Periodontitis modulates insulin signaling, c-Jun N-terminal kinase activity, IKK activity, and insulin receptor substrate-1 ser 307 phosphorylation in muscle and liver or Wistar rats

A influência da periodontite na via de sinalização da insulina, na ativação da JNK e da IKK e na fosforilação em serina 307 do IRS-1, em ratos machos Wistar

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Objective – To evaluate the effect of periodontal disease in insulin sensitivity in the modulation of proteins of the insulin signaling pathway such as IR, IRS-1, IRS-2, Akt, ERK and activation of the JNK and IKK in liver and muscle of rats. Methods – In this study, we evaluated the regulation of insulin signaling, JNK and IKK activation and IRS-1ser307 phosphorylation by immunoprecipitation and immunoblotting in the liver and muscle of rats with periodontitis induced by ligature for 28 days. Results – Chronic inflammatory periodontal disease (periodontitis) leads to gingival inflammation, destruction of periodontal tissues and loss of alveolar bone. Periodontitis increased blood glucose and plasma insulin levels during the OGTT, and reduced the glucose disappearance rate in the insulin tolerance test, characterized a state of insulin resistance. In periodontitis rats we observed a reduction in insulin-induced IR, IRS-1, IRS-2, Akt phosphorylation in liver and muscle, compared to controls. JNK and IKK activity and IRS-1ser307 phosphorylation were higher in the tissues of rats with periodontitis. Conclusions – In summary, the insulin resistance, induced by periodontitis, is accompanied by activation of the JNK and IKK pathways and IRS-1ser307 phosphorylation. The impairment of the insulin signaling in these tissues may lead to insulin resistance in periodontitis.

Descriptors: Insulin resistance; Periodontitis; Insulin

Resumo

Objetivo – Avaliar o efeito da doença periodontal na sensibilidade à insulina na modulação das proteínas da via de sinalização da insulina como o IR, IRS-1, IRS-2, Akt, ERK e a ativação da JNK e da IKK em fígado e músculo de ratos. Métodos – Neste estudo, avaliou-se a regulação da sinalização de insulina, ativação da JNK e IKK e fosforilação do IRS-1ser307 por imunoprecipitação e immunoblotting no fígado e músculo de ratos com periodontite induzida por ligadura durante 28 dias. Resultados – A doença inflamatória crônica periodontal (periodontite) leva à inflamação gengival, destruição dos tecidos periodontais e perda óssea alveolar. A periodontite aumentou os níveis de glicose no sangue e os níveis de insulina no plasma durante o OGTT, e reduziu o taxo de desaparecimento da glicose no teste de tolerância à insulina, caracterizando um estado de resistência à insulina. Em ratos com periodontite observou-se uma redução da fosforilação induzida por insulina nas proteínas IR, IRS-1, IRS-2, Akt no fígado e no músculo, em comparação com o grupo controle. As atividades das proteínas JNK e IKK e na fosforilação em serina 307 do IRS-1 foram mais elevadas nos tecidos dos ratos com periodontite. Conclusão – A resistência à insulina induzida pela periodontite, é acompanhada por ativação das vias JNK e IKK e na fosforilação em serina 307 do IRS-1. Uma alteração da sinalização de insulina nestes tecidos pode levar à resistência à insulina em animais com periodontite.

Descritores: Resistência à insulina; Periodontite; Insulina

Introduction

Many studies provide evidence that inflammatory states may induce a chronic state of insulin resistance, even if subjects have not previously had diabetes². Chronic inflammatory periodontal disease [periodontitis] represents a primarily anaerobic Gram-negative oral infection that leads to gingival inflammation, destruction of periodontal tissues, loss of alveolar bone and eventual exfoliation of teeth in severe cases³₂⁹. Recent studies have indicated that periodontitis may produce alterations in systemic health and severe periodontal disease often coexists with severe diabetes mellitus²⁹. It is possible that periodontal disease may aggravate diabetes because periodontal infection may induce a state of insulin resistance⁴. However, the molecular mechanism by which periodontitis induces insulin resistance has not yet been investigated. At the molecular level, insulin signaling initiates when activation of the IR results in tyrosine phosphorylation of IRS proteins. Phosphorylated IRSs then bind proteins containing Src homology 2 domains, such as the p85 regulatory subunit of phosphatidylinositol 3 kinase [PI-3K]⁵. Downstream from PI-3K, the serine/threonine kinase Akt triggers insulin effects on the liver, such as glycogen synthesis and the suppression of hepatic glucose production. Akt on the muscle effects glycogen synthesis and migration of GLUT 4 to the cellular membrane.

In addition, a second postreceptor insulin signaling pathway involves the phosphorylation of Shc and activation of Ras, Raf, MEK and mitogen-activated protein
vator protein-1 (AP-1) proteins, including c-Jun and JNK-1, -2, and -3, belong to the MAPK family and regulate multiple activities in cellular signaling and JNK and NFκB inhibition of insulin signaling, including JNK and inhibitor of NFκB kinase (IKKβ). The 3 members of the JNK group of serine/threonine kinases, JNK-1, -2, and -3, belong to the MAPK family and regulate multiple activities in development and cell function, mainly through their ability to control transcription by phosphorylating activator protein-1 (AP-1) proteins, including c-Jun and JunB. JNK has recently emerged as a central metabolic regulator, playing an important role in the development of insulin resistance in obesity. In response to stimuli such as stress, cytokines, and fatty acids, JNK is activated, where upon it associates with and phosphorylates IRS-1 on Ser307, impairing insulin action. IKKβ can affect insulin signaling through at least 2 pathways. Firstly, it can directly phosphorylate IRS-1 on serine residues. Secondly, it can phosphorylate inhibitor of NFκB, thus activating NFκB, a transcription factor that, among other targets, stimulates production of multiple inflammatory mediators, including TNF and IL-6. Mice heterozygous for IKKβ are partially protected against insulin resistance due to lipid infusion, high-fat diet, or genetic obesity.

It’s known that periodontitis increases production of TNFα and interleukins, which have been reported to lower insulin sensitivity, probably through activation of JNK and NFκB pathways. In this study, we evaluated the effect of periodontitis on insulin sensitivity, insulin signaling and JNK and NFκB activation in the liver and muscle of male Wistar rats.

Methods

Materials

Male Wistar rats were provided by the State University of Campinas Central Breeding Center [Campinas, Brazil]. Anti-pY, anti-iR3, anti-IRS-1, anti-IRS-2, anti-Akt1/2, anti-phospho-JNK, and anti-JNK1, anti-IκB-α, anti-phospho-ERK, anti-ERK1/2 and anti-IKKβ antibodies were from Santa Cruz Technology [Santa Cruz, CA]. Anti-phospho-Akt was from Cell Signaling Technology [Beverly, MA]. Anti-phospho-IRS-1 was obtained from Upstate Biotechnology, Inc. [Lake Placid, NY]. Human recombinant insulin was from Eli Lilly and Co. [Indianapolis, IN]. Routine reagents were purchased from Sigma Chemical Co. [St. Louis, MO] unless specified elsewhere. [125I]Protein A was obtained from Amersham [Amersham Biosciences Group, Little Chalfont, UK].

Experimental animals

Six-week-old male Wistar rats were divided into two groups with similar body weights [156 ± 4 g], control group and periodontitis group and both were assigned to receive a standard rodent chow and water ad libitum.

Protocol of experimental periodontal disease

Animals were subjected to experimental periodontitis. After general anesthesia with intramuscular injection of 0.05ml/100g body weight of ketamine [Francotar®, Virbac do Brazil Ind. e Com. Ltda, São Paulo, Brazil] and 0.1ml/100g body weight of Diazepam, a cotton thread ligature was surgically placed around the cervix of the mandibular first molar on the left side. The ligature was knotted on the vestibular side, so that it remained subgingivally in the palatinal side.

Radiographic analysis

The mandibles were carefully removed, and soaked in 4% paraformaldehyde for 48h. Then, in order to measure the amount of bone, standardized digital radiographs were obtained with the use of a computerized imaging system, CDR® [Francotar®]. Electronic sensors were exposed at 65KV and 10mA. The source-to-film distance was always set at 50cm. The amount of alveolar bone loss, expressed by the distance from the alveolar bone crest to the cemento-enamel junction, was measured [in mm] three times, in different days and by the same examiner, for each mesial surface of the mandibular first molars on each radiograph.

Experimental animals and research protocols

Six-week-old male Wistar rats [R. norvegicus] from the University of Campinas Central Animal Breeding Center were used in the experiments. The rats were allowed ad libitum access to standard rodent chow and water. Food was withdrawn 12 h before the experiments. All experiments were conducted in accordance with the principles and procedures described by the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and were approved by the State University of Campinas Ethical Committee.

Glucose tolerance test (GTT)

Oral GTT was performed, after 25 days of ligature-induction. Food was withdrawn 6 h before the test and the rats were anesthetized. An unchallenged sample was collected (time 0), and then a solution of 25% glucose (1.75g/kg body weight) was administered by gavage. Blood samples were collected from the tail at 15, 30, 60, 90, and 120 min for determination of glucose and insulin concentrations.

Insulin tolerance test (ITT)

To perform an intraperitoneal ITT, food was with-
drawn 6h before the test and the rats were anesthetized. An unchallenged sample was collected (time 0), and then insulin (150U/kg body weight) was injected into the peritoneal cavity, and blood samples were collected at 5, 10, 15, 20, 25 and 30 min from the tail for serum glucose determination. The constant rate for glucose disappearance (Kitt) was calculated using the formula, $0.693/t^{1/2}$. Glucose half-time $t^{1/2}$ was calculated from the slope of the least-square analysis of plasma glucose concentrations during the linear decay phase. \[19\]

**Tissue extraction and immunoprecipitation**

Rats 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 0.2ml normal saline with or without insulin (200μg) were injected. At 30 sec after the insulin injection, the liver was removed, and 90 sec later, muscle was removed, minced coarsely, and homogenized immediately in extraction buffer, as described elsewhere. Extracts were then centrifuged at 11,000 rpm and 4°C for 20 min to remove insoluble material, and the supernatants were used for immunoprecipitation with α-IR, α-IRS-1 and -2, and protein A-Sepharose 6MB (Pharmacia, Uppsala, Sweden).

**Protein analysis by immunoblotting**

The precipitated proteins and/or whole-tissue extracts were treated with Laemmli sample buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 5 min, after which they were subjected to SDS-PAGE in a Bio-Rad miniature slab gel apparatus (Mini-Protean). For total extracts, 250μg of proteins 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-Protean transfer apparatus. Nonspecific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h in blocking buffer (5% non-fat dry milk, 10mM Tris, 150mM NaCl, 0.02% Tween 20). The nitrocellulose blot was incubated with specific antibodies overnight at 4°C and then incubated with $^{125}$I-labeled protein A. The results were visualized by autoradiography with preflashed Kodak XAR film. Band intensities were quantified by optical densitometry (Hoefer Scientific Instruments, San Francisco, CA; model GS300).

**Statistical analysis**

Experiments were always carried out by studying the groups of animals in parallel (control animals vs. periodontitis animals). Data are means ± SEM accompanied by the indicated number of independent experiments. For comparisons, one-way ANOVA was used; where F ratios were significant, further comparisons were made using the Bonferroni test. The significance level was set at a p value of less than 0.05.

**Results**

**Animal characteristics**

The body weights were similar in the two groups (c: 235±36g vs p: 241±23g). The periodontitis rats demonstrated an increase in the area of blood glucose (c: 12899±11,7mg/dL.min vs p: 14873±9,9mg/dL.min p<0.05) (Fig. 1A, 1B). Animals with periodontitis were more insulin resistant than the control rats, as expressed by their higher area of serum insulin concentrations (c: 31.4±0,08 vs p: 72.3±0,13ng/ml.min p<0.05) (Fig. 2A, 2B) and lower plasma glucose disappearance rates measured by the thirty minute insulin tolerance test (Kitt c: 4.88±0.56%/min vs Kitt p: 2.17±0.38%/min, n=5 each, p=0.05) (Fig. 3). At 28 days of ligature, this group showed significant increases in mean bone loss when compared with the group control (c: 0.016±0.001 vs p: 0.032±0.001, p<0.0001) (Fig. 4A,4B).

**Figure 1A.** Graph of glycemia obtained from the glucose tolerance test in Wistar rats. Glycemia mean ± SEM, n = 5.

**Figure 1B.** Incremental area under curve (IAUC) of glycemia obtained from the glucose tolerance test. AREA ± SEM, *p<0.05,n=5.
Table 1. Characteristics of ligature and control groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Area of blood glucose (mg/dl.min)</th>
<th>Area of serum insulin (ng/ml.min)</th>
<th>Kitt (%/min)</th>
<th>Mean alveolar bone loss (mm)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>235.2±36.82</td>
<td>12898.5±11.7</td>
<td>31.37±0.08</td>
<td>4.88±0.56</td>
<td>0.016±0.001</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>241.2±23.40</td>
<td>14873.4±9.9</td>
<td>72.30±0.13</td>
<td>2.17±0.38</td>
<td>0.032±0.001</td>
</tr>
</tbody>
</table>

Data are given as means ± SEM. p values of less than 0.05 were considered to be significantly different.

ap<0.0001 vs young controls – b p<0.05 vs young controls

Figure 2A. Graph of serum insulin obtained from the glucose tolerance test in Wistar rats. Serum insulin mean ± SEM, n=5.

Figure 2B. Incremental area under curve (IAUC) of serum insulin concentrations obtained from the glucose tolerance test. Area ± SEM, p<0.05, n=5.

Figure 3. Plasma glucose disappearance rates (kITT) measured by the thirty minute insulin tolerance test (ITT). Area ± SEM, p<0.05, n=5.

Insulin signalling in skeletal muscle from control and animals with periodontitis

There was no difference in the IRβ protein expression in the muscle of control and periodontitis rats (Fig. 5B). Animals with periodontitis showed a significantly reduced insulin-stimulated IR tyrosine phosphorylation in muscle when compared with the control group (p: 85±7% vs c: 100±4%, n=5; Fig. 5A).

There was no difference in the IRS-1 protein expression in the muscle of control and periodontitis rats (Fig. 5D). Animals with periodontitis showed a significantly reduced insulin-stimulated IRS-1 tyrosine phosphorylation in muscle when compared with the control group (Fig. 5C, c: 100±5% vs p: 68±2%, n=5, p<0.05).

There was no difference in the IRS-2 protein expression in the muscle of control and periodontitis rats (Fig. 5F). Animals with periodontitis showed a significantly reduced insulin-stimulated IRS-2 tyrosine phosphorylation in muscle when compared with the control group.
Insulin-stimulated Akt serine phosphorylation was significantly decreased in animals with periodontitis when compared with the control group (c: 100±4% vs p: 66±1%, n=5, p<0.05; Fig. 5G). Akt protein levels did not differ among the groups in this study (Fig. 5H).

Insulin-stimulated ERK1/2 tyrosine phosphorylation showed a significant decrease in muscle from animals with periodontitis when compared with the control group (c: 100±4% vs p: 80±3%, n=5; Fig. 5I). ERK 1/2 protein levels did not differ among the groups in this study (Fig. 5J).

Figure 5 (A-K). Evaluation of liver phosphorylation expression of IR, IRS-1, IRS-2, p-Akt, P-ERK, Ser-307, p-JNK, and IκBα by immunoblot (n=5;*, P<0.05 vs. respective control).

Figure 6 (A-K). Evaluation of liver phosphorylation expression of IR, IRS-1, IRS-2, p-AKT, P-ERK, SER-307, P-JNK, and IκBα by immunoblot (n=5;*, P<0.05 vs. respective control).
IRS-1 serine 307 phosphorylation in skeletal muscle from animals with periodontitis demonstrated a significant increase when compared with control group (c: 100±8% vs p: 160±3%, n=5, p<0.05; Fig. 5K).

JNK ½ phosphorylation was significantly increased in muscle from animals with periodontitis when compared with the control group (p: 140±1% vs c: 100±1%, n=5; Fig. 5L).

The expression of IkB-α was reduced in muscle in the periodontitis group when compared with the control group (p: 30±5% vs c: 100±1%, n=5; Fig. 5M).

Insulin signalling in liver from ligation rats and control animals

There was no difference in the IRβ protein expression in the liver of control and periodontitis rats (Fig. 6B). Animals with periodontitis showed a significantly reduced insulin-stimulated IR tyrosine phosphorylation in liver when compared with the control group (p: 38±3% vs c: 100±5%, n=5; Fig. 6A).

There was no difference in the IRS-1 protein expression in the liver of control and periodontitis rats (Fig. 6D). Animals with periodontitis showed a significantly reduced insulin-stimulated IRS-1 tyrosine phosphorylation in liver when compared with the control group (c: 100±4% vs p: 55±1%, n=5; Fig. 6C).

There was no difference in the IRS-2 protein expression in the liver of control and periodontitis rats (Fig. 6F). Animals with periodontitis demonstrated a significantly reduced insulin-stimulated IRS-2 tyrosine phosphorylation in muscle when compared with control group animals (c: 100±2% vs p: 45±1%, n=5; Fig. 6E).

Insulin-stimulated Akt serine phosphorylation was significantly decreased in animals with periodontitis when compared with the control group (c: 100±8% vs p: 69±5%, n=5, p<0.05, Fig. 6G). Akt protein levels did not differ among the groups in this study (Fig. 6H).

Insulin-stimulated ERK1/2 tyrosine phosphorylation was significantly decreased in liver of animals with periodontitis when compared with the control group (c: 100±8% vs p: 78±4%, n=5, Fig. 6I). ERK 1/2 protein levels did not differ among the groups in this study (Fig. 6J).

IRS-1 serine 307 phosphorylation in liver from animals with periodontitis showed a significant increase when compared with the control group (c: 100±8% vs p: 140±12%, n=5, p<0.05). (Fig. 6K, n=5).

JNK ½ phosphorylation showed a significant increase in liver from animals with periodontitis when compared with the control group (p: 150±3% vs c: 100±2%, n=5; Fig. 6L).

The expression of IkB-α was reduced in the liver of the periodontitis group when compared with the control group (p: 30±5% vs c: 100±1%, n=5; Fig. 6M).

Discussion

Chronic inflammatory periodontal disease (periodontitis) represents a primarily anaerobic gram-negative oral infection that leads to gingival inflammation, destruction of periodontal tissues and loss of alveolar bone. In the present study, we induced periodontitis in rats and showed that these animals developed a state of insulin resistance, characterized by altered insulin signaling in liver and muscle.

We induced periodontitis in rats through a cotton ligature in the lower left first molar to induce this experimental disease. The periodontitis was characterized by a loss of alveolar bone. Animals with periodontitis demonstrated metabolic characteristics of insulin resistance. They presented higher insulin levels during the OGGT and reduced glucose disappearance rate in the ITT, characterizing a state of reduced insulin sensitivity. The mechanism underlying this insulin resistance is not fully understood, thus, in the present study we evaluated the in vivo insulin stimulated tyrosine phosphorylation of the insulin receptor, IRS-1, IRS-2 and Akt in the liver and muscle of rats with periodontitis.

In rats with periodontitis there was a decrease in insulin-induced IRS-1 and IRS-2 tyrosine phosphorylation accompanied by a decrease inAkt phosphorylation in liver and muscle. These findings may be of biological significance since IRS-1/2 /Akt pathway is linked to activation of glucose transport in muscle and glycogen synthesis in liver and muscle. A reduction in the activation of this pathway in rats with periodontitis may have a role in insulin resistance in these animals.

There are a number of possible mechanisms that may lead to an impairment of the insulin-signaling pathway in rats with periodontal disease. Our results show that increased serine phosphorylation of IRS-1 may be one of these mechanisms. Serine phosphorylation of IRS proteins is believed to be a major mechanism of suppression of IRS-1 activity that contributes to insulin resistance. Regulation of serine phosphorylation of IRS proteins has been one of the fields of interest in the search for the molecular mechanism of insulin resistance that may occur, at least in part, by inducing IRS-1 serine phosphorylation.

JNK and IKKβ are serine kinases that mediate interleukin and TNF-α signaling. Recently, these kinases have been linked to the regulation of insulin signaling by several studies. It is suggested that JNK and IKKβ may contribute to insulin resistance by phosphorylating IRS-1 at serine 307, and that this phosphorylation leads to the inhibition of the IRS-1 function. In this study, we observed that JNK and IKKβ were activated in animals with periodontitis suggesting a possible mechanism for the observed increase in IRS-1 serine phosphorylation at Ser307, indicating that these serine kinases may have a role in periodontitis-induced insulin resistance.

Based on results from a previous study and those presented here, our results demonstrated that ligation-induced periodontitis is associated with insulin resistance and downregulation of the IR/IRS/Akt pathway in the liver and muscle, accompanied by an increase in JNK and IKKβ activation and in IRS-1 serine phosphorylation at Ser307, suggesting a possible molecular mechanism for the reduced insulin insulin sensitivity.
Conclusion

In summary the insulin resistance, induced by periodontitis, is accompanied by activation of the INK and IKK pathways and IRS-1 ser 307 phosphorylation. The impairment of the insulin signaling in these tissues may lead to insulin resistance in periodontitis.

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References