Aspirin inhibits serine phosphorylation of insulin receptor substrate 1 in growth hormone treated animals

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Abstract In this study, we demonstrate that pretreatment with aspirin inhibits GH-induced insulin resistance. GH was observed to lead to serine phosphorylation of IRS-1, a phenomenon which was reversed by aspirin in liver, muscle and WAT in parallel with a reduction in JNK activity. In addition, our data show an impairment of insulin activation in the IR/IRS/PI(3)kinase pathway and a reduction in IRS-1 protein levels in rats treated with GH, which was also reversed in the animals pretreated with aspirin. Overall, these results provide new insights into the mechanism of GH-induced insulin resistance.

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1. Introduction

Insulin stimulation initiates intracellular signaling by activation of insulin receptor (IR) tyrosine kinase and tyrosine phosphorylation of endogenous substrates [1]. IR substrate (IRS)-1/2 are major IR substrates that recruit various Src homology 2 (SH2) domain – containing signaling molecules, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) [2,3]. In insulin target cells, such as adipose and skeletal muscle cells, insulin-stimulated glucose transport is mostly achieved by the translocation of GLUT4 from the intracellular storage pool to the plasma membrane (PM), which is mediated by a PI 3-kinase-dependent pathway [4,5].

The chronic effects of growth hormone (GH) are considered to be anti-insulin, and they include increased blood glucose concentrations, decreased peripheral insulin sensitivity despite higher levels of insulin secretion, decreased insulin-stimulated glucose uptake in muscle, increased hepatic glucose production and stimulation of lipolysis [6]. In vivo studies indicate that chronic administration of GH in rats induced insulin resistance, which was accompanied by decreases in insulin-stimulated autophosphorylation of IR and tyrosine phosphorylation of IRS-1/2 in skeletal muscle [7]. However, the mechanism that modulates IR and IRS-1 in chronic GH-treated rats has not yet been described.

Many mechanisms may contribute to the deregulation of the insulin-signaling pathway, including serine phosphorylation of IRS proteins by protein kinases such as c-jun N terminal kinase (JNK) [8–10]. JNK is a member of the MAP kinase family [11] and can be activated by TNF-α [12] and IL 1β [13]. In addition, JNK might serve as a feedback inhibitor during insulin stimulation [9]. There are three JNK isofoms described, named JNK1, 2 and 3 [14], of which JNK1 is the most involved in the pathophysiology of obesity and insulin resistance [12]. JNK activation induces inhibitory serine 307 (Ser307) phosphorylation of IRS-1, as shown in previous studies [9,15]. 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 [15].

Recent evidence indicates that aspirin improves insulin sensitivity, probably by reducing serine phosphorylation of IRS-1. This may be the consequence of reduced JNK and/or IKKβ serine kinase activity, induced by aspirin. Since JNK is stimulated by GH and inhibited by aspirin [16–18], the current study was designed to investigate the molecular events of GH-associated insulin resistance, and aspirin was used to test the influence of GH on the IRS-1 serine phosphorylation. Results demonstrate that aspirin is able to partially inhibit GH-induced insulin resistance and IRS-1 serine phosphorylation.

2. Materials and methods

2.1. Animals

We studied six-week-old male Wistar rats. All experiments involving animals were in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the ethics committee at the University of Campinas. Room temperature was maintained between 21 and 23 °C, rats were subjected to a standard light–dark cycle (06:00:18:00/18:00–06:00) and provided with standard rodent chow and water ad libitum.

2.2. GH and aspirin pretreatment

Rats were treated with growth hormone (GH; 1 mg/kg sc) twice daily over 2.5 days. Starting at 12:00 h on the day before the experiment, salicylate (42 mg/kg; Sigma Chemical Co., St. Louis, Missouri, USA) or saline (for the control and GH groups; matching volume) was fed
using oral gavage at 6-h intervals (i.e., at 12:00, 18:00, and 24:00 h). The food was removed from the cage at 17:00 h for the overnight fasting of animals.

2.3. Protein analysis by immunoblotting

Rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg body weight) and the experiments were performed after the loss of corneal and pedal reflexes. The abdominal cavity was opened, the cava vein was exposed, and 0.5 ml of normal saline (0.9% NaCl) with or without 10^{-6} mol/l of insulin was injected. Liver, muscle and WAT were removed after 30, 90 and 90 s, respectively, minced coarsely and homogenized immediately in the solubilization buffer containing 100 mM Tris (pH 7.6), 1% Triton X-100, 150 mM NaCl, 0.1 mg aprotinin, 35 mg PMSF/ml, 10 mM Na_{2}VO_{4}, 100 mM NaF, 10 mM Na_{4}P_{2}O_{7}, and 4 mM EDTA, using a politron PTA 20S generator operated at maximum speed for 30 s and clarified by centrifugation. Equal amounts of protein were used for immunoprecipitation followed by Western blot analysis with the indicated antibodies and 125I-Protein A. 125I-Protein A bound to the antiphosphotyrosine and anti-peptide antibodies was detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −80 °C for 12–48 h. Band intensities were quantitated by optical densitometry (Hoefer Scientific Instruments, San Francisco; model GS 300) of the developed autoradiographs. The antibodies against IR (sc-711), IRS-1 (sc-559), IRS-2 (sc-8299), SOCS3 (sc-9023), phospho-JNK (sc-6254), phosphor-JUN (sc-822) and anti-phosphotyrosine (sc-508) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the p85 subunit of PI 3-kinase and IRS-1 phosphorylated at Ser 307 were from Upstate Biotechnology (Lake Placid, NY).

2.4. Insulin tolerance test

Rats were fasted overnight and submitted to an intravenous insulin tolerance test (IVITT; 1 U/kg body weight of insulin, i.v.) and samples for blood glucose measurements were collected at 0 (basal), 4, 8, 12, and 16 min after injection. Rats were anesthetized, then 40 μl of blood were collected from their tails and blood glucose concentration was measured by the glucose oxidase method. The rate constant for plasma glucose disappearance (K_{glu}) was then calculated using the formula

\[
{K_{glu}} = \frac{{\ln 2}}{{t_{1/2}}}.
\]

The plasma glucose t_{1/2} was calculated from the slope of the least squares analysis of the plasma glucose concentrations during the linear phase of decline [19].

2.5. Statistical analysis

Where appropriate, the results were expressed as the mean ± S.E.M. accompanied by the indicated number of rats used in experiments. Comparisons among groups were made using parametric 2-way ANOVA, where F ratios were significant, further comparisons were made using Newman–Keuls test.

3. Results

3.1. Metabolic characteristics

GH treated animals were more insulin resistant than the control rats, as expressed by their lower plasma glucose disappearance rates measured by the insulin tolerance test (K_{glu} Control: 4.65 ± 0.45%/min vs. GH: 1.84 ± 0.22%/min; P < 0.05). Aspirin partially reversed the GH-induced insulin resistance (K_{glu} GH: 1.84 ± 0.22%/min vs. GH + Aspirin: 3.91 ± 0.18%/min; P < 0.05).

3.2. In vivo effect of insulin on tyrosine phosphorylation of IR in the liver, muscle and WAT

The effect of in vivo intravenous insulin infusion on IR tyrosine phosphorylation was examined in the liver, muscle and WAT of controls, GH and GH plus aspirin pretreated rats. Fragments of tissues from insulin or vehicle-treated rats were submitted to immunoprecipitation with anti-IR antibody and then blotted with anti-phosphotyrosine antibody. In the liver, IR tyrosine phosphorylation was observed to increase in control animals by 8.5-fold following insulin administration compared with a 2.9-fold increase in the liver of GH-pretreated rats and a 6.7-fold increase in the liver of GH-plus-aspirin-pretreated rats (Fig. 1A). In the muscle, IR tyrosine phosphorylation increased by 9.3-fold in control animals following insulin administration, compared with a 4.2-fold increase in the muscle of GH-pretreated rats and a 9.9-fold increase in the muscle of GH-plus-aspirin-pretreated rats (Fig. 1B). In WAT, IR tyrosine phosphorylation increased by 8.5-fold in control animals following insulin administration, compared with a 4.4-fold increase in the WAT of GH-pretreated rats and 9.2-fold increase in the WAT of GH-plus-aspirin-pretreated rats (Fig. 1C). The protein concentration of IR in liver, muscle and WAT did not change with GH or aspirin treatment (Fig. 1, lower panels).

3.3. In vivo effect of insulin on tyrosine phosphorylation of IRS-1 in the liver, muscle and WAT

In the liver, IRS-1 tyrosine phosphorylation and IRS-1/PI-3 kinase association were observed to increase in control

Fig. 1. IR tyrosine phosphorylation and protein levels in liver, muscle and WAT of rats pretreated with vehicle, GH or GH + aspirin. Insulin-stimulated tyrosine phosphorylation of IRS-1. Liver, muscle and WAT from rats treated with insulin or vehicle were lysed and tissue extracts were immunoprecipitated with anti-IR antibodies and blotted with anti-phosphotyrosine antibody (pY, upper panel) or anti-IR (lower panel). Data (mean ± S.E.M., n = 6) are expressed relative to control. \(^*\) P < 0.05 insulin control vs. insulin GH. \(^{\#}\) P < 0.05 insulin GH vs. insulin GH + aspirin.
animals by 9.0- and 9.1-fold following insulin administration, respectively, compared with 3.1- and 3.2-fold increases in the liver of GH-pretreated rats and 9.1- and 7.0-fold increases in the liver of GH-plus-aspirin-pretreated rats (Fig. 2A). In the muscle, IRS-1 tyrosine phosphorylation and IRS-1/PI 3-kinase association increased by 7.9- and 8.1-fold, respectively, in control animals following insulin administration compared with 3.6- and 2.5-fold increases in the muscle of GH-pretreated rats and 7.9- and 6.4-fold increases in the muscle of GH-plus-aspirin-pretreated rats (Fig. 2B). In WAT, IRS-1 tyrosine phosphorylation and IRS-1/PI 3-kinase association increased by 7.9- and 8.2-fold, respectively, in control animals following insulin administration compared with 3.1- and 2.6-fold increases in the WAT of GH-pretreated rats and 7.6- and 5.7-fold increases in the WAT from GH plus aspirin pretreated rats (Fig. 2C). In addition, the IRS-1 protein concentrations were reduced in liver, muscle and WAT from GH-treated rats and pretreatment with aspirin partially restored IRS-1 protein levels (Fig. 2 lower panels). With respect to IRS-1 phosphorylation and PI 3-K association, when the data were corrected for the change in the number of IRS-1 molecules, the results showed a slight (but not statistically significant) decrease in the apparent stoichiometry of IRS-1 tyrosine phosphorylation and PI 3-K association (data not shown).

3.4. In vivo effect of insulin on tyrosine phosphorylation of IRS-2 in the liver, muscle and WAT

In the liver, IRS-2 tyrosine phosphorylation and IRS-2/PI-3 kinase association were observed to increase in control animals by 7.3- and 9.6-fold following insulin administration, respectively, compared with 2.3- and 3.1-fold increases in the liver from GH pretreated rats and 5.5- and 6.7-fold increases in the liver of GH-plus-aspirin pretreated rats (Fig. 3A). In the muscle, IRS-2 tyrosine phosphorylation and IRS-2/PI 3-kinase association increased by 10.6- and
8.8-fold, respectively, in control animals following insulin administration compared with 5.6- and 2.5-fold increases in the muscle of GH-pretreated rats and 8.3- and 7.6-fold increases in the muscle of GH-plus-aspirin pretreated rats (Fig. 3B). In WAT, IRS-2 tyrosine phosphorylation and IRS-2/PI 3-kinase association increased by 6.6- and 7.6-fold, respectively, in control animals following insulin administration compared with 2.6- and 2.3-fold increases in the WAT of GH-pretreated rats and 7.6- and 6.7-fold increases in the WAT of GH-plus-aspirin-pretreated rats (Fig. 3C). The protein concentration of IRS-2 in the liver, muscle and WAT did not change with GH or aspirin treatment (Fig. 1, lower panels).

3.5. Aspirin inhibits Ser\textsuperscript{307} phosphorylation of IRS-1 in GH-treated animals

Among the serine residues that become phosphorylated in response to risk factors of insulin resistance, Ser\textsuperscript{307} has been studied extensively and Ser\textsuperscript{307} phosphorylation has become a molecular indicator of insulin resistance [9,12,15,20]. It has been reported that aspirin could inhibit IRS-1 serine phosphorylation [21], but the effect of aspirin on GH-induced IRS-1 serine phosphorylation has not been identified. To address this issue, we tested Ser\textsuperscript{307} phosphorylation in liver, muscle and white adipose tissue (WAT). As shown in Fig. 4 (upper panels), there was a significant increase in IRS-1 serine phosphorylation in rats pre-treated with GH, which was associated
with a reduction in IRS-1 protein levels (Fig. 4 lower panels) in liver muscle and WAT. In animals pretreated simultaneously with aspirin and GH, aspirin was found to reduce Ser307 phosphorylation by 70–85% and increase IRS-1 protein levels greatly in all tissues.

3.6. Aspirin inhibits JNK activity in GH-treated animals

JNK activation was determined by monitoring phosphorylation of JNK (Thr183 and Tyr185) and c-Jun (Ser63), which is a substrate of JNK. JNK phosphorylation was detected in control animals, however a significant increase was observed in animals pretreated with GH (Fig. 5 upper panels). JNK phosphorylation was reduced by 65–80% in rats pretreated with GH plus aspirin. Consistent with JNK activation, c-Jun phosphorylation was induced by GH treatment and reversed by aspirin in a similar fashion to JNK activity (Fig. 5 middle panels). GH treatment induced SOCS 3 expression in liver, muscle and WAT, however pretreatment with aspirin did not change the modulation in SOCS 3 expression by GH in these tissues.

4. Discussion

Studies in humans and animal models show that chronic GH excess has an anti-insulin effect on carbohydrate and lipid metabolism [6]. In this study, we demonstrate that pretreatment with aspirin inhibits GH-induced insulin resistance. GH was observed to lead to serine phosphorylation of IRS-1 and aspirin reversed this phenomenon in liver, muscle and WAT, in parallel with a reduction in JNK activity. In addition, our data show an impairment in insulin activation of IR, IRS-1 and IRS-2 tyrosine phosphorylation and IRS-1/Pi 3-K and IRS-2/Pi3-K associations in rats chronically treated with GH and, consistent with the reduction of IRS-1 serine phosphorylation observed in animals pretreated with aspirin and GH, there was an increase in IRS-1 protein levels and tyrosine phosphorylation.

There are a number of possible mechanisms that may lead to an impairment of the insulin-signaling pathway in GH-chronically treated rats. Our results show that increased serine phosphorylation of IRS-1 may be one of these mechanisms. Our results also show that GH treatment induced the expression of SOCS3, pointing to another possible mechanism GH-induced insulin resistance. Previous studies demonstrated that overexpression of SOCS3 decreases insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation levels, inducing insulin resistance [22]. However, this modulation of SOCS3 by GH was not reversed by aspirin.

Serine phosphorylation of IRS proteins is believed to be a major mechanism of suppression of IRS-1/2 activity that contributes to insulin resistance [23,24]. Regulation of serine phosphorylation of IR and IRS-1/2 proteins has been one of the focuses in the search for the molecular mechanism of insulin resistance. The reduction in IRS-1 protein levels in the liver, muscle and WAT of GH-treated rats may also contribute to the insulin resistance observed in these animals, since the decrease in IRS-1 tyrosine phosphorylation is probably due to the diminished IRS-1 expression. These reductions in IRS-1 protein expression may be secondary to the increased degradation of these proteins, such as the consequence of increased serine phosphorylation levels [25], and/or may be under transcriptional control. In contrast, the protein levels of IRS-2 remained unaltered in the presence of GH. A recent report demonstrated that hyperinsulinemia mediates reductions of IRS-1/2 protein expression via different mechanisms [26]. It is possible that in GH-induced insulin resistance in animals, which has also hyperinsulinemia, different mechanisms of IRSs degradation may be operating to explain the differences in protein expression observed. Differences in IRS-1 or IRS-2 protein expression may have a role in tissue specific insulin resistance, since IRS-1 has a more important role in mediating insulin signaling in muscle and IRS-2 in liver [27]. However, the insulin induced tyrosine phosphorylation levels of these proteins and interaction with p85 were similarly altered in the liver and muscle of GH-treated animals. In accordance, functional studies demonstrated that elevated GH levels induces insulin resistance in liver and muscle [28,29].

Three recent studies provide clear evidence that aspirin promotes peripheral glucose disposal by enhancing insulin sensitivity [21,30,31]. In these studies, aspirin was found to increase insulin-stimulated glucose uptake and to reduce serine phosphorylation of IRS-1. Our results show a marked reduction in IRS-1 serine phosphorylation after aspirin treatment in GH-treated rats in parallel with an increase in IR autophosphorylation. A previous study demonstrated that treatment of cultured murine adipocytes with TNF-α induces serine phosphorylation of insulin receptor substrate 1 (IRS-1) and converts IRS-1 into an inhibitor of the IR tyrosine kinase activity in vitro [32]. The IRS-1-mediated inhibition of IR
tyrosine kinase activity could occur by direct or indirect inter-
actions between the IR and IRS-1 [33,34]. Serine-phosphory-
lated IRS-1 might associate with the IR to block the
autophosphorylation reaction, alternatively, serine-phosphory-
lated IRS-1 might act indirectly on the IR through an associ-
atlon with an inhibitor that acts on the IR in a stoichometric
or catalytic fashion [32].

Taken together, these data suggest that GH mediates insulin
resistance, at least in part, by inducing IRS-1 serine phos-
phorylation and decreasing IRS-1/2 tyrosine phosphorylation
and that this effect is inhibited by aspirin. Since the IR/IRS-1/2
pathway is involved in glucose uptake in adipocytes and mus-
cle and glycogen synthesis in liver and muscle we may suggest
that aspirin, by acting on this pathway reverses insulin resis-
tance of animals treated with GH.

In previous studies from our laboratory, GH treatment did
not decrease IR tyrosine phosphorylation in rat liver nor
IRS-1 expression in muscles [7]. The reasons for these discrep-
ancies are not clear, but may be related to methodological dif-
fences in the duration of GH treatment.

JNK is a serine kinase that is responsible for activation of
the transcription factors, c-Jun and ATF2, by phosphorylating
these two proteins [35,36]. Recently, JNK has been linked to
the regulation of insulin signaling by several studies
[9,12,15,37,38]. It is suggested that JNK contributes to insulin
resistance by phosphorylating IRS-1 at serine 307, and this
phosphorylation leads to inhibition of the IRS-1 function
[9,15,37,38]. It has recently been observed that aspirin inhibits
JNK activity [17,18]. In this study, we observed that aspirin
inhibited GH-induced JNK activity, and that this inhibition
was accompanied by a reduction in IRS-1 serine phosphoryla-
tion at Ser307, indicating that this serine kinase is one of the
causes of GH-induced insulin resistance.

In summary, aspirin pretreatment improves insulin sensiti-
ity in GH-treated rats by reversing GH-induced decreases in
insulin-stimulated IR, IRS-1 and IRS-2 tyrosine phosphoryla-
tion, and also IRS-1 protein expression. The effect of aspirin
on insulin action is further supported by our findings that rats
pretreated with aspirin show a reduction in IRS-1 serine phos-
phorylation and are also protected from GH-induced insulin
resistance. Overall, these results provide important new
insights into the mechanism of GH-induced insulin resistance.

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