Low salt intake modulates insulin signaling, JNK activity and IRS-1\(^{ser307}\) phosphorylation in rat tissues

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Abstract

A severe restriction of sodium chloride intake has been associated with insulin resistance and obesity. The molecular mechanisms by which the low salt diet (LS) can induce insulin resistance have not yet been established. The c-jun N-terminal kinase (JNK) activity has been involved in the pathophysiology of obesity and induces insulin resistance by increasing inhibitory IRS-1\(^{ser307}\) phosphorylation. In this study we have evaluated the regulation of insulin signaling, JNK activation and IRS-1\(^{ser307}\) phosphorylation in liver, muscle and adipose tissue by immunoprecipitation and immunoblotting in rats fed with LS or normal salt diet (NS) during 9 weeks. LS increased body weight, visceral adiposity, blood glucose and plasma insulin levels, induced insulin resistance and did not change blood pressure. In LS rats a decrease in PI3-K/Akt was observed in liver and muscle and an increase in this pathway was seen in adipose tissue. JNK activity and IRS-1\(^{ser307}\) phosphorylation were higher in insulin-resistant tissues. In summary, the insulin resistance, induced by LS, is tissue-specific and is accompanied by activation of JNK and IRS-1\(^{ser307}\) phosphorylation. The impairment of the insulin signaling in these tissues, but not in adipose tissue, may lead to increased adiposity and insulin resistance in LS rats.

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Introduction

Reduction in daily sodium intake has been recommended as a non-pharmacological approach to the treatment of hypertension (Chobanian & Hill 2000, Sacks et al. 2001, Vollmer et al. 2001, Chobanian et al. 2003). However, a severe restriction of sodium chloride intake has shown adverse effects on glucose and lipid metabolism (Iwaoka et al. 1988, Sharma et al. 1990, Egan et al. 1991, Ruppert et al. 1991, Catanozi et al. 2001). In previous reports, chronic salt restriction in Wistar rats induced increases in body weight and adiposity (Prada et al. 2000, Xavier et al. 2003, Okamoto et al. 2004) which were associated with a decrease in whole body glucose uptake evaluated with the euglycemic hyperinsulinemic clamp (Prada et al. 2000). In addition, other studies have shown that a severe salt restriction induces increased plasma triacylglycerol and total cholesterol concentration in humans as well as in rats (Sharma et al. 1990, Ruppert et al. 1991, Catanozi et al. 2001). Taking these reports together, it seems that a severe sodium restriction could dysregulate insulin sensitivity and increase adiposity. However, the molecular mechanisms by which a low salt diet can induce insulin resistance are not yet completely understood. Under such conditions, insulin resistance may be tissue-specific to the liver and muscle, whereas adipose tissue remains sensitive to insulin, as seen in a previous study (Hirata et al. 2003).

At the molecular level, insulin initiates its biological effects by activating the insulin receptor, resulting in tyrosine phosphorylation of several substrates, including the insulin receptor substrate (IRS) proteins, such as IRS-1 and IRS-2 (Saltiel & Pessin 2002). Following tyrosine phosphorylation, IRS-1 and IRS-2 bind and activate the enzyme phosphatidylinositol 3-kinase (PI3-K) (Foldi et al. 1992, Saltiel & Pessin 2002). The activation of PI3-K increases serine phosphorylation of Akt (protein kinase B)
which, in turn, stimulates the glucose transport in the muscle and adipose tissue, stimulates glycogen synthesis in the liver and muscle, and stimulates lipogenesis in the adipose tissue. Therefore, the PI3-K/Akt pathway has an important role in the metabolic effects of insulin.

Many mechanisms may contribute to the dysregulation of the insulin signaling pathway, including serine phosphorylation of IRS proteins by protein kinases such as c-jun N-terminal kinase (JNK) (Lee et al. 2003). JNK is a member of the MAP kinase family (Davis 1999, Weston et al. 2002) and can be activated by Tumor Necrosis Factor (TNFα; Hirosumi et al. 2002), Interleukin 1β (IL 1β; Major & Wolf 2001, Nikulina et al. 2003) and following a high fat diet (Hirosumi et al. 2002). There are three JNK isoforms described: JNK1, 2 and 3 (Ip & Davis 1998). JNK1 activity has been implicated in obesity and insulin resistance (Hirosumi et al. 2002). JNK activation induces inhibitory serine 307 (Ser307) phosphorylation of IRS-1, as shown in previous studies (Aguirre et al. 2002, Lee et al. 2003). Ser307 is located next to the phosphotyrosine-binding (PTB) domain in IRS-1 and its phosphorylation inhibits the interaction of the PTB domain with the phosphorylated NPEY motif in the activated insulin receptor, causing insulin resistance (Aguirre et al. 2002).

Here, we investigate the effects of low salt intake on insulin signaling, in JNK activation and in IRS-1\textsuperscript{Ser307} phosphorylation in liver, skeletal muscle and white adipose tissue of Wistar rats.

**Materials and Methods**

**Materials**

Male Wistar rats were provided by the Institutional Animal Facility (University of São Paulo School of Medicine, São Paulo, Brazil). Antiphosphotyrosine (αPY), anti-IRβ (αIRβ), anti-IRS-1, anti-IRS-2, anti-Akt1/2, anti-pJNK and anti-JNK1 antibodies were from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-pAkt and anti-pFoxo1 were from Cell Signaling Technology (Beverly, MA, USA). Anti-PI3-K, anti-IRS-2 and anti-phospho-IRS-1\textsuperscript{Ser307} were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Human recombinant insulin (Humulin R) was purchased from Eli Lilly. Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified elsewhere. \textsuperscript{125}I-Protein A was from Amersham.

**Animals**

All experiments reported herein are in accordance with the guidelines of the Ethics Committee of the University of São Paulo School of Medicine and the Ethics Committee of the State University of Campinas, Brazil.

Male Wistar rats were fed a low salt-diet (LS, 0-06% Na, TD 92141-Harlan Teklad) or normal salt-diet (NS, 0-5% Na, TD 92140) from weaning (3 weeks old) to adulthood (12 weeks old). Rats were housed in a controlled-temperature environment (25 °C), with a 12-hour light/dark cycle and free access to chow and tap water.

**Metabolic characterization**

Body weight (BW) was measured at weaning and at 12 weeks of age when epididymal, retroperitoneal and mesenteric fat pads were excised and weighed. Fasting blood samples were collected for blood glucose and plasma insulin measurements. Insulin resistance was assessed from fasting insulin and glucose levels, using the previously validated homeostasis model of assessment (HOMA-IR) (Matthews et al. 1985, Bonora et al. 2000); HOMA-IR = fasting glucose (mmol/l) × (fasting insulin (µU/ml)/22.5).

Blood glucose was measured by the glucose oxidase method. Plasma insulin was assayed using commercial rat specific radioimmunoassay kits (Linco Research Inc, St. Louis, MO, USA).

**Blood pressure measurement**

In other groups of animals fed on the NS or LS diets, a carotid catheter was inserted under anesthesia (sodium thiopental – 50 mg/kg BW, i.p.) and exteriorized at the dorsal face of the neck. The animals were then housed in individual cages and allowed to recover from surgery. Four to five days later, the mean blood pressure (MBP) was measured in conscious animals through the carotid artery catheter, which was attached to a pressure transducer (Gould Statham Instruments Inc., model P23DB, Hato Rey, Puerto Rico, USA) and was connected to an amplifier (Stemtech Inc., GPA-4 model 2, Menomonee Falls, WI, USA) that provided the analog blood pressure signal, which was digitized by a computer-based monitoring system (DATAQ Instruments Inc., Akron, OH, USA). MBP measurement was registered during 30 min. The mean of all values obtained during each determination was considered for calculations.

**Tissue extraction and immunoprecipitation**

After 6 h of fasting, animals were anesthetized with sodium thiopental and used 10–15 min later. As soon as anesthesia was assured by the loss of pedal and corneal reflexes, the abdominal cavity was opened, the portal vein was exposed and 0.2 ml of normal saline with or without insulin (2 µg) was injected. Thirty seconds after insulin injection, the liver was removed and 90 seconds later gastrocnemius and epididymal adipose tissue were removed, minced coarsely and homogenized immediately in extraction buffer, as described elsewhere (Torsoni et al. 2003). Extracts were then centrifuged at 30 000 g and 4 °C, during 40 min to remove insoluble material and the supernatants were used for immunoprecipitation with...
αIR, αIRS-1, αIRS-2 and Protein A-Sepharose 6 MB (Pharmacia).

Protein analysis by immunoblotting

The precipitated proteins and/or whole tissue extracts were treated with Laemmli sample buffer (Laemmli 1970) containing 100 mM DTT (dithiothreitol) and heated in a boiling water bath for 5 min, after which they were subjected to SDS-PAGE (sodium dodecyl sulphate – polyacrylamide gel electrophoresis) in a Bio-Rad miniature slab gel apparatus (Mini-Protein). For total extracts, 200 µg protein were subjected to SDS-PAGE.

Electrotransfer of proteins from the gel to nitrocellulose was performed for 120 min at 120 V in a Bio-Rad Mini-Protein transfer apparatus (Towbin et al. 1979) with the addition of 0·02% SDS to the transfer buffer. Non-specific protein binding to the nitrocellulose was reduced by pre-incubating the filter for 2 h in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, 0·02% Tween20). The nitrocellulose blot was incubated with specific antibodies overnight at 4 °C and then incubated with 125I-Protein A. The results were visualized by autoradiography with preflashed Kodak XAR film (Eastman Kodak Co., Rochester, NY, USA). Band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, San Francisco, USA; model GS300).

Statistical analysis

Values are expressed as mean ± S.E.M. All groups of animals were studied in parallel. Comparisons between different groups were performed by Student’s t-test for unpaired samples. The level of significance was P<0·05.

Results

Animal characteristics

The body weight at weaning was similar in the two groups (NS: 53 ± 2·2 g vs LS: 50 ± 1·6 g, n=8 each, P>0·05). However, as shown in Table 1, 9 weeks after starting the diets, the body weight was higher (P<0·05) in the LS than in the NS group (P<0·05). The epididymal, retroperitoneal and mesenteric fat pads were higher in LS compared with NS (P<0·05). Fasting blood glucose and plasma insulin were higher in LS compared to NS (P<0·001). The HOMA-IR index, calculated from fasting glycemia and insulinenia, was increased in LS compared to NS rats (P<0·001). The MBP measured in the carotid artery was similar in both groups (P>0·05).

Insulin signaling in liver of animals fed on a LS

There were no differences in the IR protein expressions in liver of NS compared to LS rats (Fig. 1A). There was a significant reduction (P<0·001) in insulin-stimulated IR tyrosine phosphorylation in the liver of animals fed on a LS when compared to NS (Fig. 1B). There were no differences in the IRS-1 protein expressions in the livers of NS compared to LS-rats (Fig. 1C). However, animals fed with LS presented a significant reduction (P<0·001) in both insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 1D) and in IRS-1/PI3-K association when compared to NS (Fig. 1E). Although, no differences were observed in the IRS-2 protein expressions in the livers of NS compared to LS rats (Fig. 1F), there was a reduction (P<0·05) in insulin-stimulated tyrosine phosphorylation of IRS-2 (Fig. 1G) and in its association with p85 subunit of PI3-K in the liver of animals fed on a LS compared to NS (Fig. 1H).

Akt protein levels did not differ among the groups in this study (Fig. 1I). Insulin-stimulated Akt serine phosphorylation was lower (P<0·05) in LS compared to NS rats (Fig. 1J).

JNK protein levels did not differ among the groups in this study (Fig. 1K). Liver from animals fed with LS showed increased JNK activation, increased IRS-1/JNK1 association and increased IRS-1/ser307 phosphorylation in the liver when compared with NS animals (P<0·05) (Figs. 1L, M and N).

Insulin signaling in muscle of animals fed on a LS

There were no differences in IR protein expression in muscle of NS compared to LS rats (Fig. 2A). Animals fed with LS showed a significant reduction (P<0·05) in insulin-stimulated IR tyrosine phosphorylation in muscle (Fig. 2B) compared with NS animals.

There were no differences in the IRS-1 protein expressions in the muscles of NS compared to LS rats (Fig. 2C). Despite this observation, animals on LS showed a significantly reduced (P<0·05) insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 2D) and in IRS-1/P13-K association (Fig. 2E) in muscle from animals fed with LS when compared with NS group.

There were no differences in IRS-2 protein levels, in insulin-stimulated tyrosine phosphorylation of IRS-2 and

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*P<0·05 vs NS, **P<0·01 vs NS, ***P<0·001 vs NS. LS, low salt diet; NS, normal salt diet.
in its association with p85 subunit of PI3-K in muscle of LS rats compared with NS rats (Figs. 2F, G and H).

Akt protein levels did not differ among the groups in this study (Fig. 2I). Insulin-stimulated Akt serine phosphorylation was lower ($P<0.05$) in animals receiving LS when compared with NS (Fig. 2J).

JNK protein levels did not differ among the groups in this study (Fig. 2K). Muscles from animals fed LS...
demonstrated higher (P<0.001) JNK activation than NS rats (Fig. 2L), accompanied by higher (P<0.05) degrees of association between IRS-1/JNK1 (Fig. 2M) and increased (P<0.05) IRS-1 ser307 phosphorylation (Fig. 2N).

**Figure 2** Insulin signaling in muscle of NS (normal salt diet) animals and rats fed with low salt diet (LS) from weaning to adulthood. The whole tissue extracts were immunoprecipitated (IP) with anti-insulin receptor (IR) and immunoblotted (IB) with anti-IR antibodies (A); IP with anti-IR and IB with antiphosphotyrosine (pY) antibodies (B); IP with anti-IRS-1 and IB with anti-IRS-1 antibodies (C); IP with anti-IRS-1 and IB with anti-pY antibodies (D); IP with anti-IRS-1 and IB with anti-PI3-K antibodies (E); IP with anti-IRS-1 and IB with anti-aKT antibodies (F); IP with anti-IRS-1 and IB with anti-pY antibodies (G); IP with anti-IRS-1 and IB with anti-PI3-K antibodies (H); IP with anti-IRS-1 and IB with anti-aKT antibodies (I); IB with antiphosphotyrosine (pY) antibodies (J); IB with anti-IRS-1 antibody (K); IB with anti-IRS-1ser307 antibody (N). Data are expressed as means ± S.E.M. Each group was composed of five animals. *P<0.05 vs NS*, **P<0.001 vs NS**.

Insulin signaling in adipose tissue of animals fed on a LS

There were no differences in IR protein levels (Fig. 3A) nor in insulin-induced IR, tyrosine phosphorylation in adipose tissue of LS and NS animals (Fig. 3B).
Despite similar levels of IRS-1 in the adipose tissue of animals fed with LS and NS diets (Fig. 3C), there was a decrease \( (P<0.001) \) in insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 3D) and in IRS-1/PI3-K association (Fig. 3E) in LS, when compared with NS group. There were no differences in IRS-2 protein levels in the adipose tissue of LS rats compared with the NS group (Fig. 3F). On the other hand, there was an increase \( (P<0.05) \) in insulin-stimulated IRS-2 tyrosine phosphorylation (Fig. 3G) and in its association with the

Figure 3 Insulin signaling in adipose tissue of NS (normal salt diet) animals and rats fed with low salt diet (LS) from weaning to adulthood. The whole tissue extracts were immunoprecipitated (IP) with anti-insulin receptor (IR) and immunoblotted (IB) with anti-IR antibodies (A); IP with anti-IR and IB with antiphosphotyrosine (pY) antibodies (B); IP with anti-IRS-1 and IB with anti-phosphotyrosine (pY) antibodies (C); IP with anti-IRS-1 and IB with anti-pY antibodies (D); IP with anti-IRS-1 and IB with anti-PI3-K antibodies (E); IP with anti-IRS-2 and IB with anti-IRS-2 antibodies (F); IP with anti-IRS-2 and IB with anti-pY antibodies (G); IP with anti-IRS-2 and IB with anti-PI3-K antibodies (H); IB with anti-Akt1/2 antibody (I); IB with anti-Akt antibody (J); IB with anti-JNK1 antibody (K); IB with anti-pJNK antibody (L); IP with anti-IRS-1 and IB with anti-JNK1 antibodies (M); IB with anti-IRS-1 Ser307 antibody (N). Data are expressed as means ± S.E.M. Each group was composed of five animals. *\( P<0.05 \) vs NS, **\( P<0.001 \) vs NS, § \( P<0.05 \) vs NS.
Akt protein levels presented no di
terences among the
groups in this study (Fig. 3I). However, there was an
increase (P<0.05) in basal (LS- vs NS-) and in insulin-
stimulated Akt serine phosphorylation in LS compared
with NS rats (Fig. 3J).

JNK protein levels did not di
ter among the groups in
this study (Fig. 3K). Adipose tissue from animals fed LS
showed similar JNK activation to that of NS rats (Fig. 3L),
accompanied by a similar degree of association between
IRS-1/JNK1 (Fig. 3M), which probably led to similar
IRS-1ser307 phosphorylation between LS and NS groups
(Fig. 3N).

Insulin-induced Foxo1 phosphorylation in the liver, muscle and adipose tissue of animals fed on a LS

Animals fed on LS presented a significant decrease
(P<0.05) in insulin-stimulated Foxo1 phosphorylation when compared with NS rats in the liver and muscle (Figs. 4A and B). In contrast, there was a significant increase
(P<0.05) in insulin-stimulated Foxo1 phosphorylation in the adipose tissue of LS compared with NS (Fig. 4C).

Discussion

The results of the present study showed that animals fed with LS had higher body weight and adiposity, which were associated with insulin resistance characterized by the HOMA-IR index. Blood pressure was normal in these animals. These metabolic characteristics were
accompanied by alterations in the insulin signaling in muscle, liver, and adipose tissue.

The effects of salt restriction on insulin sensitivity have already been studied (Rocchini 1994, Prada et al. 2000, Okamoto et al. 2004). However, there is no consensus regarding the molecular mechanism by which sodium restriction induces decreased insulin sensitivity.

Our data showed that, in LS rats, there were significant decreases in insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation and a decrease in Akt activation in liver, suggesting impairment in insulin signaling in this tissue. In parallel, significant decrease was observed in the insulin-stimulated IRS-1/PI3-K/Akt pathway in muscle. In fact, previous data have shown that IRS-1 is more important than IRS-2 in muscle in mediating the e
eff
ect of insulin on carbohydrate and lipid metabolism in vivo (Yamauchi et al. 1996, Previs et al. 2000). The PI3-K/Akt pathway has
been implicated in glucose transport in muscle and in glycogen synthesis in muscle and liver (Cross et al. 1995). Recently, it has been demonstrated that Akt phospho-
rylates the forkhead transcription factors (Foxo) in the nucleus and inhibits their transcriptional activity (Kops & Burgering 1999, Kaestner et al. 2000). The insulin-
induced phosphorylation/inactivation of Foxo may also contribute to the control of glucose and lipid metabolism in liver (Altomonte et al. 2003, 2004). As such, the alterations in insulin signaling, through this pathway, in liver and in muscle may have a role in the insulin resistance of LS rats.

In adipose tissue of LS rats there was a differential modulation of IRSs activation, with a decrease in IRS-1 and an increase in IRS-2 tyrosine phosphorylation,
resulting in an increase in insulin-induced Akt activation. A previous study points out that the IRS-2 regulation may predominate over IRS-1 in downstream insulin signaling in adipose tissue (Kido et al. 2000). It has also been demonstrated that, in the adipose tissue, the phosphorylation/inactivation of Foxo1 increases the adipocyte differentiation (Nakae et al. 2003). Thus, the increased insulin-induced phosphorylation/inactivation of Foxo1, described in our study, might have a role in the enhanced adiposity in the LS group.

Furthermore, another study did not find any significant difference between GLUT4 gene expression and GLUT4 protein translocation before and after insulin injection in adipose tissue from low compared to normal salt rats (Okamoto et al. 2004). Taking this report together with the present data, it may be suggested that the increase in the IRS-2/Pi3-K/Akt pathway in adipose tissue may be linked more to lipogenesis by inactivating Foxo1, in turn increasing the visceral adiposity described in LS rats.

For instance, in other models of insulin resistance as seen in monoglutamate (MSG) -insulin-resistant rats (Hirata et al. 2003), an increase in the IRS-2/Pi3-K/Akt pathway was demonstrated in adipose tissue as well as an increased adiposity, suggesting that this pathway may have an important role in increasing the central fat depot.

There are probably a number of mechanisms that may lead to an impairment of the insulin-signaling pathway in muscle and liver. Previous studies clearly demonstrated that an increase in IRS serine phosphorylation could induce marked insulin resistance, indicating that this is an important mechanism in the control of insulin signaling (Tanti et al. 1994, Hotamisligil et al. 1996, Mothe & Van Obberghen 1996). It has been reported that activation of JNK induces serine 307 phosphorylation of IRS-1 (Aguirre et al. 2002), leading to a decrease in the insulin-stimulated Pi3-K activity. Our data showed an increase in JNK phosphorylation, in agreement with altered insulin signaling in the liver and in muscle of LS-rats, suggesting that this serine kinase may have an important role in downregulating insulin signaling in these tissues. Moreover, we demonstrated an increase in IRS-1 (ser307) phosphorylation with similar regulation and tissue distribution to JNK activation. These data suggest that JNK may have an important role in the altered insulin signaling in tissues of rats fed with LS.

Although there was a reduction in IRS-1 tyrosine phosphorylation in adipose tissue, this was not accompanied by an increase in IRS-1 (ser307) phosphorylation or JNK phosphorylation. This suggests that JNK may not have a role in reduced IRS-1 tyrosine phosphorylation, and hence the increased involvement of other serine kinases in the modulation of IRS-1 in adipose tissue.

In addition, at least two other mechanisms may have a role in reduced IRS-1 tyrosine phosphorylation. First, there is a possibility that an increase in phosphorysine phosphatases activity, such as PTP1B, may dephosphorylate IRS-1 (Goldstein et al. 2000). Recently, we described a new mechanism of modulation of IRS-1 through nitration of this protein, which reduces its tyrosine phosphorylation level (Carvalho-Filho et al. 2005). These two possibilities deserve further investigation in LS rats.

In summary, our data suggest that insulin resistance, induced by LS, is tissue-specific. In addition, the insulin resistance induced by LS in liver and muscle was accompanied by activation of JNK and IRS-1 (ser307) phosphorylation. This altered Pi3-K/Akt signaling pathway in the muscle, liver and adipose tissue may lead to the development of increased adiposity and insulin resistance in the LS rats.

Further studies are required to test the hypotheses derived from the observations in the current report, such as the effect of LS diet on JNK1/ -/- mice.

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