isoforms with several and not completely known functions. In particular Ob-Rb is expressed in a wide range of tissues including hypothalamus (Tartaglia, 1997). Hypothalamus is the major site of energy homeostasis regulation. The arcuate nucleus (ARC) is a key hypothalamic site involved in food intake and body weight. Some ARC neurons express NPY whose injection into the third ventricle or paraventricular nucleus (PVN) potently increases food intake. These neurons also express Ob-Rb and are inhibited by leptin. A separate population of ARC neurons expresses proopiomelanocortin (POMC), the precursor of alpha-melanocyte-stimulating hormone (alpha-MSH) which powerfully inhibits feeding through hypothalamic melanocortin-3 (MC3-R) and melanocortin-4 (MC4-R) receptors. POMC is also colocalized with Ob-Rb, and is stimulated by leptin. These opposing neuropeptide systems interact at different levels. Firstly, the NPY neurons coexpress Agouti related peptide (AgRP), an antagonist at MC4-R

which reinforces the action of NPY by inhibiting the action of alpha-MSH. Secondly, NPY neurons are thought to inhibit POMC neurons via NPY Y1 receptors, while the POMC neurons may inhibit NPY expression and release via MC3-R (Rocha et al., 2003).

It has recently been reported that ghrelin, a gastric derived peptide, plays an important role in the reproductive function both in animals (Gualillo et al., 2002; Caminos et al., 2003) and humans (Gaytan et al., 2003).

In the CNS this hormone induces GH release and interacts with hypothalamic nuclei, stimulating feeding and determining body weight gain (Wren et al., 2000). Ghrelin augments neuropeptide Y gene expression and blocks leptin-induced feeding reduction, implying that there is a competitive interaction between ghrelin and leptin in feeding regulation (Nakazato et al., 2001) (figure 4). Expression of ghrelin and GHS-R genes has been described in non-pregnant and decidualized endometrium, and ghrelin has been involved as paracrine/autocrine regulator of decidualization of human endometrial stromal cells, and tentatively, in the cross-talk between endometrium and embryo during implantation (Tanaka et al., 2003). Notably, ghrelin levels in uterine fluid dramatically increased during fasting in mice, and ghrelin has been recently reported to inhibit the development of mouse preimplantation embryos in vitro (Kawamura et al., 2003). In good agreement, it has recently observed that chronic ghrelin treatment during the first half of pregnancy in the rat induced a significant reduction in the litter size (Fernandeez-Fernandez et al., 2005).

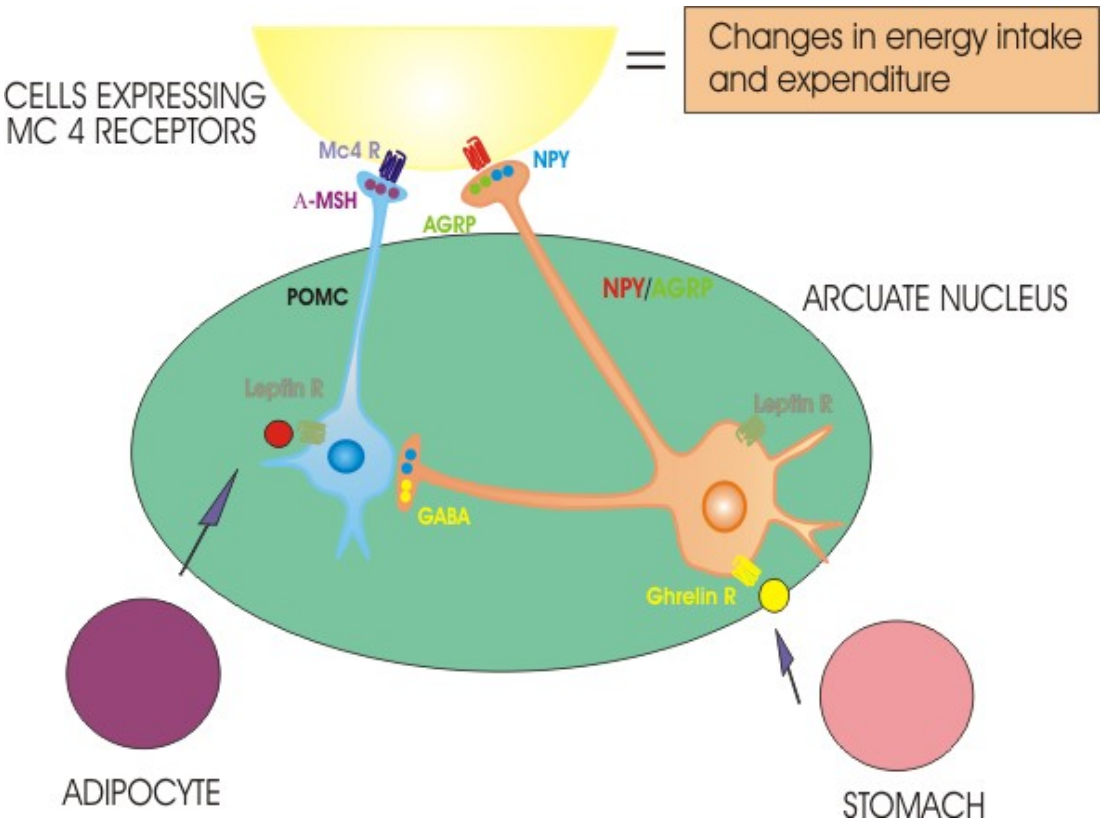

Fig. 4 Leptin and ghrelin act on neuronal populations in the arcuate nucleus which then transmit the information regarding current appetite status

In addition, ghrelin has been detected in human and rat placenta (Gualillo et al., 2001), and ghrelin has been demonstrated in human fetal circulation (Cortelazzi et al., 2003). The role of placental and fetal ghrelin in the regulation of gestational growth and metabolism remains to be fully elucidated.

Ghrelin mRNA expression was persistently detected in rat ovary throughout pregnancy, with higher levels in early pregnancy and lower expression during the later part of gestation (Caminos et al., 2003) and its secretion might influence the fetal growth and maturation. This reduced ghrelin expression is less evident in undernutrited animals in pregnancy; in this situation plasmatic levels and ghrelin

gastric mRNA are up-modulated, showing a role of the hormone in mediating the physiological responses to undernutrition and could represent an adaptative response to prevent long-lasting alterations in energy balance and body weight homeostasis (Gualillo et al., 2002).

Recently, ghrelin has also been shown to participate in cardiovascular and sympathetic regulation. Intravenous injection of human ghrelin elicits a decrease in blood pressure without an increase in heart rate in healthy men. Ghrelin has direct vasodilatory effects possibly through GH or nitric oxide-independent mechanisms (Okumura et al., 2002). However, because the depressor response was not accompanied by tachycardia, it is likely that the mechanisms other than direct vasodilating effects, at least in part, are involved in this depressor response of ghrelin. Intracerebroventricular administration of ghrelin suppresses renal sympathetic nerve activity and decreases arterial pressure in conscious rabbits (Matsumura et al., 2003). Therefore, depressor effect induced by intravenous injection of ghrelin is partly explained by the central inhibition of sympathetic activity. In addition, intracerebroventricular infusion of subdepressor dose of ghrelin augments the baroreflex sensitivities assessed by renal sympathetic nerve activity and heart rate compared with those of vehicle infusion (Matsumura et al., 2003). Ghrelin acts at the central nervous system to modulate sympathetic activity in these two different manners; however, brain region where ghrelin acts have not been well determined.

Furthermore, ghrelin plasmatic levels are significantly higher in patients with pregnancy-induced hypertension (PIH) indicating a significant correlation between

ghrelin concentration and systemic blood pressure in these patients (Makino et al., 2002). Therefore we speculate a role of this hormone in cardiovascular control during pregnancy and related pathophysiology.

Ghrelin is also synthesized by placenta and its secretion might influence the fetal growth and maturation. During rat pregnancy the maternal concentration of plasma ghrelin is significantly lower than that of non-pregnant animals, even if ghrelin peptide concentrations in the stomach did not change significantly during pregnancy (Shibata et al., 2004).

2. THE AIM OF THE STUDY

On the basis of previous research data, here we studied the adaptative mechanisms responsible for the improvement of hypertensive status during pregnancy observed in SHR.

To this purpose, we evaluated the modifications of several cardiovascular and inflammatory parameters in pregnant or not SHR compared to respective normotensive WKY: i.e (i) the changes of AT1 and AT2 receptor expression in kidney, (ii) the vascular response to Ang II in the mesenteric plexus, (iii) the kidney modification of pro-inflammatory transcription factor NF-κB and related gene expression, such as COX-2 and iNOS and (iv) the oxidative stress, as malondialdehyde (MDA) and protein nitrotyrosilation.

Moreover, for the first time, we determined the modifications of AT1, ACE, nitric oxide synthase isoforms (eNOS, iNOS), as regulators of vascular tone in placenta. In this tissue we also evaluated COX-2 expression and MMPs activity, as indicators of labour onset. Finally, to asses fetal development in SHR, we also evaluated placental expression of PPARα and PPARγ, as regulators of fetal growth and placental functions.

In order to evaluate prospective metabolic and hormonal differences or modifications, in another set of experiments, we compared plasma leptin and its protein expression in placenta and adipose tissue at the end of gestation (20d) in normal (WKY) and hypertensive (SHR) animals. Differential regulation of the Ob-R expression in peripheral tissues and in the hypothalamus in WKY and SHR rats was

also evaluated. The plasma ghrelin level and mRNA in the stomach and placenta, were also measured.

3. MATERIALS AND METHODS

3.1 Animals

Non pregnant (-NP) or pregnant (-P) SHR and Wistar Kyoto normotensive rats were used. Twenty-week-old female normotensive WKY (body weight 211±2.6g) and SHR (body weight 210±2.3 g; n=8 each group, Harlan Italy, San Pietro al Natisone, Udine, Italy) were mated at oestrous, and the day of mating, determined by the presence of spermatozoa after a vaginal smear, was considered day 0 of pregnancy. Same number of virgin WKY (body weight 206±2.4g) and SHR (body weight 214±1.7g) of the same age served as controls. At the end of gestation animal body weight was also determined.

All procedures involving animal were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 and associated guidelines in the European Communities Council Directive of November 24, 1986 (86/609/ECC), using the number of animals as small as possible.

3.2 Measurement of arterial blood pressure and heart rate in conscious rat

The systolic blood pressure (BP) and heart rate (HR) was measured in conscious rats by noninvasive common indirect method using a tail-cuff device in combination with blood flow sensor and recorder (Ugo Basile, Biological Research Apparatus, 21025 Comerio, Italy) (Mattace Raso et al., 2007). Briefly, rats were housed for 30 min in a warmed room (28-30°C), then a tail cuff placed about 2 cm from the base of the tail for measuring systolic blood pressure without physical restraint of the animal.

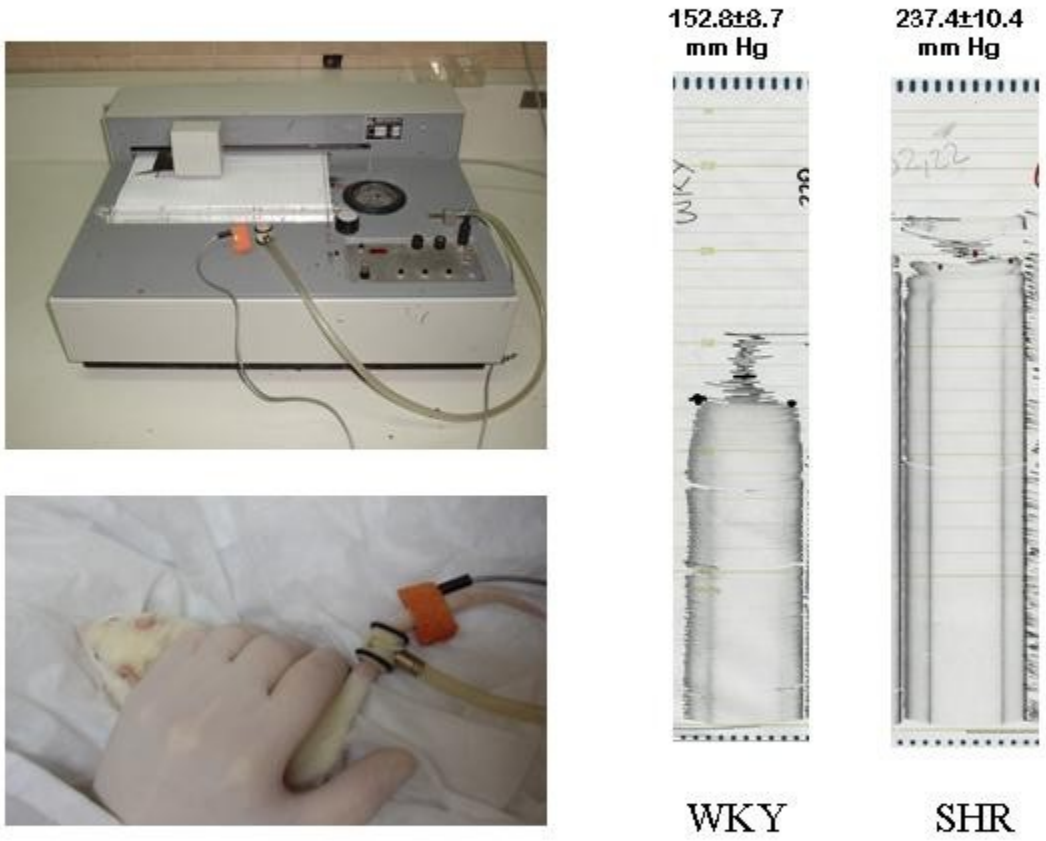

Fig. 5 Measurement of arterial blood pressure and heart rate in conscious rat using an tail-cuff device, blood flow sensor and recorder.

Care was taken in selecting an appropriate cuff size for each animal. Rats were allowed to habituate to this procedure for 2 weeks before experiments were performed. Heart rate was detected by a pulse rate counter placed after the tail cuff and monitored with the audio signal (figure 5). Before mating and at 6, 14 and 20 days of pregnancy and in control non pregnant rats, BP and HR values were recorded and were averaged from at least three consecutive readings obtained from each rat.

3.3 Body weight, food intake, and body gain in fat

Throughout pregnancy body weight and food intake were monitored once a week (6, 14, and 20 d). At the end of the experimental period, food intakes were cumulated. Bioelectrical impedance analysis (BIA) was applied to body composition assessment at d 20 by a BIA 101 analyzer, modified for rat (Akern, Florence, Italy). Free fat mass was calculated by the BIA (50 kHz) prediction equation of Ilagan et al. (1993) and fat mass content was obtained as the difference between body weight and free fat mass.

3.4 Tissue collection and blood parameters

At d 20 of pregnancy animals were anesthetized by enflurane and sacrificed by cervical dislocation. Control animals were sacrificed at the second day of diestrus. Blood collected by cardiac puncture was centrifuged at 1500xg, at 4 C for 15 min and sera were stored at –70 C for later biochemical and hormonal measurements.

Glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, and cholesterol were quantified using nonfasting blood sample. The serum leptin and ghrelin (the bioactive form of the hormone, n-octanoyl modified in Ser^3) concentration was measured by RIA kits according to manufacturer's instruction (Linco Research, Inc., St Charles, MO).

Subcutaneous white adipose tissue, stomach, placenta, and hypothalamus [dissected according to the map of Glowinski & Iversen (1966)] kidney, heart, and mesenteric plexus were excised and used for experimental procedures or immediately frozen in liquid nitrogen.

3.5 Western blot analysis

The subcutaneous adipose tissue, hypothalamus, and placenta obtained from each animal were disrupted by homogenization on ice in lysis buffer (Tris-HCl, 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml leupeptin and trypsin inhibitor). After 1 h, cell lysates were obtained by centrifugation at 100,000 g for 15 min at 4°C. Protein concentration of the samples was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Milan, Italy), using bovine serum albumin as the standard.

For western blot analysis, 35-100 μg protein of tissue lysate was dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to SDS-PAGE (8%, 12% or 15% polyacrylamide). The blot was performed by transferring proteins from a slab gel to nitrocellulose membrane at 240 mA for 40 min at room temperature. The filter was then blocked with 1x PBS, 5% non fat dried milk for 40 min at room temperature and probed with rabbit polyclonal antibodies against AT1, AT2, IκB-α, ACE, PPARα, PPARγ, Ob (Santa Cruz Biotechnology, Santa Cruz, CA) 3-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY; 1:5000) or COX-2 (1:1000, Cayman Chemical, Ann Arbor, MI) or with mouse monoclonal antibody anti-p65 NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) or against eNOS, or iNOS (DB Transdaction Laboratories, Lexington, KY, USA), or with a goat polyclonal anti-C-term Ob-Rb antibody (Santa Cruz Biotechnology, Santa Cruz,

CA), dissolved in 1x PBS, 5% non fat dried milk, 0.1% Tween 20 at 4°C, overnight. The secondary antibody (anti-rabbit or anti mouse or anti-goat IgG-horseradish peroxidase conjugate) was incubated for 1 h at room temperature. Subsequently, the blot was extensively washed with PBS, developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions, and exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY). Western blot for β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (in cell lysates) or lamin protein (in nuclei lysates) (Sigma; St. Louis, MO) was performed to ensure equal sample loading. The protein bands on x-ray film were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories).

3.6 RT-PCR analysis

Total RNA was extracted by a modified method of Chomczynski & Sacchi (1987), using TRIzol Reagent (Life Technologies, Milan, Italy) according to the manufacturer's instructions. Reverse transcription was performed by a standard procedure (Brenner et al. 1989) using 2 μg of total RNA. After reverse transcription, 2 μl of RT products were diluted in 48 μl of PCR mix, to give a final concentration of 50 U/ml of Taq DNA polymerase (Life Technologies, Milan, Italy), 4 μM of 5' and 3' primers, 50 μM of each dNTP, 1.5 mM $MgCl_2$, and 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). The following oligonucleotides were used: ghrelin [forward primer 5' TTGAGCCCAGAGCACCAGAAA-3' and reverse primer 5'-AGTTGCAGAGGAGGCAGAAGCT-3'] and β-actin [forward primer 5'-

TACAACTCCTTGCAGCTCC-3' and reverse primer 5'-ATCTTCATGAGGTAGTCAGTC-3']. PCR primers were synthesised by "Servizio di Biologia Molecolare, Stazione Zoologica A. Dohrn" (Naples, Italy). The amplification profile for rat ghrelin and β-actin was: denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. Thirty five- cycle amplification was completed with an additional step at 72°C for 10 min. The amplification was performed in an automatic thermal cycler (Biometra, Germany). To assure that PCR was performed in the linear amplification range, samples were taken after 15, 20, 25, 30, 35, and 40 cycles, showing that the reaction was linear over this range (data not shown). Then 10 μl of RT-PCR products were separated by 1.5% agarose gel electrophoresis in TBE 1× (Tris–base 10.089 M, boric acid 0.089 M) containing 0.2 μg/ml of ethidium bromide. Fragments of DNA were seen under UV light. β-actin was used as an internal reference. PCR generates a single 347-bp product for rat ghrelin and a single 603-bp product for the β-actin gene. The bands of ghrelin were scanned and densitometrically analyzed with a model GS-700 imaging densitometer (Bio-Rad Laboratories, Milan, Italy).

3.7 Isolated and perfused mesenteric bed

Mesenteric bed preparation was performed according to Warner (1990). Briefly, at d 20 of pregnancy or at the second day of diestrum, rats were anaesthetized by enflurane and killed by cervical dislocation. The superior mesenteric artery was cannulated and the mesenteric vascular bed perfused via the artery for 5 min at 2ml/

min with Krebs buffer containing heparin (20 I.U./ml). The intestine was separated from the mesentery by cutting close to the intestinal border of the mesentery and the preparation was perfused at 2 ml/min by an infusion pump (Harvard pump type 22, Watson-Marlow) with warmed (37°C) and gassed Krebs buffer containing indomethacin (INDO, 10μM, Sigma, Milan, Italy). Changes in perfusion pressure were measured by a pressure transducers Bentley 800 Trantec (Basile, Comerio, Italy) connected to a recorder (Basile Unirecord, Comerio, Italy).

After an equilibration time (30 min), mesenteric arterial reactivity was evaluated adding Ang II, 0.1 nmol on basal tone. To evaluate the endothelium-dependent and independent relaxation, Ach (10 nmoles) and SNP (0.1 nmol) were injected on MTX (an alpha1-adrenoceptor selective agonist, 100μM) pre-constricted mesenteric bed.

3.8 MDA measurement

MDA levels in the renal and heart tissues were determined as an indicator of lipid peroxidation (Mullane et al., 1988). Tissues were homogenized in 1.15% KCl solution. An aliquot (200 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid (TBA), and 600 μl of distilled water. Samples were then boiled for 1 h at 95 C and centrifuged at 3,000*g* for 10 min. The supernatant absorbance was measured by spectrophotometry at 550 nm.

3.9 MMPs activity

Gelatin-zymography was performed to determine the MMP activity of the pro-enzyme forms (pro-MMP-9 and pro-MMP-2) and MMP-2 active form, as previously described (Okada et al., 2001). Briefly, two placentas obtained from each animal were pooled and their standardised homogenates were subjected to electrophoresis in (2 mg/ml) gelatin-containing polyacrylamide gels in the presence of SDS under non-reducing conditions. After electrophoresis, the gels were incubated at 37°C overnight in 0.1 M Tris–HCl gelatinase-activation buffer (pH 7.4) containing 10mM $CaCl_2$ and subsequently stained with 0.5% Coomassie Blue. After intensive destaining, proteolysis areas appeared as clear bands against a blue background.

The lack of active MMP-9 in the zymograms may be due to its high level of instability and the removal of active enzyme during the washing of specimens, as it has been previously suggested (Deleve et al., 2003).

MMPs were identified by their molecular weight compared with standards (Medina et al.,2003). To measure the activities of the detected enzymes, zymograms were read using a ScanJet 3c scanner (Hewlett-Packard, Boise, ID). Quantitative evaluation of both surface and intensity of lysis bands, on the basis of grey levels, was performed comparing gelatinolytic activity of WKY and SHR placentas.

3.10 Statistical analysis

All data were presented as mean ± SEM. The different groups were compared for variables by the student' t test or ANOVA, with Bonferroni's test for the post-hoc

analysis. A p value of ≤ 0.05 was considered significant. The statistical analysis was performed using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA).

4. RESULTS

4.1 Systolic blood pressure and heart rate in pregnant or not WKY and SHR

The mean values of systolic blood pressure (mmHg) and heart rate (bpm) detected during the experimental period for both SHR and WKY are shown in figure 6 (panel A and B, respectively). No differences in blood pressure were observed in WKY-P compared to WKY-NP rats (panel A). In SHR-P rats this parameter was significantly reduced at d 14 of pregnancy compared to SHR-NP ($P<0.05$) and the reduction was more marked at d 20 ($P<0.001$), where blood pressure reading was not significantly different compared to that of normotensive pregnant rats.

Similarly, no changes were observed in heart rate among WKY-NP and WKY-P (panel B), while in SHR-P during pregnancy a significant decrease of heart rate was shown both at 14 and 20 d ($p<0.001$ vs SHR-NP). At the end of pregnancy also heart rate value of SHR-P became close to normotensive pregnant rats.

4.2 AT1 and AT2 expression in kidney from WKY and SHR

Since we observed an improvement in cardiac parameters in SHR strain during pregnancy, our goal was to investigate if this positive modification was associated to the RAS changes. In this regards kidney was used for AT1 and AT2 analysis. SHR-NP kidney presented a more marked expression of AT1 compared to that of WKY controls ($t=2.9$, Fig. 7 panel A). For the first time in hypertensive animals, we showed at d 20 of pregnancy a significant decrease of renal AT1 expression ($p<0.001$ vs SHR-NP). Moreover, no significant modification was evidenced for renal AT2

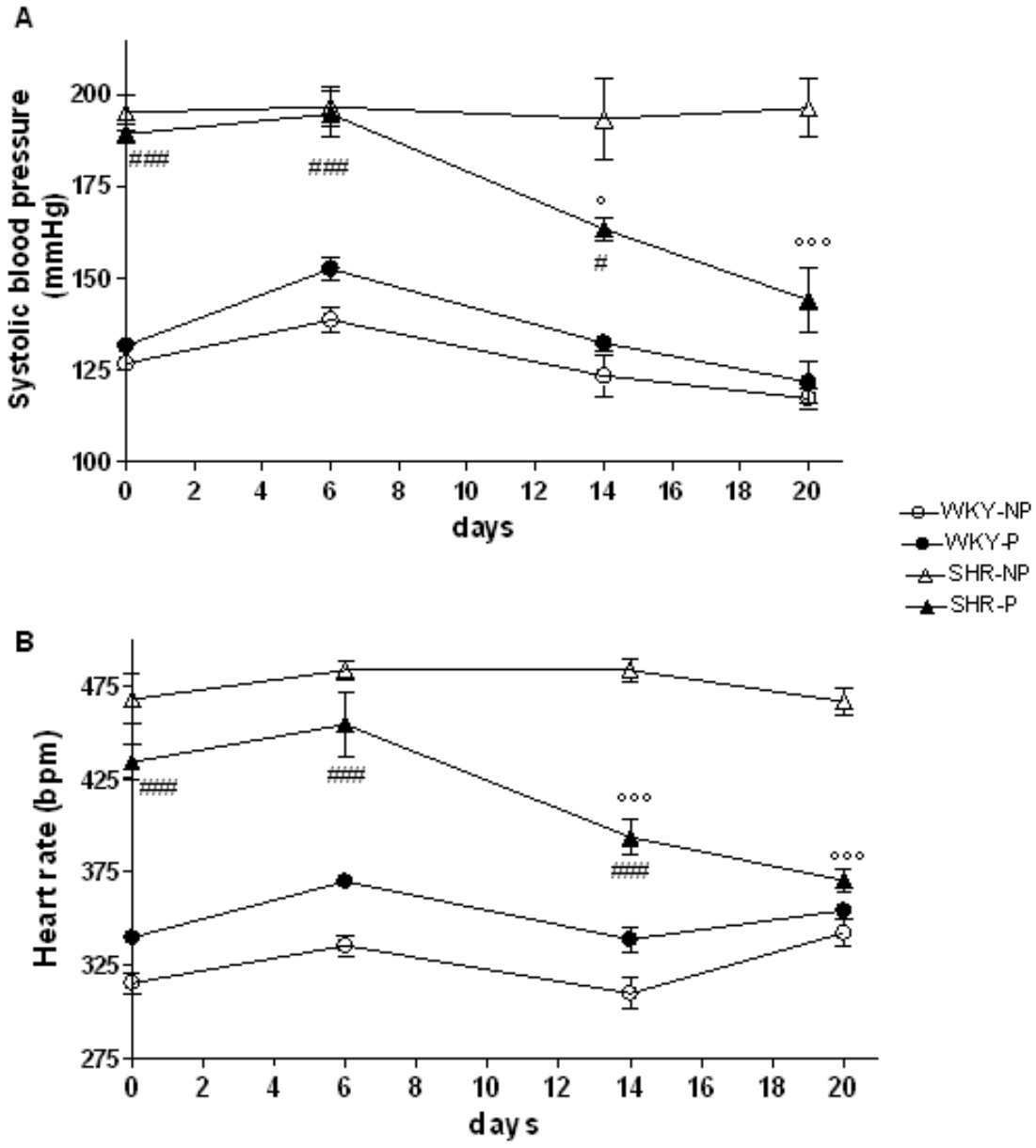

Fig. 6 Systolic blood pressure and heart rate of non pregnant (-NP) or pregnant (-P) hypertensive (SHR) and normotensive (WKY) rats measured at 0, 6, 14 and 20 days. The results are expressed as mean ± SEM of 8 animals.
°$p<0.05$, and °°°$p<0.001$ vs SHR-NP; #$p<0.05$, and ###$p<0.001$ vs WKY-P.

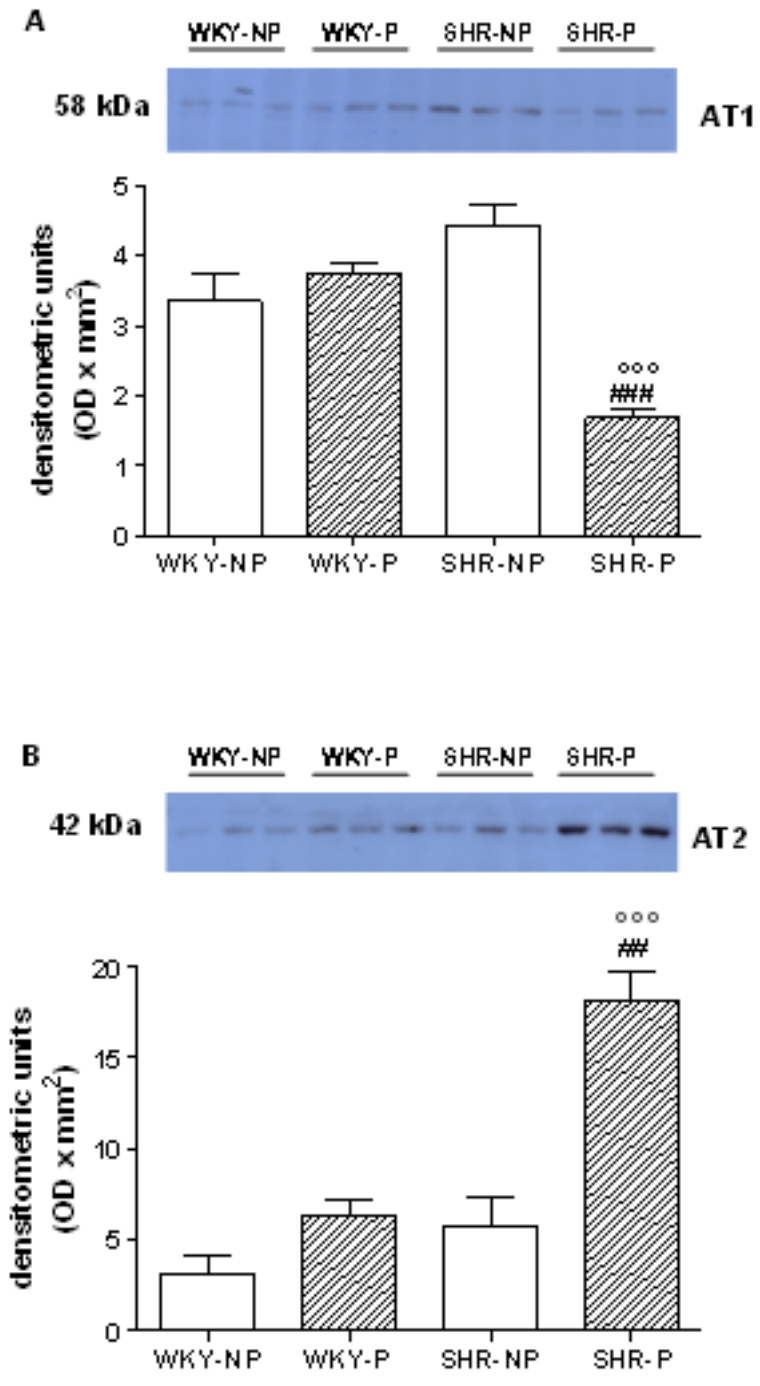

Fig. 7 AT1 (panel A) and AT2 (panel B) expression in kidney from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 6 animals. °° °p<0.001 vs SHR-NP; ##p<0.01 and ###p<0.001 vs WKY-P.

expression in SHR-NP compared to WKY, while during pregnancy a marked and significant increase of AT2 band was found in SHR-P (panel B).

4.3 Effect of Ang II on isolated and perfused mesenteric bed

Basal perfusion pressure in mesenteric artery bed was 19.0±2.1, 16.7± 5.3, 21.6±4.1, 26.3±2.8 mmHg (n=6) for WKY-NP, WKY-P, SHR-NP and SHR-P, respectively without significant differences. The ANG II challenge caused an increase in perfusion pressure in all groups examined (fig. 3). Albeit a trend of increase in pressure perfusion was observed in SHR-NP compared with WKY-NP, on the other hand in SHR pregnancy a significant reduction in ANG II response was registered ($p<0.05$ *vs* SHR-NP). The vascular response to ANG II of SHR-P was similar to that observed in WKY-P, indicating that the pregnancy established the physiological status. In order to evaluate the vasorelaxant activity, Ach, as endothelium-dependent, and SNP, as endothelium-independent activator, were used on stable tone induced by MTX. We did not observe differences either in MTX increase in perfusion pressure or in Ach- or SNP-induced relaxation among groups (data not shown).

4.4 p65 NF-κB and IκB-α, COX-2 and iNOS expression in kidney from WKY and SHR

A higher inflammatory status in tissues from SHR has also been associated to AT1 receptor activation, in fact AT1 antagonism has been shown to inhibit vessel wall inflammation (Kaufmann et al., 2003).

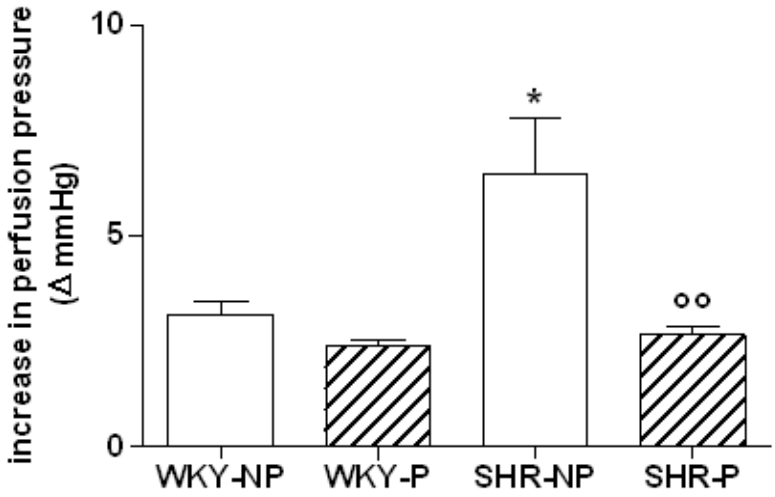

Fig. 8 Ang II (0.1 nmoles bolus injection) effect on basal tone of isolated and perfused mesenteric vascular bed in presence of indomethacin (10μM) from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Perfusion pressure (mmHg) was reported as mean ± S.E.M. of 6 animals each group.
*p<0.05 *vs* WKY-NP and °° p<0.01 *vs* SHR-NP.

To confirm that in SHR pregnancy the reduction in AT1 expression was also associated to an antinflammatory status p65 NF-κB expression was evaluated. A trend of decrease in p65 NF-κB expression was shown in pregnant WKY compared to WKY-NP (Fig. 9, panel A). In kidney of SHR-P we observed a significant ($p<0.001$) reduction of p65 NF-κB expression compared to that of SHR-NP. Since NF-κB activation is regulated by the inhibitory protein IκB-α, we also evaluated its expression. As expected, a weak upregulation of the inhibitory protein was detected in normotensive rats during pregnancy and a similar trend was significant in hypertensive animal (Fig. 9, panel B).

The improvement of inflammatory status in SHR pregnant animals was confirmed by the direct evaluation of the expression of the main proinflammatory enzyme COX-2 and iNOS. As shown in fig. 10 A and B, in normotensive animals pregnancy did not modify significantly both enzyme expression, anyway a trend of decrease was shown in iNOS protein band. Differently, in SHR pregnant animals a significant reduction of both COX-2 and iNOS was shown.

4.5 Kidney lipid peroxidation and protein nitrotyrosilation

Hypertension is often associated to oxidative stress and since it has been shown that AT1 receptor signalling is linked to the process of oxidative stress-induced vascular injury (Akishita et al., 2005), we thought to evaluate kidney lipid peroxidation and protein nitrotyrosylation. To this purpose we evaluated the major reactive product MDA and several MDA-like aldehydes and ketones, which react with TBA.

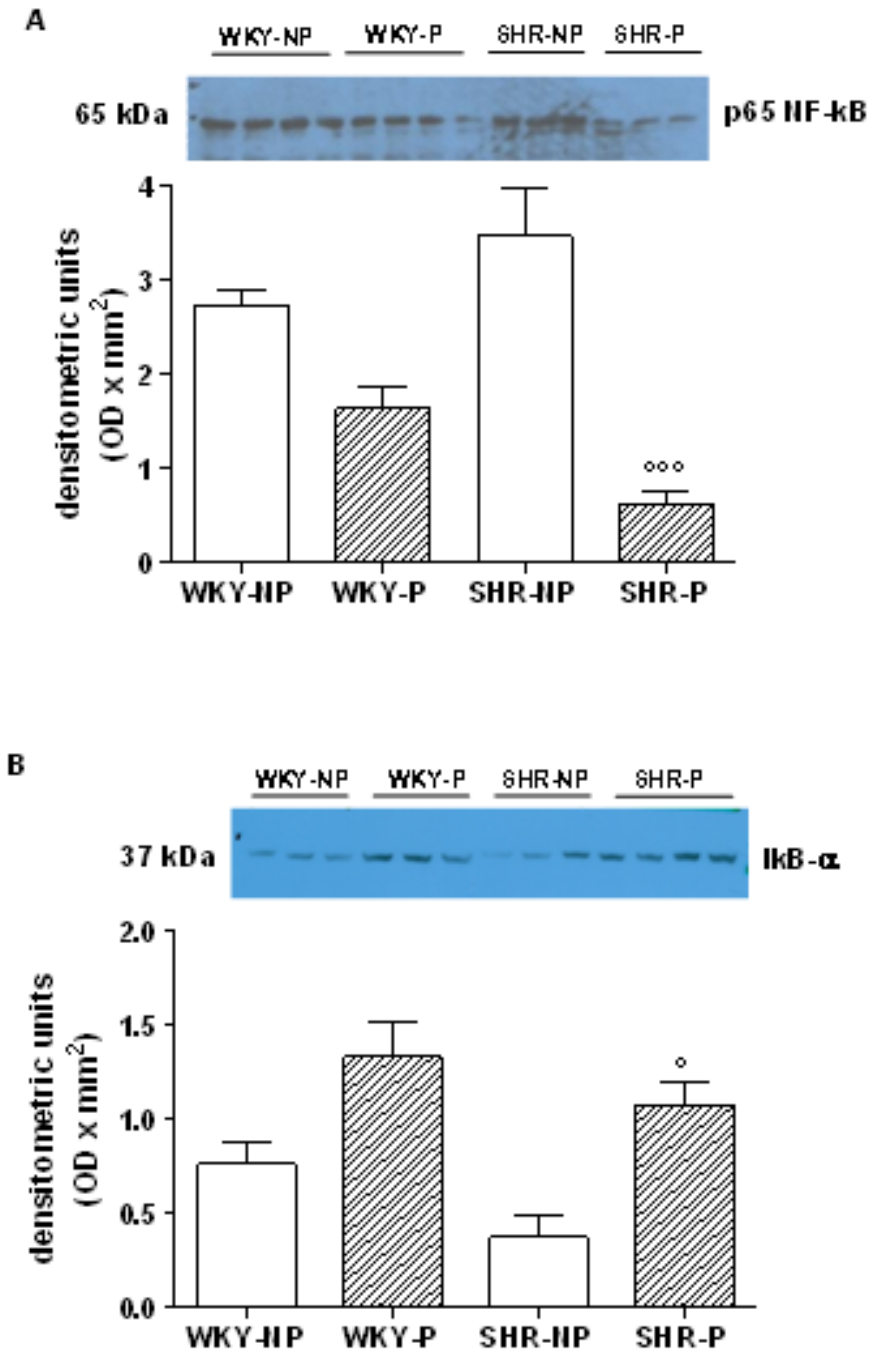

Fig. 9 p65 NF-kB (panel A) and Ik-B-α (panel B) expression in kidney from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 6 animals. °p<0.05 and °°°p<0.001 vs SHR-NP.

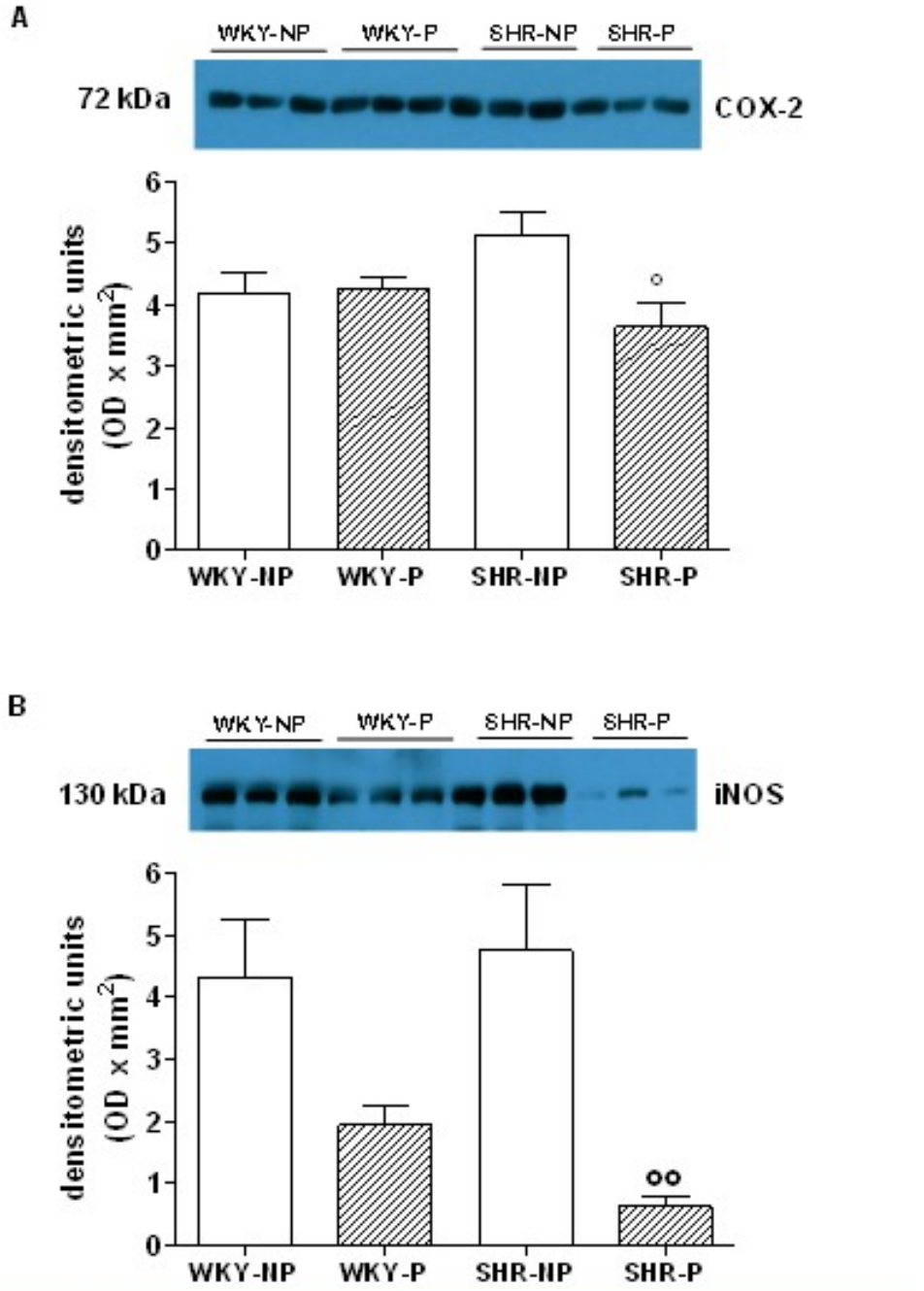

Fig. 10 COX-2 (panel A) and iNOS (panel B) expression in kidney from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 6 animals. °$p<0.05$ and °°$p<0.01$ vs SHR-NP.

As shown in figure 11 A, pregnancy induced a significant reduction of kidney lipid peroxidation both in normotensive ($p<0.05$ vs WKY-NP) and more significantly in hypertensive animals ($p<0.001$ vs SHR-NP). The hypertensive status in SHR strain induced an increase of MDA content also in the heart ($p<0.05$; data not shown), a tissue particularly exposed to oxidative damage, due to a constitutive deficiency of defensive systems.

Since prolonged production of NO by iNOS may result in kidney damage and inflammation and considering that peroxynitrite is a specific marker of iNOS activity, we have also determined the levels of nitrotyrosine (downstream reaction products of peroxynitrite) of kidney proteins. As reported, the increase of iNOS expression results an increase of protein nitrotyrosylation in kidney of SHR animals as compared with WKY. Pregnancy completely inhibited both iNOS expression and nitrotyrosylation pattern in hypertensive animals, while no difference was evidenced in normotensive rats (fig. 10B and fig. 11B).

4.6 AT1, ACE, iNOS and eNOS expression in placentas from WKY and SHR

To evaluate the pressor sensivity to Ang II, a local modulator of placental function, AT1 receptor protein expression in placentas was measured. Moreover, we also evaluated the placental source of Ang II, determining ACE protein expression. Fig. 12A shows that placenta from SHR presented an increase of AT1 expression, compared to that of WKY ($P<0.05$). Conversely, ACE in WKY tissue lysates was expressed at higher levels than that of SHR ($P<0.01$, Fig. 12B).

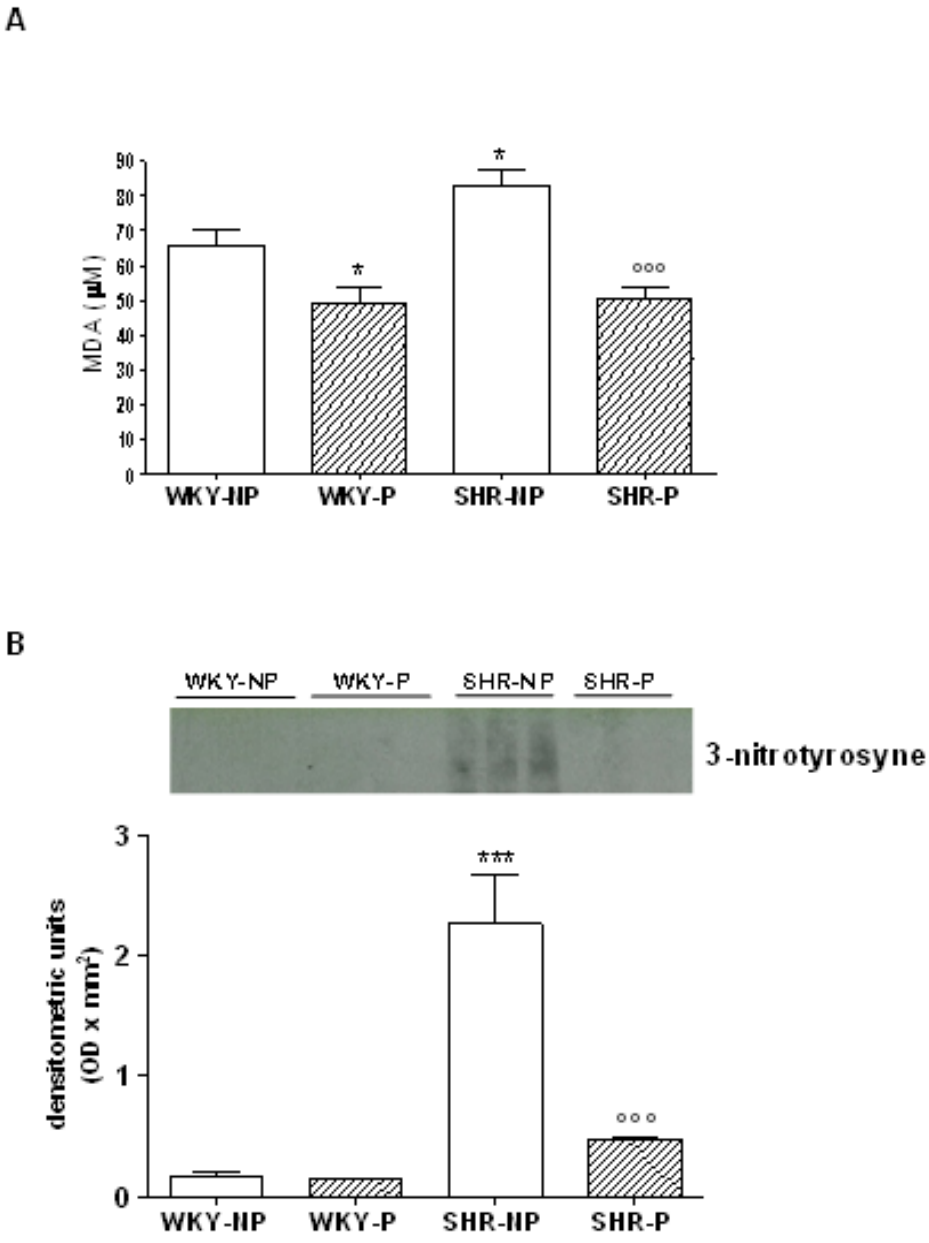

Fig. 11 MDA content (panel A) and protein nitrotyrosylation (panel B) in kidney lysates from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 8 animals.
*p<0.05 and *** p<0.001 vs WKY-NP; °°°p<0.001 vs SHR-NP.

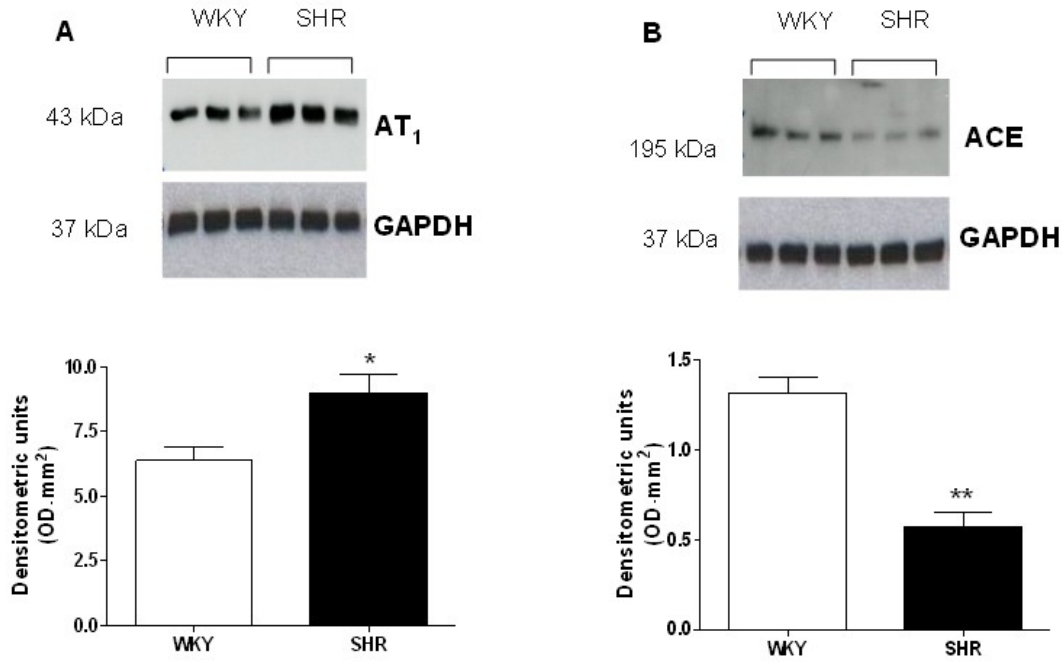

Fig. 12 Representative immunoblot of AT1 (A) and ACE (B) protein expression in placentas (two/each animal) from pregnant hypertensive (SHR) or normotensive (WKY) rats. Equal loading was confirmed by GAPDH staining. Densitometric analysis values are mean ± SEM of five animals. *p<0.05, ** p<0.01 vs WKY.

Since NO regulates placental functions, such as maintenance of blood flow, we evaluated the expression of iNOS and eNOS to investigate the placenta capability to synthesize NO. As appears in Fig. 13A, iNOS expression in placenta of SHR was increased in comparison with WKY ($P<0.001$). The expression of eNOS was similar in both strains (Fig. 13B).

4.7 Activity of MMPs and expression of COX-2 in placentas from WKY and SHR

Pro-MMP-9, pro-MMP2 and the active MMP-2 activity was measured by gelatin-zymography (Fig. 14A). The activity of pro-MMP-9, whose active product MMP-9 is considered a molecular marker in the initiation of labour, was not significantly modified in placentas from SHR (Fig. 14A.1). The lack of active MMP-9 in the zymogram could be due to its high level of instability and the removal of active enzyme during the washing of specimens, as reported above. Conversely, both pro-MMP-2 and the active MMP-2 present a higher activity in SHR in comparison to WKY (Fig. 14A.2 and A.3, $p<0.01$ and 0.05, respectively). Due to the profound deleterious reproductive effects and increased neonatal mortality in absence of COX-2, this enzyme was evaluated, revealing a deficiency of its expression in SHR placentas ($p<0.05$, Fig. 14B and 14B.1).

4.8 PPARα and PPARγ expression in placentas from WKY and SHR

PPARα and PPARγ, involved in guiding proper placental and fetal development through fatty acid/lipid homeostasis were evaluated. The expression patterns of

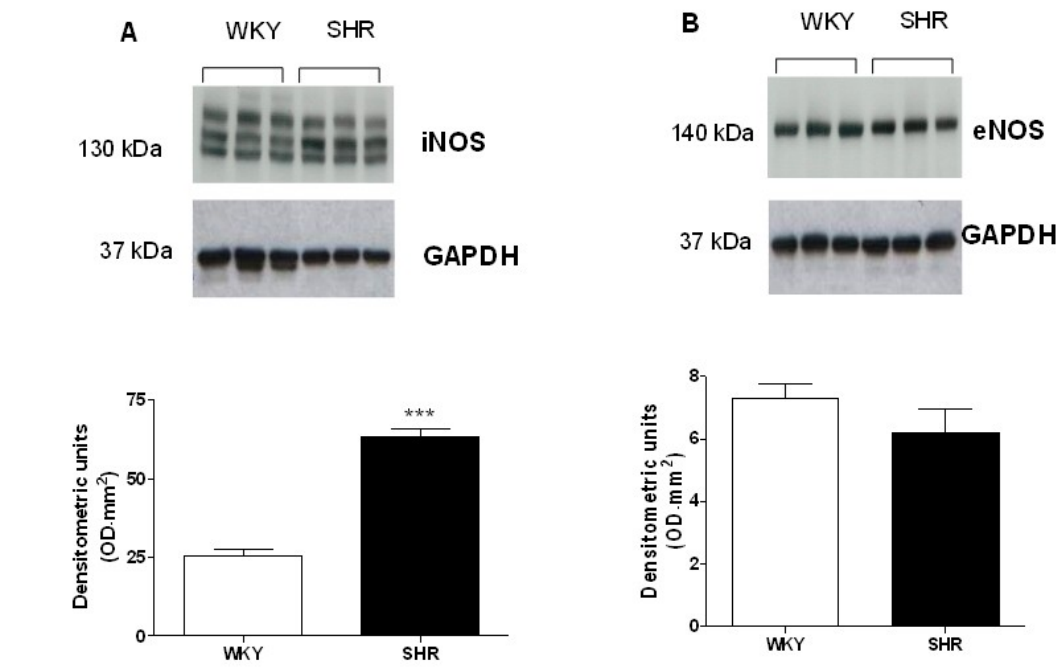

Fig. 13 Representative immunoblot of iNOS (A) and eNOS (B) protein expression in placentas (two/each animal) from pregnant hypertensive (SHR) or normotensive (WKY) rats. Equal loading was confirmed by GAPDH staining. Densitometric analysis values are mean ± SEM of five animals. *** $p<0.001$ vs WKY.

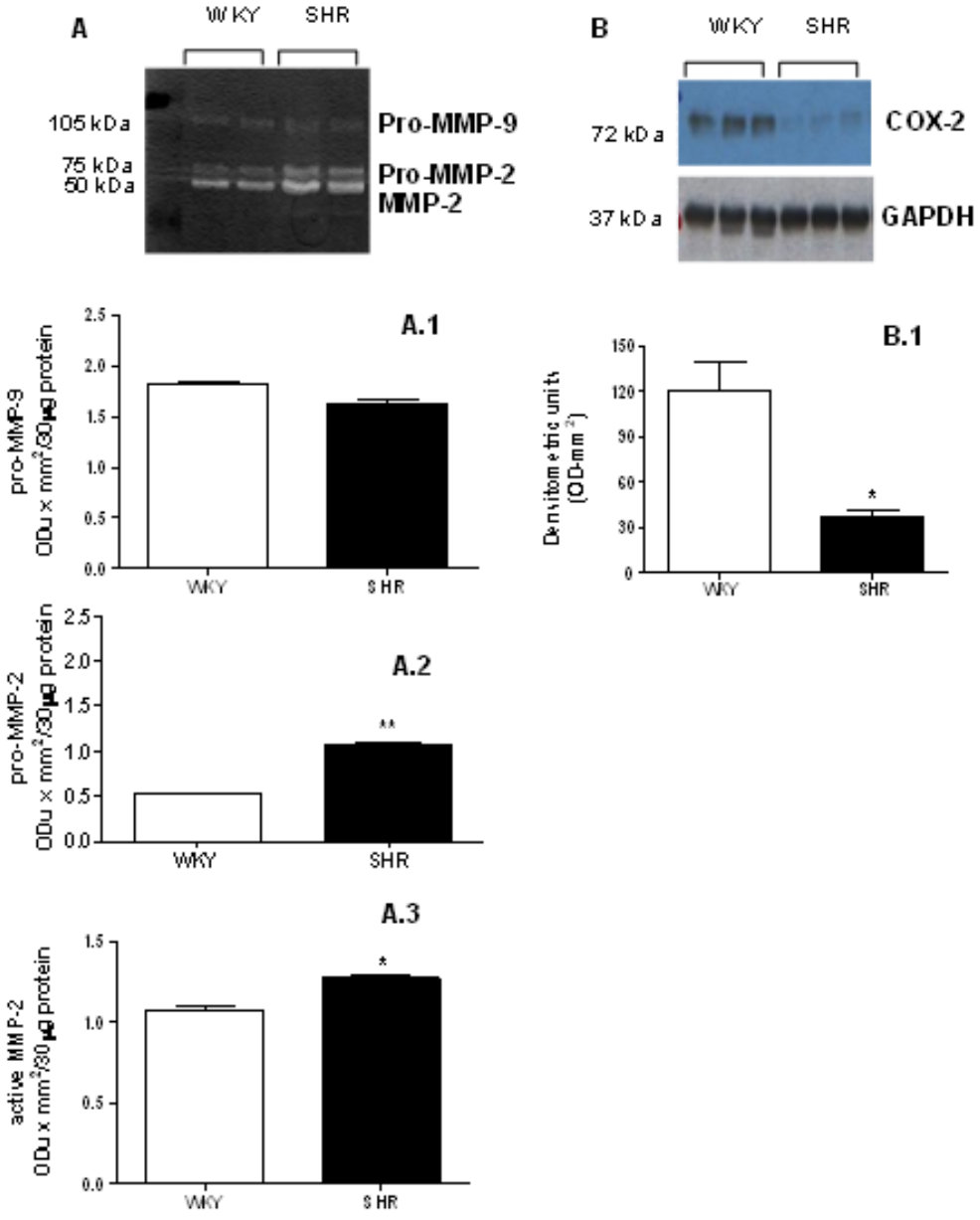

Fig 14 Representative zymogram of placental pro-MMP-9, pro-MMP-2 and active MMP-2 (A) activities and representative immunoblot of COX-2 protein expression (B) in placentas (two/each animal) were shown. Densitometric analysis values are given as means ± SEM of five rats. *$p<0.05$, ** $p<0.01$ vs WKY. Equal loading for Western blot analysis was confirmed by GAPDH staining.

PPARα and PPARγ are reported in Fig. 15 A and B, respectively. Placentas from SHR showed a reduced expression of both PPARα and PPARγ, compared to that of normotensive WKY ($P<0.05$).

4.9 Body weight gain, food intake, and body fat modifications in hypertensive and normotensive rats during pregnancy

Body weight gain, food intake, and fat mass of the normotensive and hypertensive rats are presented in figure 16. Pregnancy in normotensive as well as in hypertensive rats induced a significant increase ($P<0.001$) in body weight gain (panel A), food intake (panel B) and fat mass (panel C).

Interestingly, SHR-NP showed a significant increase of food intake compared to WKY-NP ($P<0.001$). This parameter was evaluated as area under curve (AUC) of the amount of food consumed in 20 days. However, in SHR-P rats there was a significant increase in body weight gain compared to that of WKY-P ($P<0.01$), that was paralleled by a significant increase in fat mass content ($P<0.001$).

4.10 Hormone levels and blood parameters

Determination of plasma leptin and ghrelin levels of all groups were shown in fig. 17 (panel A and B, respectively). In our experimental conditions SHR strain animals presented a lower level of plasma leptin versus that of the respective WKY controls ($P<0.001$). Leptin level (ng/ml), measured at gestational d 20, did not significantly

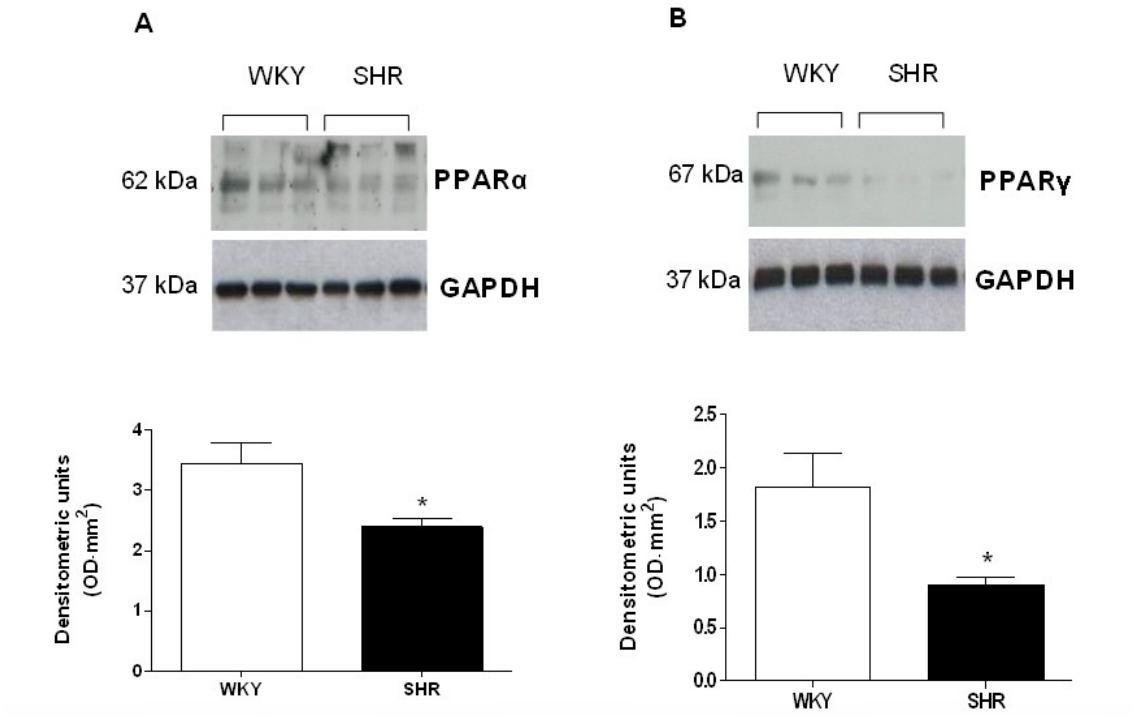

Fig. 15 Representative immunoblot of PPARα (A) and PPARγ (B) protein expression in placentas (two/each animal) from pregnant hypertensive (SHR) or normotensive (WKY) rats. Equal loading was confirmed by GAPDH staining. Densitometric analysis values are mean ± SEM of five animals. *$p<0.05$ vs WKY.

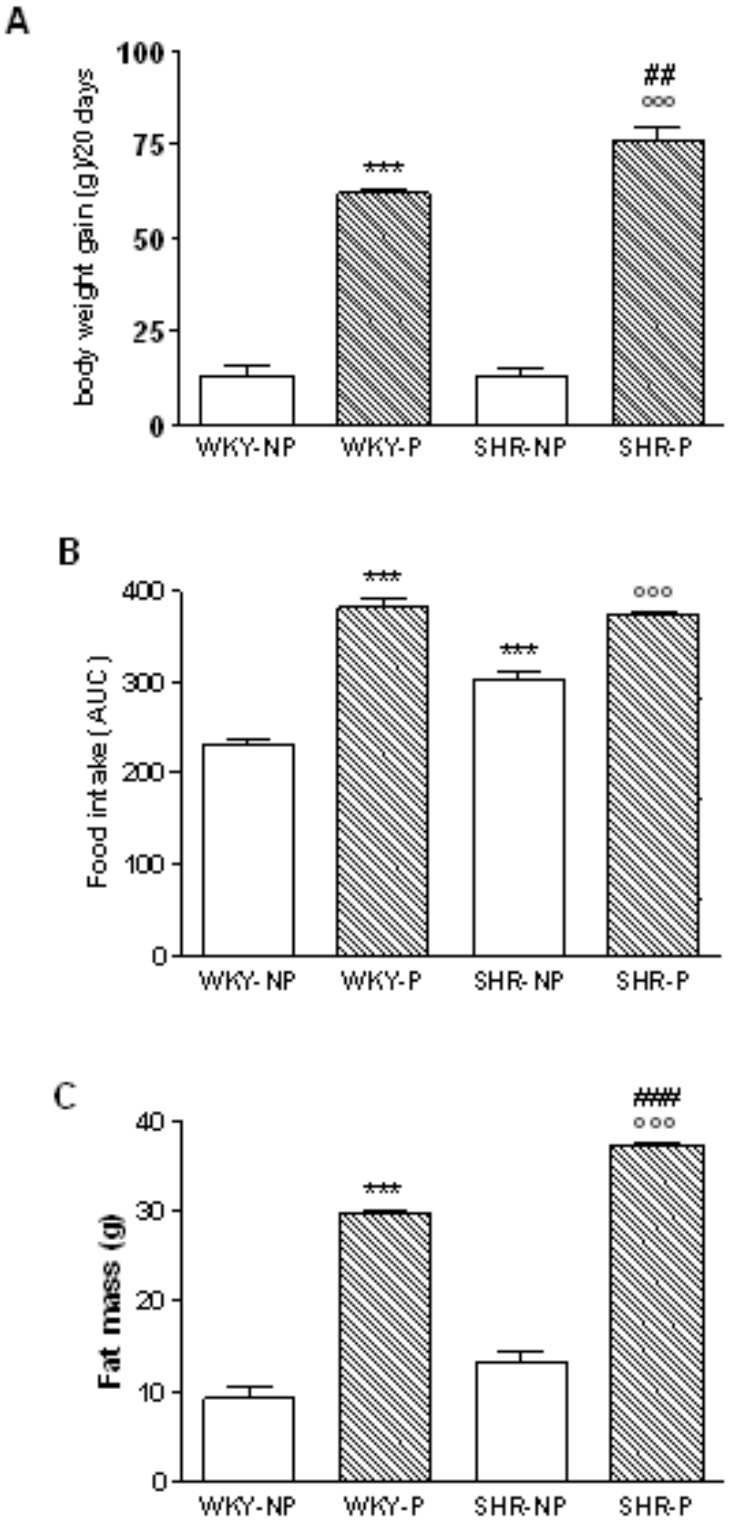

Fig. 16 Changes in body weight gain (A), food intake (B), and fat mass (C) of non pregnant (-NP) or pregnant (-P) hypertensive (SHR) and normotensive (WKY) rats. Values are mean ± SEM of 5 animals. AUC, Area under the curve. ***p<0.001 vs WKY-NP; °°°p<0.001 vs SHR-NP; ##p< 0.01 and ###p<0.001 vs WKY-P.

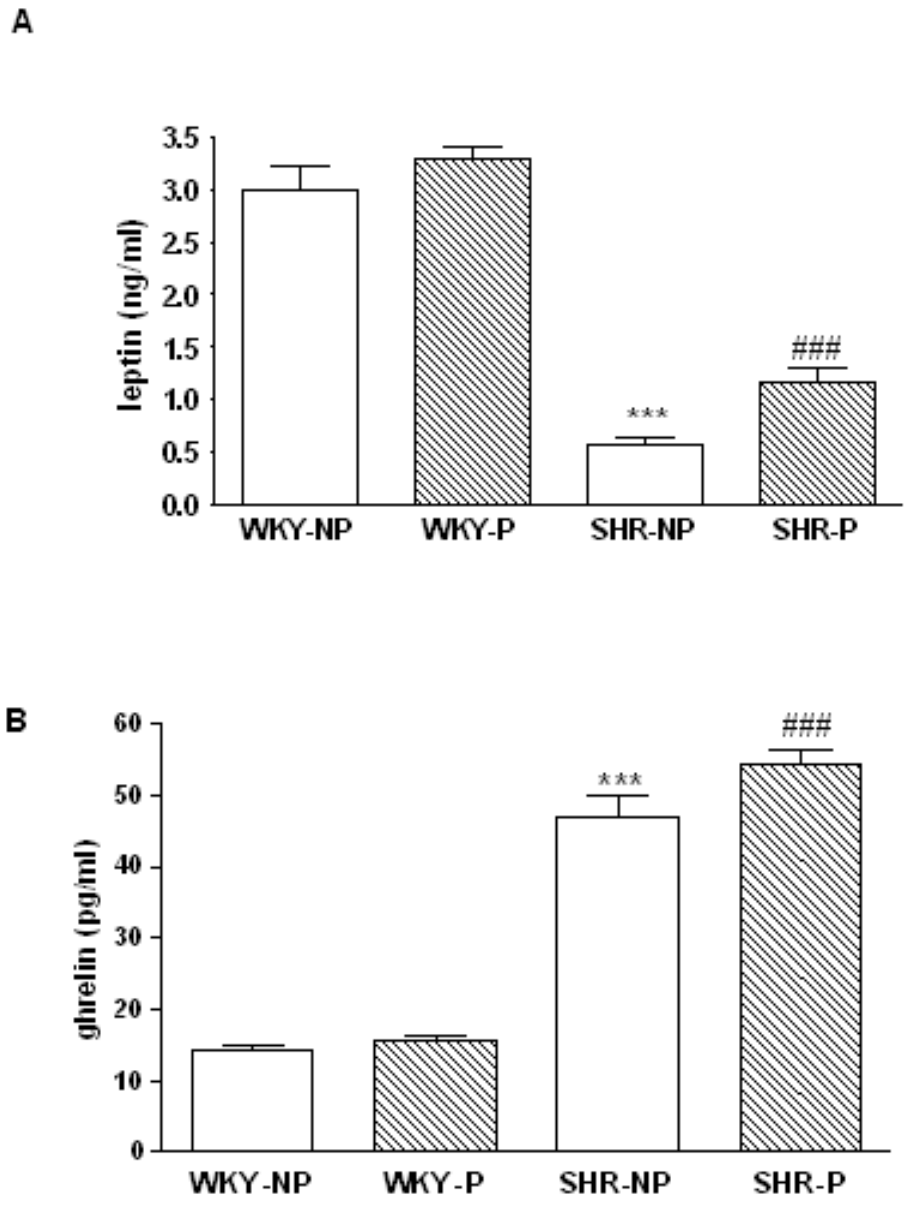

Fig. 17 Changes in circulating plasma leptin (panel A) and ghrelin (panel B) in non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 5 animals. ***p<0.001 vs WKY-NP; and ###p<0.001 vs WKY-P.

change in comparison with non-pregnant respective controls (3.30±0.10 vs 3.00±0.21 in WKY rats, and 1.16±0.14 vs 0.56±0.57 in SHR rats). Conversely, plasma ghrelin is higher in SHR compared to WKY animals (P<0.001). Neither pregnant group had different plasma ghrelin levels than their respective non-pregnant controls.

In Table 1, modifications of serum parameters are reported. Cholesterol, triglycerides and glucose were similar in non pregnant WKY and SHR rats. Conversely, SHR-NP animals presented a significant reduced plasma LDL cholesterol and a higher HDL cholesterol compared to that of WKY-NP (P<0.05, and P<0.01, respectively). In normotensive animals, pregnancy induced a significant decrease of total cholesterol (P<0.01), LDL cholesterol (P<0.001) and glucose (P<0.05), and an increase of plasma triglycerides (P<0.05). In SHR-P all these parameters were similarly modified; in particular a very strong increase of triglycerides was observed compared to WKY-P (P<0.001).

Groups	Cholesterol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	Tryglicerides (mg/dl)	Glucose (mg/dl)
WKY-NP	124.0±4.7	77.8±4.4	29.8±0.9	82.2±8.1	152.20±12.00
WKY-P	102.8±1.4**	37.6±0.8***	33.0±1.0	165.4±2.2*	99.60±2.25*
SHR-NP	120.0±4.1	63.0±2.5*	37.8±1.5**	86.2±6.7	165.40±12.45
SHR-P	97.6±3.5**	NC	31.6±1.5	440.8±40.0°°°###	124.60±8.29

Table 1. Modifications of serum parameters of non pregnant (-NP) or pregnant (-P) SHR and WKY rats. Values are the mean ± SEM.
*p<0.05, **p<0.01, and ***p<0.001 vs WKY-NP °°°p<0.001, vs SHR-NP ###p<0.001 vs WKY-P
NC Not Calculable

4.11 Ob expression in adipose tissue and placenta from WKY and SHR animals

Fig. 18 (panel A) shows that subcutaneous adipose tissue from SHR animals presented a slight reduction of leptin expression, compared to that of WKY rats; moreover, pregnancy in normotensive as well as in hypertensive rats induced a significant increase ($P<0.01$ and $P<0.05$, respectively) in Ob expression vs their respective non pregnant controls. Ob protein in WKY-P tissue lysates was more expressed than in SHR-P. Conversely, as appears in fig. 18 (panel B), Ob expression in placentas of SHR-P rats was increased ($P<0.01$) in comparison with WKY-P animals.

4.12 Ob-Rb expression in subcutaneous adipose tissue and hypothalamus from WKY and SHR animals

The expression of the functional isoform of Ob-R, Ob-Rb, in the adipose tissue and hypothalamus is reported in figure 19, panel A and B. In adipose tissue there was no significant modulation of Ob-Rb expression in any group, but a trend of Ob-Rb decreased expression was revealed in both pregnant groups vs their respective controls (panel A). Hypothalamus from SHR strain animals had a reduced expression of Ob-Rb ($P<0.05$) compared to that of normotensive WKY rats. The same pattern was found during pregnancy, where Ob-Rb continued to be underexpressed compared to WKY-P ($P<0.05$).

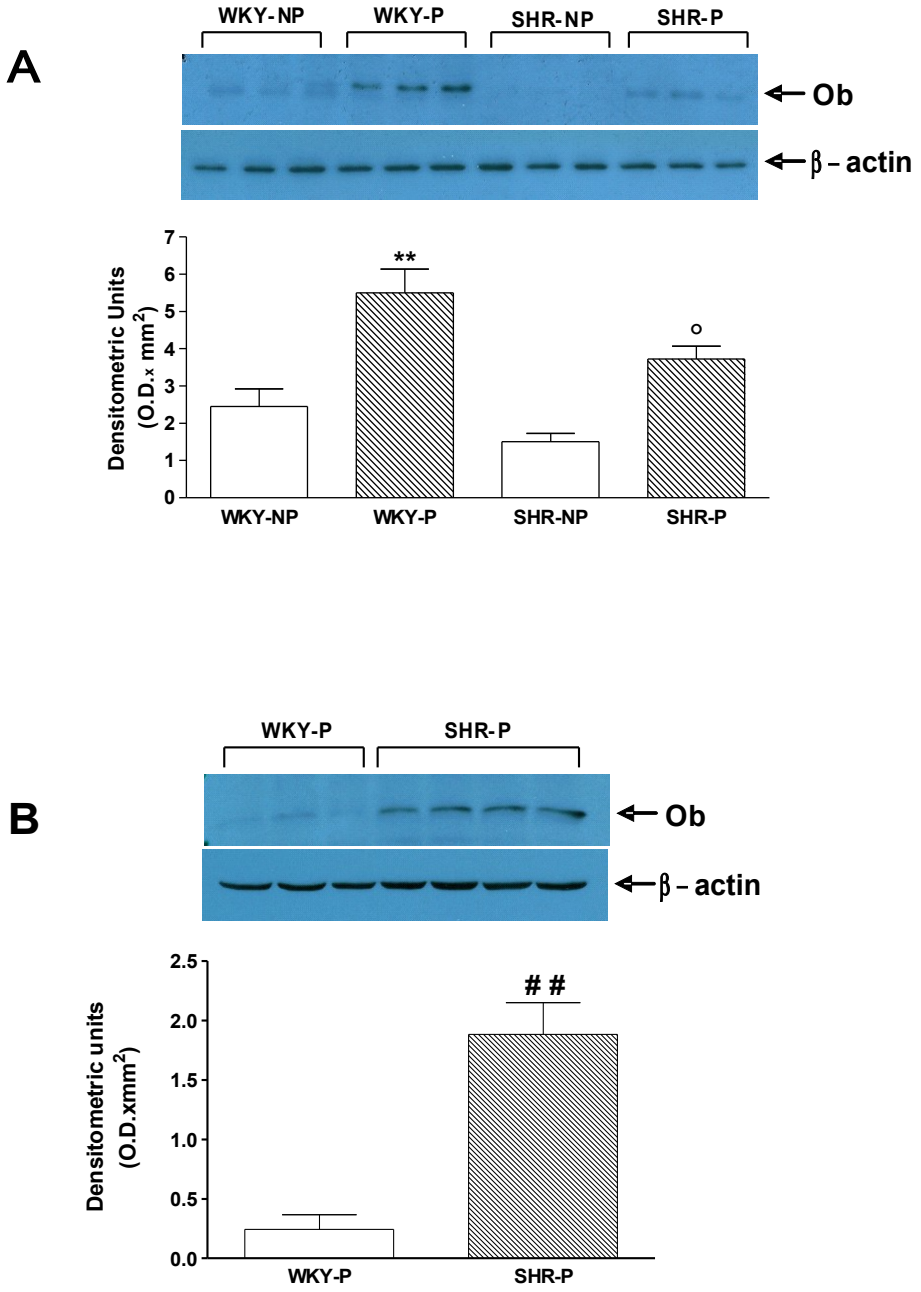

Fig. 18 Western blot analysis of Ob expression and densitometric analysis of protein bands in subcutaneous adipose tissue (panel A) and placenta (panel B). Panel A shows the modulation of Ob expression in adipose tissues prepared from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. In panel B the modulation of Ob expression was shown in placentas from pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 5 animals. Equal loading was confirmed by β-actin staining.
**p<0.01 vs WKY-NP; °p<0.05 vs SHR-NP; ##p<0.01 vs WKY-P.

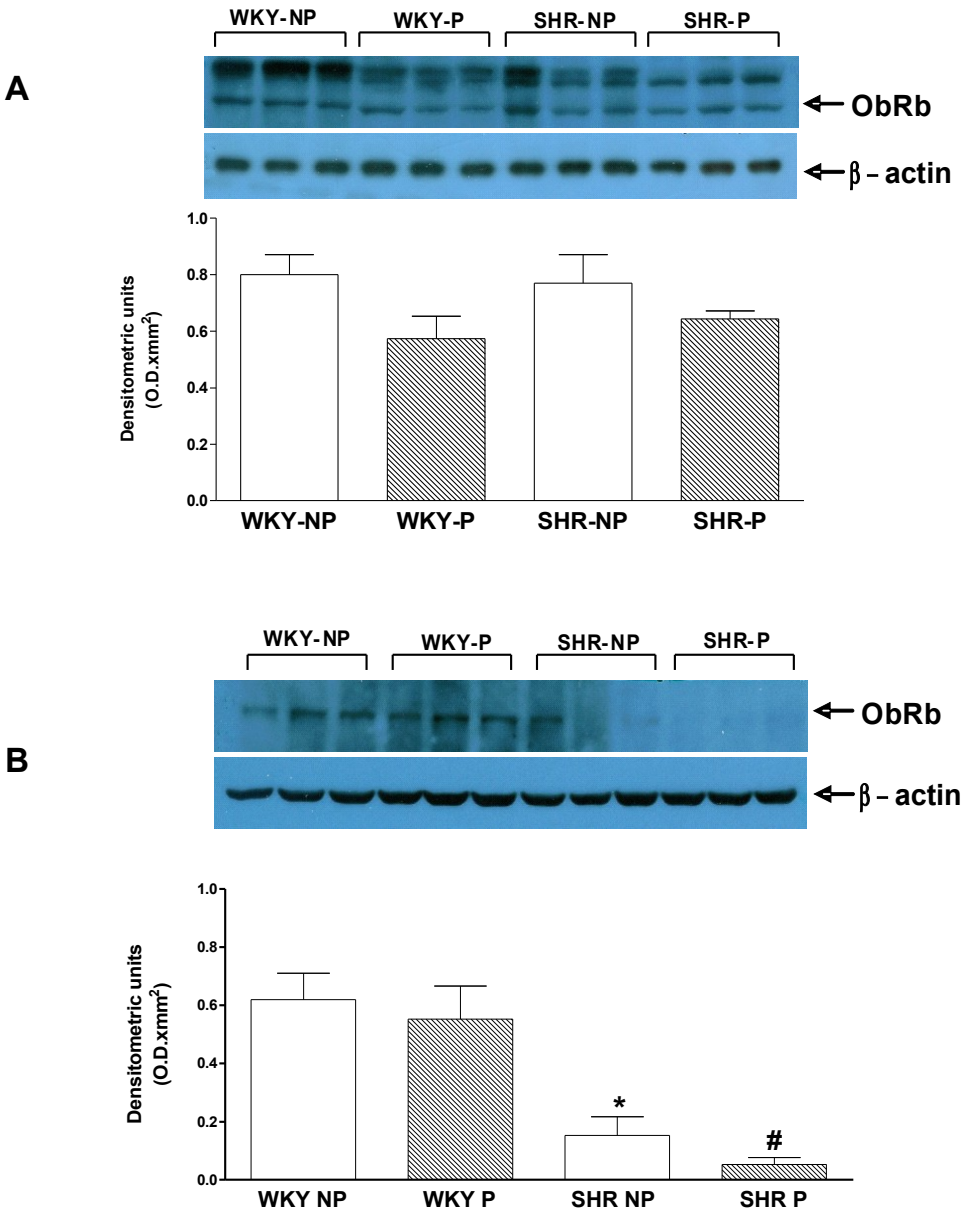

Fig. 19. Western blot analysis of Ob-Rb expression and densitometric analysis of protein bands in subcutaneous adipose tissue (panel A) and hypothalamus (panel B) prepared from non pregnant (-NP) or pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 5 animals. Equal loading was confirmed by β-actin staining.
*p<0.05 vs WKY-NP; #p<0.05 vs WKY-P.

4.13 Ghrelin mRNA expression in stomach and placenta from pregnant WKY and SHR animals

In stomach no difference in ghrelin mRNA expression was observed between normotensive and hypertensive rats (fig. 20, panel A). Conversely, a significant reduction ($P<0.05$) of ghrelin expression in placenta from SHR-P rats was evidenced (fig. 20, panel B).

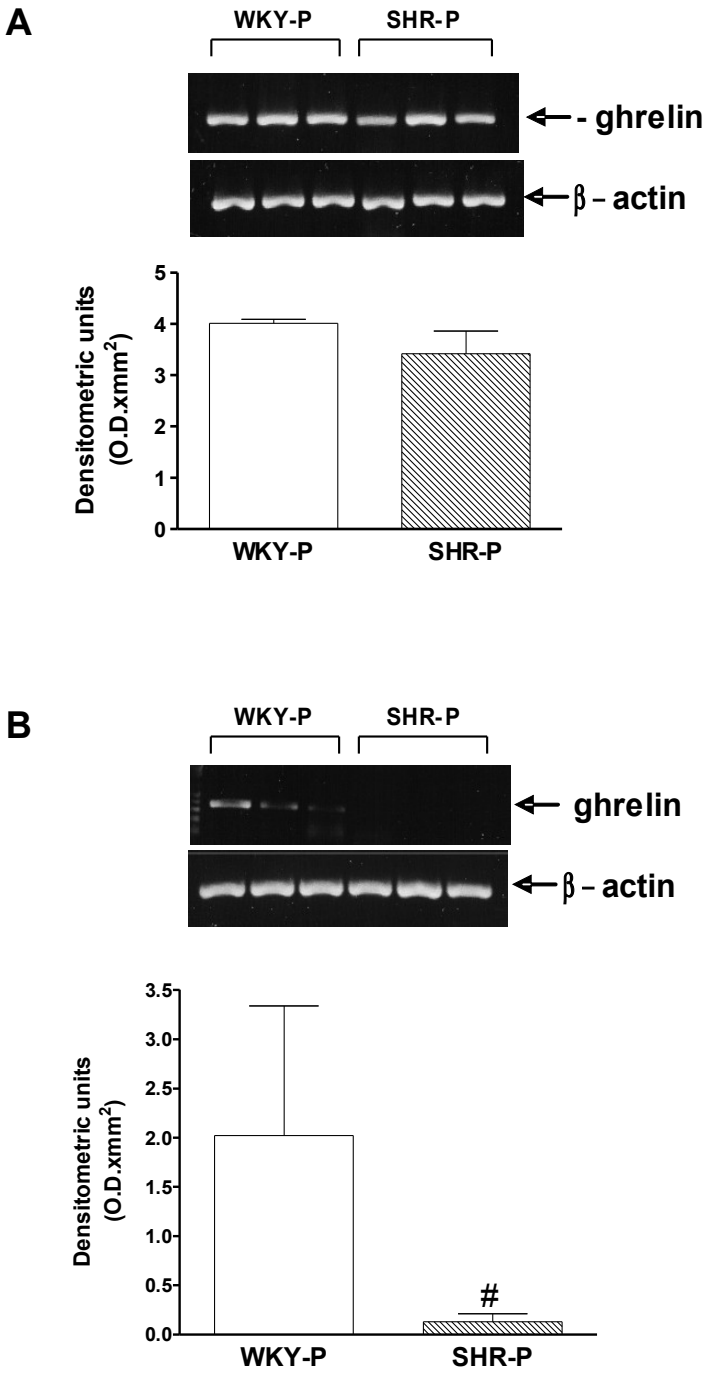

Fig. 20 RT-PCR analysis of ghrelin mRNA and its densitometric analysis in stomach (panel A) and placenta from pregnant (-P) hypertensive (SHR) or normotensive (WKY) rats. Values are mean ± SEM of 5 animals. βactin was used as internal reference.
#p<0.05 vs WKY-P.

5. DISCUSSION

In this study the SHR have been used to obtain an experimental model of pregnancy associated to hypertension, in order to investigate pressure adaptations, cardiovascular modification, hormonal and placental changes in comparison to normotensive animals, in order to complete pregnancy and ease parturition.

In normal mammalian pregnancy peripheral vascular resistance and blood pressure decrease, and extracellular fluid volume increases, while glomerular filtration rate and blood flow increase. Here, we found that in WKY blood pressure did not significantly change during pregnancy. Conversely, confirming previous data, in SHR pregnancy remarkably reduced blood pressure and heart rate in proximity of the delivery (Aoi et al., 1976; Mattace Raso et al., 2007). Even if the fall in the heart rate can contribute to the reduction of blood pressure, we investigated further mechanisms underling the normalization of pressure values at the end of pregnancy: for the first time we evaluated the modification of Ang receptor subtypes (AT1 and AT2) expression in the kidney of these animals.

Among many systems regulating the blood pressure, RAS and its main active component Ang II play an important role in the functional and vascular alterations associated with hypertension. As well known, in the kidney most of the vascular actions of Ang II are thought to be mediated via AT1 receptor and its down-regulation, that we observed in SHR at the end of pregnancy, is consistent with the normalization of blood pressure. As expected, no relevant variations were found in AT1 receptor between pregnant or not WKY.

Although the actions of the AT2 receptors have not been strengthened, evidence suggest that this receptor mediates functions opposing the vasoconstricting activity of AT1 (Ichiki et al., 1996; Munzenmaier et al., 1997). The AT1 receptor is widely distributed in adults, whereas the AT2 is highly expressed in the foetal kidney but decreased rapidly after birth (Ozono et al., 2000). Furthermore, ablation of the AT2 receptor in mice results in hyperesponsiveness to the pressor response to Ang II (Hein et al., 1995; Ichiki et al., 1995) while its over-expression in the vasculature causes vasodilatation and counteracts the AT1-mediated pressor effect (Tsutsumi et al., 1999). In addition, AT2 expression increases in pathological conditions, such as vascular injury (Hutchinson et al., 1999) All these data suggest that the deregulated expression of Ang II receptor subtypes may be involved in the altered regulation of blood pressure and cardiovascular homeostasis. In our experimental condition the significant increase of renal AT2 receptor in pregnant SHR, supports the protective role of AT2 associated to the strongly marked decrease of AT1. Moreover, it is conceivable that the increase in AT2 could support the normalization in blood pressure by subtracting Ang II from AT1 interaction and vasoconstriction.

Apparently, our data are in contrast with previous results indicating that Ang II receptors at 14 day of pregnancy are differently modified in glomeruli of SHR as compared to WKY. In particular, in the glomeruli Ang II receptor numbers increased significantly compared to their respective non–pregnant controls (Yang et al., 1994). These data cannot be completely compared with our results because of the different experimental time (14 d *vs* 20 d), methodology used (ligand binding *vs* Western blot analysis) and the differential analysis of AT1 and AT2 receptor subtypes here

reported. In our experimental conditions, we evidenced a balance between the two subtypes, whose total number could be unchanged.

To evaluate vascular reactivity of SHR compared to that of normotensive WKY, we studied the functional modification of mesenteric microcirculation, evaluating Ang II responsiveness in these animals. Interestingly, the vasoconstrictor response to Ang II was increased in SHR-NP and significantly reduced by pregnancy. The vascular response in SHR-P at the end of pregnancy (20 d) became similar to that of WKY. The vasoconstrictor response of mesenteric resistance vessels to sympathetic nerve activation (by MTX) or the vasodilator response to endothelium-dependent (by Ach) or –independent (by SNP) agents did not show significant difference among the examined groups (data not shown). Taken together, these data strongly support a role of RAS in pressure adaptations and cardiovascular modifications evidenced before delivery in SHR.

A growing body of evidence suggests that inflammatory mechanisms are involved in the physiopathology of hypertension. In fact, Ang II has been implicated in vascular inflammation related to hypertension (Cheng et al., 2005) and inhibition of AT1 receptors not only normalizes blood pressure, but also reduces inflammation in SHR (Sanz-Rosa et al., 2005). In the stroke-prone SHR the beneficial effects of AT1 blockade have been recently shown to be largely due to the broad anti-inflammatory effects of this therapy (Sironi et al., 2004). In SHR an elevated serum levels and tissue expression of cytokines (IL-6, IL-1 β and TNF-α) were evidenced. The inflammatory process appears to be mediated by Ang II, as well as by an increase in haemodynamic forces associated with hypertension, through the up-regulation of the

transcription factor NF-κB and down-regulation of its inhibitory protein IκB-α (Sanz-Rosa et al., 2005).

The present study demonstrated that in SHR strain pregnancy is associated to a marked decrease in p65 NF-κB and a significant increase in IκB-α. It is well known that NF-κB activation is regulated by the IκB-α, that retains NF-κB in the cytoplasm. Proteolytic processing of IκB-α by the proteasome complex (26S) allows NF-κB to enter into the nucleus to direct transcription of specific inflammatory genes (Ghosh et al., 1998; Karin et al., 2000). The reduction of renal NF-κB activation also contributes to ameliorate hypertensive status in pregnant SHR. These data are in agreement with Rodriguez-Iturbe et al (Rodriguez-Iturbe et al., 2005), which demonstrated that a long term treatment with PDTC, an inhibitor of NF-κB, completely abrogated hypertension, hypothesizing that NF-κB-induced renal inflammation seems to be involved in the genesis and in the maintenance of hypertension in SHR.

As well known, NF-kB controls the expression of genes encoding the pro-inflammatory cytokines, chemokines, adhesion molecules, growth factors and inducible enzymes, such as COX-2 and iNOS, all of which play critical roles in controlling most inflammatory processes. As here evidenced, pregnancy blunted the inflammatory status of SHR, reducing the expression of these enzymes at renal level. Our data are also in agreement with a recent study showing that gene expression of pro-inflammatory factors, such as IL-1β, TNF-α, ICAM-1 and iNOS, are elevated in several tissues (kidney, liver and heart) of SHR strain (Sun et al 2006).

Furthermore, inflammatory damage is often associated with the over generation of ROS, such as O_2^-, HO and H_2O_2 which results in oxidative stress. Several theories have been put forward to explain the increase in superoxide production, and it has been reported that antioxidant therapy can be beneficial (Rodriguez-Iturbe et al., 2003; Ulker et al., 2003). Among other products, peroxidation of phospholipids generates MDA and here, we observed, that this product is significantly increased in kidney of SHR strain. However, our data show that pregnancy ameliorates oxidative stress tissue damage, reducing significantly MDA content in both strain, and this protective effect is also evident and significant in heart of hypertensive animals (data not shown). Since peroxynitrite is a marker of iNOS activity, as well as of oxidative stress, the protective effect of pregnancy was also confirmed with the reduction of protein nitrotyrosylation at renal level. Our data are in agreement with a previous study showing that the reduction in immune cell infiltration in kidney by administration of mycophenolate mofetil, an immunosuppressive anti-inflammatory drug, results in amelioration of hypertension coupled with a decline in renal MDA content and in the number of superoxide positive cells in the kidney of male SHR (Rodriguez-Iturbe et al., 2002).

In summary, the haemodynamic alterations and the increased pro-inflammatory parameters, evidenced in SHR, are strongly modulated by pregnancy. Our results indicate that the improvements induced by this status could be related to the modification of AT1 and AT2 expression, to the reduction of inflammatory status, as well as of oxidative stress associated to tissue damage.

Beyond the role of RAS at renal level, also in human the placenta has been considered to possess a local RAS, which may play a physiopathological role in the regulation of uteroplacental blood circulation (Poisner, 1998). Therefore, for the first time we evaluated in SHR animals the modification of several placental proteins that could be involved in the mechanisms underling the placental adaptation to hypertensive status. Indeed, although a relationship between placental function and fetal well-being is well established (Godfrey et al., 2002; Kaufmann et al., 2003; Pardi et al., 2002; Regnault et al., 2002), the complex regulation of placental growth and development in SHR remains poorly investigated.

Examining AT subtype receptors at placental level, we found an increase of AT1 compared to WKY without any modification of AT2 receptor (data not shown). However, a significant reduction of placental ACE expression was observed. Differently from humans, that have alternative pathways for Ang II synthesis other than that of ACE, in rats ACE has been shown to be the most important enzyme for Ang II formation (Okunishi et al., 1993). Therefore, the reduced expression of ACE is most likely associated to a decrease of placental Ang II local level. This placental ACE reduction might be related to a lower placental Ang II production, repairing the up-regulated vasoconstrictor response due to the increase of placental AT1. In fact, the local level of Ang II is involved in the complex regulation of the uteroplacental blood flow and uterine contraction (Hagemann, 1997).

There is increasing evidence that, in addition to RAS system, NO plays a role in the control of placental blood flow. Pregnancy is associated in rats, as in human, with an up-regulation of NOS (Conrad et al., 1997; Melillo et al., 1996) and hence with an

increase of NO. In our condition, we found an up-regulation of the inducible iNOS in placentas from SHR and no modification of the constitutive eNOS. The increase of local iNOS-derived NO, reducing vasculature tone, may be involved in maintaining the uteroplacental circulation in a vasodilated state to allow fetal perfusion. This effect is strengthened by the finding that iNOS gene promoter contains a hypoxia-responsive element that, through feedback regulation, increases inducible enzyme expression and compensates the lack of NO or low blood flow (Melillo et al., 1996). On the other hand, the overproduction of NO from a sustained expression of inducible enzyme found in placentas from SHR is related to an impairment of labour onset, since its first step is related to a placental NO dramatic withdrawal, that shifts the balance from uterus quiescence to contraction (Purcell et al., 1997).

In SHR a significant reduction in uterus, fetal and placental weight was evidenced compared to normotensive rats, without affecting the number of implantation sites and litter size (Fernandez Celadilla et al., 2004). It is well known that COX isoforms are expressed in rat and human placentas guiding proper pregnancy, labour, and fetal development. In particular, at the end of pregnancy, COX-2 might be primarily responsible for sustained myometrial contractility and other events that culminate in fetus delivery (Xu et al., 2005), moreover the deficiency of this isoform is associated to deleterious reproductive effects and increased neonatal mortality (Loftin et al., 2002).

In our experimental condition the down-regulation of COX-2 expression supposes a reduction of PGs production, and hence the decrease of vascular permeability and

impairment of myometrial contractility, that regulates parturition and results complicated in SHR strain.

It is also known that the fetal membranes undergo striking changes in structure before delivery, that involve catabolism of extracellular matrix (Moore et al., 2006). The strength of the amnion and chorion is, in large part, a result of several different genetic types of collagen arranged in a complex framework and their degradation is controlled by matrix MMPs. Proteinases facilitate both normal and pathological (i.e. preterm) fetal membrane rupture, in particular it has been demonstrated that MMP-9 is induced in rat amnion immediately before delivery and that there is also an increase in MMP-2 activity (Lei et al., 1995; Vadillo-Ortega et al., 2005). In SHR strain no modification of pro-MMP-9 activity was evidenced, conversely, the constitutive MMP-2 presents a higher activity in SHR in comparison to WKY.

Moreover, here we observed a significant reduction both of PPARα and PPARγ expression that might be related to the well known compromised fetus development and growth observed in these rats. First evidence for a key role of PPARγ in placenta development has been demonstrated by generating PPARγ null mice (Barak et al., 1999; Kubota et al., 1999). This experimental approach has revealed unexpected functions for PPARγ in murine placental differentiation. The PPARγ null mutant fetuses only survive until midgestation and die by day 10 of development presenting essentially placental alterations. PPARγ null mutant placentas exhibit vascular anomalies with failure of vascular labyrinth formation. In the labyrinth of these mice the trophoblast fails to differentiate with no or poor compaction, poor syncytium formation and fails to accumulate lipid droplets. These placental alterations, leading

to severe placental dysfunctions, might be involved in embryo lethality. In our experiment the evidence of one embryo lethality was revealed in SHR, while the number and weight of fetuses were the same among strains (data not shown).

In contrast to PPARγ and PPARβ/δ null mice, no placental abnormality has been observed in PPARα null mice (Michalik et al., 2002). However, our study confirmed that the rat placenta expresses PPARα, and its expression is down-regulated in placenta of SHR strain. Recently, in normal rat the expression of PPARα and PPARγ was found to be increased specifically within the labyrinth zone toward term (Hewitt et al., 2006). Although the role of placental PPARα remains to be determined, it may impact on fatty acid transport and metabolism either alone or in combination with PPARγ. Wang et al. (2002) suggested that the presence of PPARα and PPARγ late in pregnancy may facilitate the necessary increase in transplacental fatty acid transfer stimulated by increased fetal demand. Consistent with this hypothesis, the reduction in labyrinth in PPARγ expression observed following glucocorticoid-induced placental growth restriction may reduce transfer of maternal fatty acids to the fetus and possibly contribute to the restricted fetal growth. In addition, placental PPARα may be important in relation to placental steroid production, since previous studies indicate that PPARα activation may promote steroidogenesis in human trophoblasts by increasing the pool of available cholesterol through fatty acid oxidation (Hashimoto et al., 2004). Although the rodent placenta exhibits only limited steroidogenic capability relative to other species (Chan et al., 1975), the rat placenta is a significant local source of progesterone within the intrauterine compartment (Benbow et al., 1995).

All the modifications of placental proteins in SHR confirm the altered physiopathological condition in these animals; in any event adptative and compensative changes of the parameters considered may contribute to re-establishment of physiological pregnancy and delivery.

However, the limitation of this animal model, reproducing human pregnancy associated with hypertension, consists in the lack of the risk of pre-eclampsia, that does not occur in SHR.

Human pre-eclampsia is widely believed to be related to the process of placentation, which is strongly different in rat, and represents a pro-inflammatory and pro-oxidant state (Walsh et al., 2007). Conversely, in SHR animals the adaptive and compensative changes may contribute to the re-establishment of physiological pregnancy and delivery.

During pregnancy in the rat, food intake is increased, whereas thermogenesis is inhibited. This produces a positive energy balance required for growing and development of fetal and maternal tissues besides building up a fat store for subsequent lactation. However, the mechanisms underlying increased food intake in gestation are poorly understood.

During pregnancy, physiological adaptation in the nutritional and hormonal setting is necessary for fetal growth and maternal well-being. When pathological conditions, such as obesity or hypertension, occur, further modifications are needed to avoid maternal complications or fetal programming.

Leptin and/or ghrelin, initially thought to be considered messengers of energy metabolism, are now considered to play a role in normal and complicated pregnancy

(Henson & Castracane 2006; Makino et al. 2002). During pregnancy the increase of leptin level in human correlates with a higher expression of the leptin gene in placenta (Masuzaki et al., 1997). Contrasting data are reported about the main source of leptin in rat; some authors indicate adipose tissue as the main source of leptin also during pregnancy because lack of leptin expression was reported in placenta (Kawai et al., 1997). Other authors provide evidence, using RT-PCR, that placenta, may be a further source of leptin in rodents and may explain the decrease of leptin levels after the delivery (Garcia et al., 2000; Hoggard et al., 1997).

As reported by Kawai et al. (1997), during rat pregnancy, maternal plasma leptin levels, measured every three days, gradually increased on day 9 to 19, when the peak leptin levels occur. Thereafter, maternal leptin levels sharply declined to less than those of non-pregnant animals. In our experimental conditions, at d 20 of pregnancy, we did not found differences between pregnant or not WKY leptinemia (3.30±0.10 vs 3.00±0.21, respectively), even if in subcutaneous adipose tissue a significant increase of leptin protein expression ($p<0.01$) was evidenced. Moreover, we observed, for the first time using hypertensive animals, at d 20, 2-fold increase of serum leptin level (1.16±0.14 SHR-P vs 0.56±0.57 in SHR-NP, $t=2.965$) that paralleled a significant increase of leptin gene expression in subcutaneous adipose tissue ($p<0.05$). This accounts for the highest amount of adipose tissue in the body and contributes to circulating leptin levels. However, SHR strain animals presented significantly lower plasma leptin compared to those determined in WKY-NP and WKY-P controls. Interestingly, in the placenta compared to the plasma or adipose tissue, the opposite seemed to happen: leptin gene expression was higher in SHR-P

than normotensive animals. The increased plasma leptin concentration during pregnancy in SHR may be due to increased placental production. In pregnant SHR, increased serum leptin concentrations and expression of placental leptin in late gestation may seem paradoxical. If the organism were to respond to this satiety signal (e.g., increased leptin), food consumption would decline, and nutritional support for the mother and fetus would be compromised. Since food intake is not diminished in late pregnancy, an adaptive process appears to have evolved to maintain maternal and fetal well-being. In SHR-P the increase of leptin in serum and tissues (adipose and placenta) is not accompanied by a food intake modification. One possible explanation of this discrepancy may be the resistance to the effects of leptin via "downregulation" of hypothalamic leptin receptors in pregnant SHR, as evidenced by the marked reduction of the hypothalamic expression of Ob-Rb protein bands. However, the possible involvement or alteration of other peptides or peripheral hormones which regulate food intake cannot be excluded. No significant modification of hypothalamic Ob-Rb expression was seen at d 20 of pregnancy in WKY, even if previous data are suggestive for leptin resistance in the hypothalamus and pituitary (Szczepankiewicz et al. 2006). Moreover in our conditions, no significant modification of Ob-Rb expression was seen in adipose tissues either in normotensive or hypertensive animals.

Previous data demonstrated that SHR is a strain which easily manifests hyperlipemia and hyperlipoproteinemia during pregnancy when compared to the Wistar rat (Yoshioka et al. 1986). In fact, many data suggest that in late pregnancy, liver lipid metabolism may be directed towards VLDL secretion at the expense of biliary

secretion. This would supply lipids, especially triglycerides, to the placenta for fetal energy requirements and to the mammary gland for milk lipids in anticipation of suckling (Smith et al. 1998). This study compared the changes in the level of serum lipid concentration in pregnant and non-pregnant SHR and WKY rats. The serum triglyceride concentration is significantly higher in the last period of pregnancy, i.e. 2-fold higher in WKY and 5-fold higher in SHR rats. It has been noted that elevated plasma lipid and lipoproteins may induce endothelial dysfunction secondary to oxidative stress and that dyslipidemia may impair trophoblast invasion, thus contributing to a cascade of pathophysiologic events that lead to the development of preeclampsia (Lorentzen & Henrisksen 1998). Consistent with our results on the lower leptin serum levels in SHR strain, Anderson et al. (2005) found a significant decrease of leptinemia in reduced uterine perfusion pressure (RUPP) animals. RUPP is an animal model of preeclampsia characterized by persistent elevation of arterial pressure, reduced litter size, fetal and placental weights.

In opposite to leptinemia, our data show an increase of ghrelin plasma level in SHR strain compared to that of WKY. In particular, in SHR rats at the end of d 20 pregnancy we determined the bioactive (n-octanoyl modificated) form of ghrelin, and showed a slight increase of plasma hormone. Ghrelin has been shown to participate in cardiovascular and sympathetic regulation (Matsumura et al. 2003). Intravenous injection of human ghrelin elicits a decrease in blood pressure (Nagaya et al. 2001) with direct vasodilatory effects possibly through GH or nitric oxide-independent mechanisms (Okumura et al. 2002). However, in our experimental condition this weak hormone increase could not account the deep fall of blood pressure evidenced

in SHR-P before delivery. Confirming previous data in normotensive animals (Shibata et al. 2004), we observed that also in SHR, as well as in WYK, the level of ghrelin peptide in the stomach did not change at the end of pregnancy in both strain. On the contrary mRNA ghrelin in the placenta of SHR rats was lower than that of normotensive rats, suggesting not only that in the fetal-placental unit there is a different production of this hormone, but also that circulating level are influenced by the release of ghrelin by other regions (i.e. intestinal tract, pancreas or hypothalamus or pituitary). Therefore, in SHR the production of placental ghrelin was not related to the plasma ghrelin concentration during pregnancy.

In conclusion, we observed an opposite profile of serum concentration of the examined hormones between WKY and SHR strains, showing a marked decrease of leptin and an increase of ghrelin level either in pregnant or not SHR. The inverse occurs in placenta expression of leptin: the higher production of leptin in pregnant SHR than WKY might compensate the down-regulated leptin system in SHR animals. During pregnancy, SHR animals presented an increase of body weight gain, paralleled to an increase of fat mass, compared to pregnant WKY animals, without a modification of food intake. Moreover, we found significant higher plasma ghrelin levels in SHR, that is not related to gastric or placental contribution. In contrast to the defined role of leptin and ghrelin in metabolic adaptations and dietary intake during pregnancy, their involvement in energy balance in hypertensive animals remains to be clarify.

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