INVOLVEMENT OF PROTEIN TYROSINE PHOSPHATASES AND INFLAMMATION IN HYPOTHALAMUS INSULIN RESISTANCE ASSOCIATED WITH AGEING: EFFECT OF CALORIC RESTRICTION

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Abstract

Aged Wistar rats present central insulin resistance associated with ageing. Several steps of the insulin signaling pathway have been described to be impaired in aged rats at hypothalamic level. In the present article we have explored possible alterations in protein tyrosine phosphatases (PTPs) involved in insulin receptor dephosphorylation, as well as pro-inflammatory pathways and serine kinases such as inhibitory kappa β kinase-nuclear factor kappa-B (IKKβ-NFκB), p38 mitogen-activated protein kinase (p38) and protein kinase C θ (PKCθ) that may also be involved in insulin signaling decrease during ageing. We detected that ageing brings about a specific increase in insulin receptor PTP activity and PTP1B serine phosphorylation. Increased association of PTP1B and leukocyte common antigen-related tyrosine protein phosphatase (LAR) with insulin receptor was also observed in hypothalamus from aged rats. Besides these mechanisms, increased activation of the IKKβ-NFκB pathway, p38 and PKCθ serine/threonine kinases were also detected. These data contribute to explain the hypothalamic insulin resistance associated with ageing. Caloric restriction ameliorates most of the effects of ageing on the above mentioned increases in PTPs and serine/threonine kinases activities and points to age-associated adiposity and inflammation as key factors in the development of age-associated insulin resistance.

Keywords:

Insulin resistance; ageing; obesity; hypothalamus; inflammation; caloric restriction.

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1. Introduction

Wistar rats, like humans (DeFronzo, 1981; Rowe et al., 1983) and other rodent models (Goodman et al., 1983), present a moderate increase in adiposity and exhibit a decrease in peripheral insulin sensitivity with ageing. In spite of no significant changes in both fasting plasma glucose and fasting insulin concentrations, aged Wistar rats present lower glucose disposal rate under euglycemic-hyperinsulinemic clamp conditions, demonstrating overall insulin resistance (Escriva et al., 1997; Nishimura et al., 1988). Besides this peripheral insulin resistance, these animals also present aged-associated central leptin resistance (Fernandez-Galaz et al., 2002) and central insulin resistance (Garcia-San et al., 2007).

A decrease in the expression of leptin receptor in hypothalamic nuclei together with an increase in suppressor of cytokine signaling-3 (SOCS-3) expression (Peralta et al., 2002) have been pointed out to be related with the above mentioned central leptin resistance associated with ageing in Wistar rats (Fernandez-Galaz et al., 2002). On the other hand, hypothalamic insulin resistance has been associated to alterations in several steps of the insulin signaling pathway. In this sense, early steps in the insulin signal transduction cascade such as insulin receptor (IR) and insulin receptor substrate-2 (IRS-2) phosphorylation, as well as further steps in the cascade like protein kinase B/thymoma viral proto-oncogene (AKT) phosphorylation, have been described to be impaired in aged Wistar rats (Garcia-San et al., 2007). Hypothalamic resistance to insulin and leptin action has been proposed to play a central role in the loss of the coordinated control of food intake and energy expenditure promoting weight gain (Schwartz et al., 2000; Schwartz and Kahn, 1999) and,

therefore, of importance in the development of overall insulin resistance in circumstances such as obesity or ageing.

Protein tyrosine-phosphatases (PTPs) catalyze the dephosphorylation of tyrosine-phosphorylated proteins. PTP1B dephosphorylates IR and Janus kinase 2 (Jak-2) (Seely et al., 1996; Zabolotny et al., 2002), being negative regulator of leptin and insulin sensitivity. The importance of this protein has been demonstrated in several animal models. Mice lacking *ptp1b* gene present increased insulin sensitivity, increased metabolic rate and resistance to increase body weight under high fat diet (HFD) (Elchebly et al., 1999; Klaman et al., 2000), and liver specific deletion of PTP1B ameliorates metabolic syndrome (Delibegovic et al., 2009). On the other hand, aged *ptp1b* null mice present a leaner phenotype than wild type mice and are protected against age-associated insulin resistance (Gonzalez-Rodriguez et al., 2011) and neuronal-specific PTP1B Knockout mice have reduced weight and adiposity as well as increased physical activity and energy expenditure (Bence et al., 2006). Tyrosine-phosphorylated insulin receptor can also be dephosphorylated by LAR (Hashimoto et al., 1992) which has been shown to be a negative modulator of insulin action implicated in insulin resistance states (Kulas et al., 1995; Zabolotny et al., 2001; Zhang et al., 1996). Nevertheless, other authors observed that the decrease of LAR protein tyrosine phosphatase induces insulin resistance and defects in glucose homeostasis (Mander et al., 2005; Ren et al., 1998).

Besides tyrosine phosphorylation, IR and IRSs can also be phosphorylated in serine residues. Depending on the site and the timing, these serine phosphorylations could have positive or negative effects on insulin

signaling (Weigert et al., 2005; Weigert et al., 2008). Inhibitory sites are phosphorylated by several serine kinases such as AKT, extracellular signal-regulated protein kinase 1/2 (ERK 1/2), mammalian targets of rapamycin (mTOR) and its downstream kinase S6-1 (p70S6K), glycogen synthase kinase-3 (GSK-3), and some members of the PKC family, as PKCθ, ε, and ζ, after insulin stimulation (Cawthorn and Sethi, 2008; Tanti and Jager, 2009). On the other hand, different stress circumstances such as inflammation and/or reticulum stress may also lead to serine phosphorylation of IRSs through N-terminal kinases (JNK), p38, the inhibitory kappa β kinases (IKKβ), or even by PKA, mTOR/p70S6K, ERK 1/2 and PKCθ, causing insulin resistance (Herschkovitz et al., 2007; Tanti and Jager, 2009). In this sense, obesity and ageing are both associated with a low-grade inflammation and insulin resistance (Tchkonia et al., 2010), and in agreement with this idea, alterations in the hypothalamic insulin signaling pathway of HFD-induced obese animals has been shown to be associated to the development of inflammation in hypothalamus and to the increase of serine phosphorylation of IR and IRSs (De Souza et al., 2005; Posey et al., 2009; Zhang et al., 2008). Moreover, these alterations have been shown to alter peripheral glucose metabolism and pancreatic function demonstrating the relationship between inflammation, central insulin resistance and peripheral glucose metabolism (Belgardt et al., 2010; Calegari et al., 2011). Similarly, aged Wistar rats also have shown to present signs of hypothalamic inflammation (Horrillo et al., 2011), increased IR and IRSs serine phosphorylation, as well as insulin and leptin resistance (Fernandez-Galaz et al., 2002; Garcia-San et al., 2007).

Thus, in the present article we have investigated the possible role of tyrosine phosphatases such as PTP1B and LAR as well as the possible role of serine kinases and/or pro-inflammatory pathways such as ERK1/2, mTOR, PKCθ, JNK, IKKβ-NFκB or p38 that could negatively modulate insulin signaling during the development of age-associated hypothalamic insulin resistance. As ageing is also associated with changes in body composition that results in fat accretion and a decrease in muscle mass, it is possible that many of the metabolic alterations associated with ageing could be due to fat accretion and its consequences, ageing by itself, or a combination of both circumstances. The fact that central leptin and insulin resistance associated with ageing in these animals is ameliorated by a caloric restriction protocol enough to decrease adiposity index to values below to those of young animals (Fernandez-Galaz et al., 2002; Garcia-San et al., 2007) also led us to investigate the effects of the same caloric restriction protocol on central mechanisms implicated in the decrement of insulin action and the possible role of age-associated adiposity.

2. Materials and methods.

2.1. Reagents and antibodies

Leupeptin, aprotinin, PMSF, benzamidine, pepstatin and protein A-agarose were purchased from Sigma Chemical Company (St. Luis, MO, USA). Complete protease inhibitor cocktail was from Roche (Mannhein, Germani). Antibodies directed towards insulin-Rβ (C-19), PKCθ (C-15), phospho-PKCθ (Thr 538), PTEN (A2B1), NFκB p65 (C-20), PTP1B (H135) and TFIID (TBP) (N-12) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody toward phospho-Erk 1/2 (Thr202/Tyr187) recombinant clone AW39R was from Milipore (Billerica, MA, USA). Antibody toward ERK 1/2 CT were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Antibodies toward phospho-p38 MAPK (Thr 180/Tyr 182), p38 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK (56G8), phospho-mTOR (Ser2448), mTOR, NFκB p50 (NLS) X, phospho-IKKα/β (Ser176/180) (16A6) and IKKβ were from Cell Signaling (Beverly, MA, USA). Anti-phospho-serine was from Chemicon International, Inc. (Temecula, CA, USA). LAR antibody was from ABGENT (San Diego, CA, USA). Anti-GAPDH was from AbCam (Cambridge, UK). Anti-mouse and anti-rabbit alkaline phosphatase linked antibodies were from Pierce Biotechnology (Rockford, IL, USA). PVDF membranes were from BioRad (Hercules, CA, USA). The rest of reagents were of analytical grade.

2.2. Experimental animals

Three, 8 and 24 month old male Wistar rats from our in-house colony (Centre of Molecular Biology, Madrid, Spain) were used throughout this study.

Rats were housed individually and placed in climate-controlled quarters with a 12h light cycle and fed *ad libitum* standard laboratory chow and water. They were handled following the NIH "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) and the European Union laws guidelines for animal care. Experimental procedures were approved by the Institutional Committee of Research Ethics.

2.3. Caloric restriction

Five and 21 month old rats were randomly assigned to undergo a caloric restriction protocol as described earlier (Perez et al., 2004). Animals were placed in individual cages and fed daily an amount of chow equivalent to 80% of normal food intake. Usually, two months after starting the nutritional restriction animals show a body weight equivalent to 85% of *ad libitum* fed aged-mates. Animals were weighed weekly and the amount of food provided was adjusted individually in order to maintain their body weight during one additional month. Caloric restricted animals were used at the age of 8 and 24 months respectively.

2.4. Extracts preparation

Whole hypothalamus extracts were prepared as previously described (Garcia-San et al., 2007). Nuclear and cytoplasmic enriched extracts were prepared as described by Andrews and Faller (Andrews and Faller, 1991) with minor modifications. Frozen hypothalamus were homogenized in an all glass Dounce homogenizer with 1 ml of cold buffer A (20 mM HEPES, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl, 1 mM, 0.5% NP-40, pH 8) supplemented

with protease inhibitor cocktail, 1 mM dithiothreitol, 50 mM sodium fluoride, 20 mM sodium β-glycerolphosphate, 1 mM sodium orthovanadate. The homogenates were allowed 5 min on ice, mixed with 200 µl of sucrose restore buffer (50 mM HEPES, 0.25 mM EDTA, 10 mM KCl and 50% sucrose, pH 8) and centrifuged for 5 min at 5,000 rpm 4 ºC to pellet nuclei. The supernatants were saved as cytoplasmic fractions. The nuclear pellets were washed with 150 µl of buffer B (20 mM HEPES, 50 mM NaCl, 0.25 mM EDTA, 0.15 mM EGTA 1.5 mM $MgCl_2$, 25% glycerol, pH 8 and supplemented as buffer A) to remove residual cytoplasmic material and resuspended in 150 µl of buffer C (20 mM HEPES, 450 mM NaCl, 0.25 mM EDTA, 0.15 mM EGTA 1.5 mM $MgCl_2$, 25% glycerol, pH 8 and supplemented as buffer A). The resulting suspensions were stirred gently with a rotator for 30 min at 4 ºC for high-salt extraction and then centrifuged for 5 min at 14,000 rpm. The supernatants were saved as nuclear fraction. In all cases protein concentration was determined by bradford.

2.5. Western blot analysis

The corresponding extracts were either directly subjected to Western blotting or immunoprecipitation before Western blotting. For direct application, equal amount of protein were subjected to SDS-PAGE and transferred onto PVDF membranes. Immunoblots were then blocked with 5% membrane blocking agent (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). After incubation with appropriate primary and secondary antibodies, PVDF membranes were washed and targeted proteins were detected using enhanced chemifluorescence reagent ECF (Amersham Pharmacia Biotech). Obtained bands were quantified using Scion Image Software. For the quantifications of

protein phosphorylation, immunoblotted membranes with phospho-PKCθ, -mTOR, -IKKβ, -JNK, -ERK 1/2 and -p38 were stripped and reblotted with the corresponding antibodies against total PKCθ, mTOR, IKKβ, JNK, ERK 1/2 and p38 MAPK protein. For the quantification of protein expression, membranes were reblotted with anti-GAPDH antibody to normalize respect total amount of protein in whole and cytosolic hypothalamus extracts, and with anti-TBP (nuclear marker) in nuclear hypothalamus extracts. For immunoprecipitation, 4 µg of anti-phospho-serine or anti-IRβ antibodies were incubated with protein A-agarose for 2 h at 4ºC. The complexes were washed with solubilization buffer and incubated with 1 mg of whole-hypothalamus extracts overnight at 4 ºC. Immunocomplexes were washed extensively, and then subjected to Western blotting and quantified as described above. For the quantification of PTP1B serine phosphorylation, membranes with phospho-serine immunoprecipitates were blotted with anti-PTP1B antibody and bands were normalized to total amount of PTP1B detected by immunoblot. For the quantification of co-immunoprecipitation of PTP1B or LAR with IRβ, membranes with IRβ immunoprecipitates were blotted with anti-PTP1B or anti-LAR antibodies and reblotted with anti-IRβ antibody to normalize respect total amount of protein. The data were expressed as percentage respect to the data of 3 month animals.

2.6. Phosphatase and tyrosine phosphatase activity

Hyphotalamus were homogenized in lysis buffer containing 50 mM Tris, 0.15 mM NaCl, 2 mM EDTA, 1% Triton X-100 (pH 7.5) supplemented with 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.1 mM PMSF, 1 µg/ml benzamidine and 1

μg/ml pepstatin. After protein determination, total PTP activity was assessed by *p*-nitrophenyl phosphate hydrolysis assays (PTP Assay Kit 1, Upstate Biotechnology). For IR tyrosine phosphatase activity determination, hypothalamic lysates were applied through a Sephadex G-25 column to remove the free phosphates. After protein content determination, PTP activity was measured in terms of phosphate release using a synthetic Tyr phosphorylated peptide from IR β-chain regulatory autophosphorylation sites (Biomol Research Laboratories, Plymouth, UK) and the malachite green assay (PTP Assay Kit 1, Upstate Biotechnology).

2.7. Statistical analysis

For the statistical analysis we used SPSS 19 software (Chicago, IL, USA). Statistical comparisons to determine the effect of age were done by one-way ANOVA followed by a Tukey`s post hoc test. For comparisons between caloric restricted and *ad libitum* aged-mates the unpaired Student's t test was used.

3. Results

3.1. Hypothalamic PTPs are increased with ageing and decrease with caloric restriction

In peripheral tissues an increase of PTP activity has been related with obesity and insulin resistance (Ahmad et al., 1995; Begum et al., 1991; Hauguel-de et al., 1993).Thus, we explored the hypothalamic PTP activity that could be involved in age-associated central insulin resistance. As shown in figure 1 A, neither ageing nor caloric restriction brought about any significant changes on total phosphatase activity in hypothalamic extracts from all groups of animals studied. However, when specific IR tyrosine phosphatase activity was analyzed using a synthetic tyrosine-phosphorylated peptide from IRβ-chain (figure 1 B), a significant increase in this parameter was observed in hypothalamus from 24 old animals. Caloric restriction showed a tendency to ameliorate this increase.

Since insulin signaling is already attenuated in aged rats at the level of insulin receptor phosphorylation, we explored the status of two PTPs involved in IR dephosphorylation: PTP1B and LAR. An increase in the expression of PTP1B in hypothalamus and other tissues associated with ageing and insulin resistance has already been described in mice and humans (Ahmad et al., 1995; Gonzalez-Rodriguez et al., 2011; Morrison et al., 2007). Figure 2 A shows a moderate, but not significant increase in the amount of PTP1B detected by immunoblot. Caloric restriction seems to decrease this parameter but no significant differences were appreciated. PTP1B activity can be regulated by serine and tyrosine phosphorylation. An increase in serine phosphorylation has

been related with an increased activity (Tao et al., 2001) and has been observed in hypothalamus of rats under HFD (Picardi et al., 2010). When PTP1B serine phosphorylation was analyzed, a significant increase in this parameter was observed with ageing. Caloric restriction significantly ameliorates this increase in aged animals (figure 2 B). Moreover, when the association of IR and PTP1B was analyzed by immunoprecipitation with anti-IRβ antibodies combined with immunoblot again PTP1B, a significant increase in this parameter was observed with ageing (figure 2C). In this case, caloric restriction also showed a tendency to decrease the association between IR and PTP1B that reached significance in the case of 24 month old caloric restricted animals.

LAR is another PTP that exhibits physical association with and de-phosphorylates IR (Ahmad and Goldstein, 1997; Hashimoto et al., 1992). Thus, this enzyme has been postulated to regulate insulin signaling and it has been implicated in the development of insulin resistance (Zabolotny et al., 2001). As in the case of PTP1B, we did not detect any significant change in the amount of this phosphatase by immunoblot (Figure 3 A), but a significant increase of its association with IR was observed in older animals (Figure 3 B). This increase showed a tendency to decrease in aged caloric restricted animals.

3.2. Activation of hypothalamic pro-inflammatory pathways increases with ageing, and decreases with caloric restriction

Inflammation, reticulum stress and oxidative stress have been related to the development of insulin resistance (Hotamisligil et al., 1993). Under these circumstances, activation of several serine/threonine kinases such as JNK,

IKKβ and p38 have been shown to increase serine phosphorylation of key elements of the insulin signaling pathway such as IR and IRSs, resulting in the impairment of insulin signaling and development of insulin resistance (Herschkovitz et al., 2007; Tanti and Jager, 2009). Moreover, inflammatory signals acting through the IKKβ-NFκB pathway may also induce resistance by transcriptional mechanisms (review in Cawthorn and Sethi, 2008).

To study the status of the IKKβ-NFκB pathway we first analyzed the phosphorylation status of IKKβ. In figure 4 A it can be observed that while no significant changes in phospho-IKKβ/IKKβ ratio was appreciated during ageing, there was a significant increase in total amount of IKKβ in hypothalamus from aged animals. Caloric restriction ameliorated this increase. To further investigate this pro-inflammatory pathway, we analyzed the status of both NFκβ isoforms, NFκβ p50 and NFκβ p65, in cytosolic and nuclear fractions during ageing and the effects of caloric restriction. As shown in figure 4 B and C, ageing was associated with an increase of NFκβ p50 and NFκβ p65 isoforms in both, cytosolic and nuclear fractions. Caloric restriction showed a clear tendency to reverse this, and the amount of NFκβ p50 and NFκβ p65 decreased in both fractions. These decreases reached significance for NFκβ p50 in the cytosolic fraction of caloric restricted 8 and 24 month old animals. Similarly, caloric restriction significantly decreased the amount of NFκβ p65 in both cytosolic and nuclear fractions of 24 old animals.

In the case of JNK we could not detect any significant changes neither in the amount nor in its phosphorylation state (data not shown). Nevertheless, in the case of p38, as shown in figures 5, ageing brought about a significant increase in both the amount of protein and the phosphorylation of this kinase in

hypothalamus from 8 and 24 month old animals. Caloric restriction significantly decreased both the amount of p38 and its phosphorylation.

3.3. Status of several serine kinases during ageing: effect of caloric restriction

Serine phosphorylation of IRSs has also been shown to be mediated by others serine/threonine kinases such as AKT, ERK1/2, mTOR/p70S6K, and GSK-3 (Tanti and Jager, 2009). We previously reported that neither ageing nor caloric restriction caused significant alterations in both, the amount of protein and the phosphorylation status of AKT and p70S6K. In the case of GSK3 although we did not detect significant changes in its phosphorylation, we observed a significant decrease in the amount of protein in old rats (Garcia-San et al., 2007). Chronic activation of the mTOR pathway has been related with increases in IRS serine phosphorylation, which contributes to insulin resistance (Tremblay et al., 2005). Nevertheless, we could not detect any significant change neither in the amount nor in the phosphorylation status of mTOR associated with ageing (data not shown). Similar results were observed for ERK1/2, and caloric restriction did not bring about any significant change in these parameters either (data not shown).

On the other hand, several members of the novel PKC isoforms also have been described to play a negative regulatory role on insulin action (Sampson and Cooper, 2006). In particular, PKCθ phosphorylates IRS-1 on serine leading to insulin resistance (Yu et al., 2002), and arcuate-specific knockdown of PKCθ attenuated diet-induced obesity and improved central insulin signaling (Benoit et al., 2009), suggesting that this enzyme may also participate in the mechanism of central insulin resistance. Therefore, we also

analyzed the status of this member of the novel PKC family. Figure 6 shows that, in spite of no significant changes in the amount of PKCθ detected by immunoblot, a significant increase in phosphorylated PKCθ was observed with ageing in both, 8 and 24 months old animals. In this case caloric restriction did not significantly decrease PKCθ phosphorylation.

4. Discussion

The present article demonstrates that, besides the alterations in different steps of the stimulatory pathway described earlier (Garcia-San et al., 2007), hypothalamic insulin signaling also presents alterations in the mechanisms involved in turning off the signal during ageing. We demonstrate that ageing brings about a specific increase in insulin receptor protein tyrosine phosphatase activity. This increase can be explained, at least in part, by the increased activation of PTP1B and LAR protein tyrosine phosphatases. Besides these mechanisms, increased activation of the IKKβ-NFκB pathway, p38 and PKCθ serine/threonine kinases also contribute to explain the hypothalamic insulin resistance associated with ageing. Caloric restriction ameliorates most of the effects of ageing on the above mentioned increases in PTPs and serine/threonine kinases activities and points to age-associated adiposity and inflammation as key factors in the development of age-associated insulin resistance.

PTP1B plays an important role at hypothalamic level by regulating insulin and leptin signals (Bence et al., 2006). Increases in the expression of PTP1B in hypothalamus and other tissues in aged (Gonzalez-Rodriguez et al., 2011; Morrison et al., 2007), obese (White et al., 2009) rodent models and humans (Ahmad et al., 1995), as well as increases in the expression of LAR in obese rodent models (Ahmad and Goldstein, 1995; Tagami et al., 2002) and humans (Ahmad et al., 1995), have been related to the decrease of insulin sensitivity. Although we could not detect any significant increase neither in the amount of PTP1B nor in the amount of LAR by immunoblot, it is possible that changes in the expression of these enzymes in specific hypothalamic nuclei involved in

insulin signaling such as arcuate can be diluted when total hypothalamus extracts are analyzed, as it is the case. Nevertheless, the increase in a specific IR tyrosine phosphatase activity, demonstrated in hypothalamic extracts from aged animals using the IR tyrosine phosphorylated peptide, clearly points to an up-regulation of the activities of these enzymes involved in the turning off mechanism of insulin action at the level of IR. Moreover, the fact that PTP1B presents increased serine phosphorylation, and both PTP1B and LAR present increased association with IR suggest that both phosphatases could be responsible of the increased IR tyrosine phosphatase activity detected in aged animals, and that these mechanisms contribute to the decreased hypothalamic insulin response described in aged Wistar rats.

The activation of PTP1B and/or LAR could be related to the development of inflammation. Activation of both PTP1B and LAR have been associated with inflammation and insulin resistant states (Cheung et al., 2000; Zabolotny et al., 2008). In this sense, hypothalamus from aged Wistar rats have been shown to present sings of inflammation such as increases in the expression of the pro-inflammatory cytokine TNFα, M1 macrophage marker CD11b and proteins related with macrophage infiltration and inflammation signaling such as chemo-attractant protein-1 (MCP1/CCL2) and toll like receptor-4 (TLR-4), respectively (Horrillo et al., 2011). On the other hand, our results that show increases in the amount of IKKβ, p50 and p65 NFκβ in both, cytosolic and nuclear fractions, together with the increase in the amount and phosphorylation of p38, support the activation of pro-inflammatory pathways, and reinforce the idea that different elements of inflammatory pathways seem to be up-regulated in hypothalamus of aged rats. The activation of these serine kinases agree well with the

increased serine phosphorylation of hypothalamic IR and IRSs described in aged rats (Garcia-San et al., 2007). In agreement with this idea, other models of central insulin resistance, such as mice under HFD, have also shown signs of hypothalamic inflammation (Belgardt et al., 2010; De Souza et al., 2005). Besides the possible activation of hypothalamus PTPs, the activation of the pro-inflammatory IKKβ-NFκβ pathway could indeed induce insulin resistance through SOCS3 (Zhang et al., 2008) which expression increases in hypothalamus from aged rats (Peralta et al., 2002).

The increase in PKCθ phosphorylation detected in hypothalamus from aged rats suggests that activation of this kinase may also contribute to the state of hypothalamic insulin resistance associated with ageing. PKCθ is expressed in several hypothalamus nuclei, including arcuate, lateral hypothalamus and perifornical area (Irani et al., 2010). Although other PKCs have been described to be expressed in hypothalamus (Irani et al., 2010; Thaler et al., 2009), PKCθ has been directly related with central insulin resistance. PKCθ knockout mice are protected from fat-induced insulin resistance (Kim et al., 2004), and arcuate-specific knockdown of PKCθ attenuated diet-induced obesity and improved central insulin signaling. On the other hand, the fact that PKCθ mediates hypothalamic insulin resistance induced by palmitic acid (Benoit et al., 2009) agrees well with the idea that the alteration of the status of PKCθ during ageing can also be a consequence of the development of adiposity and/or a low-grade inflammation.

As ageing is associated with changes in body composition that result in fat accretion and a decrease in muscle mass, it is possible that some metabolic alterations associated with ageing such as central insulin resistance could be

due, at least in part, to fat accretion and its consequences, as it is the case of animals under HFD. The fact that central leptin and insulin resistance associated with ageing in these animals is ameliorated by caloric restriction (Fernandez-Galaz et al., 2002; Garcia-San et al., 2007) also led us to investigate whether or not caloric restriction reverse any of the effects of ageing on PTP1B, LAR and the different serine/threonine kinases above mentioned. Although some of the changes observed with ageing in parameters such as PKCθ phosphorylation and IKKβ protein expression were not reverted/altered by caloric restriction, the fact that caloric restriction ameliorated the increases of specific IR tyrosine phosphatase activity, PTP1B serine phosphorylation, and IR association with PTP1B and LAR clearly points to age-associated adiposity as a key factor in the development of central insulin resistance, at the level of IR tyrosine phosphorylation. As this is an early step in the insulin signaling pathway its consequences can propagate downstream along the pathway, having a wide array of consequences. This agrees well with the fact that weight loss in humans improves insulin sensitivity by the reduction in PTP activity and PTP1B and LAR abundance in adipose tissue (Ahmad et al., 1997). Fat tissue accretion has been described to increase not only macrophage infiltration but also macrophage polarization towards a more pro-inflammatory secretory profile (Olefsky and Glass, 2010). This would lead to a self fed cycle of inflammation resulting in increased cytokine and fatty acids production, which, finally, interfere with insulin signaling, not only in adipose tissue but also in other tissues, establishing a global state of insulin resistance. Aged Wistar rats present signs of inflammation in several tissues, including hypothalamus, and caloric restriction ameliorates, at least in part, this state of inflammation (Horrillo

et al., 2011) improving central and peripheral insulin resistance.

The present data provide support for the involvement of protein tyrosine phosphatases, PTP1B and LAR, in the development of central insulin resistance associated with ageing. Increased activities of IKKβ-NFκB pathway, as well as p38 and PKCθ serine/threonine kinases also seem to contribute to explain the hypothalamic pro-inflammatory state and insulin resistance associated with ageing. Caloric restriction ameliorates, at least in part, several of these alterations pointing to the key role of age associated adiposity and inflammation in the development of hypothalamus insulin resistance. These results together with the fact that mice deficient in PTP1B have been shown to be protected from age-associated obesity, inflammation and insulin resistance (Gonzalez-Rodriguez et al., 2011) and, that the same caloric restricted protocol decrease hypothalamic pro-inflammatory state (Horrillo et al., 2011) let us to conclude that caloric intervention could be a useful tool to ameliorate or prevent age-associated development of central and peripheral insulin resistance.

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Figure legends

Fig. 1. Effect of ageing and caloric restriction (CR) on hypothalamic PTP and IR total PTP activity. Total phosphatase activity in whole hypothalamus extracts was assay as described in the 2.6 section (A). IR tyrosine phsphatase activity was measured using a synthetic tyrosine-phosphorylated peptide from IRβ-chain as described in the 2.6 section (B). Data are means ± SEM (n=6-8) expressed in percentage respects 3 month old equaled 100. The data *ad libitum* were evaluated by one-way ANOVA test, followed by a Tukey's post hoc test. *, $p<0.05$ vs. 3 month old animals. CR was compared with its *ad libitum* age-mate group using non paired Student's *t*-test.

Fig. 2. Effect of ageing and caloric restriction (CR) on hypothalamic PTP1B protein expression, serine-phosphorylation and its association with IRβ. PTP1B protein expression was determined by western blot with an antibody against PTP1B, and anti-GAPDH antibody as control for equal loading of protein (A). Phospho-serine-PTP1B was determined by immunoprecipitation (IP) with an antibody against phospho-serine (P-Ser) following immunoblotting (IB) with anti-PTP1B antibody, and corrected by PTP1B levels (B). The association of PTP1B with IRβ was analyzed by IP with anti-IRβ antibody then blotted with anti-PTP1B antibody, and corrected by IRβ contents (C). Representative western blots are shown in the upper panels. Bars, in the lower panels, represent means ± SEM (n=5-6) expressed as arbitrary units (a.u.) in percentage respects 3 month old equaled 100. The data *ad libitum* were evaluated by one-way ANOVA test, followed by a Tukey's post hoc test. **, $p<0.01$, ***, $p<0.001$ vs. 3 month old.

CR was compared with its *ad libitum* age-mate group using non paired Student's *t*-test. +, $p<0.05$, ++, $p<0.01$ vs. its *ad libitum* age-mates.

Fig. 3. Effect of ageing and caloric restriction (CR) on hypothalamus LAR protein expression and its association with IRβ. LAR protein expression was determined by western blot with antibody against LAR, and anti-GAPDH antibody as control for equal loading of protein (A). The association of LAR with IRβ was analyzed by immunoprecipitation (IP) with anti-IRβ antibody following immunoblotting (IB) with anti-LAR antibody, and corrected by IRβ levels (B). Representative western blots are shown in the upper panels. Bars, in the lower panels, represent means ± SEM (n=5-6) expressed as arbitrary units (a.u.) in percentage respects 3 month old equaled 100. The data *ad libitum* were evaluated by one-way ANOVA test, followed by a Tukey's post hoc test. *, $p<0.05$ vs. 3 month old animals. CR was compared with its *ad libitum* age-mate group using non paired Student's *t*-test.

Fig. 4. Effect of ageing and caloric restriction (CR) on hypothalamus IKKβ protein expression and phosphorylation, and NFκβ p50 and p65 content in cytosolic and nuclear hypothalamus extracts. Whole hypothalamus extracts were subjected to SDS-PAGE and immunoblotted with antibodies against phospho-IKKβ (P-IKKβ) and IKKβ, and anti-GAPDH antibody as control for equal loading of protein (A). Hypothalamus cytosolic and nuclear extracts were subjected to western blot analysis using antibodies against p50 (B) or p65 (C) NFκB, with anti-GAPDH and anti-TBP antibodies as controls for equal loading of protein in the cytosolic and nuclear fractions respectively. Upper panels show

representative western blots. Bars, in the lower panels, represent means ± SEM (n=4-6) expressed as arbitrary units (a.u.) in percentage respects 3 month old equaled 100. The data *ad libitum* were evaluated by one-way ANOVA test, followed by a Tukey's post hoc test. *, $p<0.05$, **, $p<0.01$ vs. 3 month old. CR was compared with its *ad libitum* age-mate group using non paired Student's *t*-test. +, $p<0.05$ vs. its *ad libitum* age-mates.

Fig. 5. Effect of ageing and caloric restriction (CR) on hypothalamus p38 protein expression and phosphorylation. Whole hypothalamus extracts were subjected to SDS-PAGE and immunoblotted with antibodies against phospho-p38 MAPK (P-p38) and p38 MAPK (p38), and anti-GAPDH antibody as controls for equal loading of protein. Upper panel shows representative western blots. Bars, in the lower panel, represent means ± SEM (n=4-6) expressed as arbitrary units (a.u.) in percentage respects 3 month old equaled 100. The data *ad libitum* were evaluated by one-way ANOVA test, followed by a Tukey's post hoc test. *, $p<0.05$, **, $p<0.01$ vs. 3 month old animals. CR was compared with its *ad libitum* age-mate group using non paired Student's *t*-test. +, $p<0.05$, ++, $p<0.01$ vs. its *ad libitum* age-mates.

Fig. 6. Effect of ageing and caloric restriction (CR) on hypothalamus PKCθ protein expression and phosphorylation. Whole hypothalamus extracts were subjected to SDS-PAGE and immunoblotted with antibodies against phospho-PKCθ (P-PKCθ), and PKCθ, and anti-GAPDH antibody as controls for equal loading of protein. Upper panel shows representative western blots. Bars, in the lower panel, represent means ± SEM (n=4-6) expressed as arbitrary units (a.u.)

in percentage respects 3 month old equaled 100. The data *ad libitum* were evaluated by one-way ANOVA test, followed by a Tukey's post hoc test. *, $p<0.05$, **, $p<0.01$ vs. 3 month old animals. CR was compared with its *ad libitum* age-mate group using non paired Student's *t*-test.