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Submandibular Gland and Caries Susceptibility in the Obese Zucker Rat

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Abstract

Background—Obesity is a prevalent disorder characterized as marked insulin resistance and low grade inflammation. We tested the hypothesis that obesity upregulates inflammatory markers in the submandibular gland in association with derangements of its architecture and predisposition to caries in obese Zucker rats. We also examined the potential impact of chromium picolinate (Cr(Pic)3), a nutritional supplement suggested to improve glycemic control, on the aforementioned parameters.

Design—Male obese Zucker rats (OZR) were treated with diets lacking and containing 5 or 10 mg/kg chromium (as Cr(Pic)3) from 6 weeks to about 6 months of age; lean Zucker rats (LZR) served as controls. Thereafter, glycemic status, salivary tissue architecture and levels of several inflammatory markers were determined in association with caries susceptibility.

Results—OZR showed reduced insulin sensitivity, increased ratio of phospho-nuclear factor kappa B (NF- κ B) to total NF- κ B and increased intercellular adhesion molecule-1 level but similar histological features compared to LZR. Importantly, compared to LZR, OZR displayed rampant caries and a tendency for reduced dentin mineral density. Treatment of OZR with Cr(Pic)3

attenuated upregulation of these proinflammatory indicators in association with reduced severity of caries without improving insulin sensitivity.

Conclusions—Obesity promotes proinflammatory changes within the submandibular gland, without affecting glandular architecture, in association with rampant caries; Cr(Pic)3 treatment provided some protective effects.

Keywords

Zucker rat; Submandibular gland; NF- κ B; Cell adhesion molecules; Caries; Chromium picolinate

Introduction

Obesity increases the risk for development of systemic disorders such as type 2 diabetes and cardiovascular diseases (1-2). Surprisingly, however, information on the impact of obesity on oral health is sparse. Since obesity represents a state of low grade inflammation (3-4), it could potentially influence oral health as exemplified by the close correlation of obesity with periodontal disease and other chronic inflammatory diseases (e.g., type 2 diabetes) (2,5-6). Indeed, reduction in salivary flow rate and increased caries susceptibility have been reported for the Prader-Willi Syndrome (PWS); PWS is an example of a monogenic disorder (i.e., abnormalities of chromosome 15) resulting in marked obesity (7). Given the limitations of clinical studies, the impact of obesity on oral health in relation to salivary glands can be better established with the use of appropriate animal models of the disease.

The obese Zucker rat lacks the leptin receptor due to an autosomal recessive mutation of the *fa*-gene. As a result, the animal is hyperphagic and becomes markedly obese compared to its lean control. Other characteristic features of the obese Zucker rat are peripheral insulin resistance, hyperinsulinemia and glucose intolerance (8-9). Utilizing this animal model, we tested the hypothesis that obesity causes derangements of the submandibular gland architecture in associated with increased expression of the key proinflammatory transcription factor, nuclear factor kappa B (NF- κ B), and its downstream mediators (e.g., vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). These studies were carried out in the context of assessment of carious lesions as a surrogate marker of oral health status. In addition, we also examined the impact of chronic chromium picolinate (Cr(Pic)3) treatment of the obese Zucker rat on aforementioned parameters. This nutritional supplement has gained popularity because of its purported beneficial influence on glycemic control (10-12). Further, a recent study suggests that Cr(Pic)3 attenuates plasma levels of several inflammatory markers (13). Accordingly, we postulated that chronic Cr(Pic)3 treatment will reduce insulin resistance and the expression of proinflammatory factors in association with preservation of salivary gland architecture and reduced caries susceptibility.

Materials and Methods

Six-week-old male lean and obese Zucker rats (i.e., LZR and OZR, respectively) were obtained from Harlan Laboratories (Indianapolis, Indiana) and were fed the regular non-cariogenic rodent diet (Harlan Teklad diet 8604). Additional OZR were fed Cr(Pic)3-enriched 8604 diet providing chromium at a dose of either 5 or 10 mg/kg diet (i.e., OZR; 5 Cr and OZR; 10 Cr; Harlan Teklad diet numbers 07602 and 07603, respectively) (14-16), providing chromium at a dose of 0.19 and 0.41 mg/kg/day, respectively. The animals (n=7-9 animals/group) had free access to food and water throughout the study. The use of animals in this study was in accordance with the NIH guide on Humane Treatment of Laboratory Animals.

At about 6 months of age, fasting plasma glucose (Beckman Glucose Analyzer) and insulin (^{125}I radio- immunoassay Kit; MP Biomedicals, LLC; Solon, OH) were measured and used to calculate the quantitative insulin sensitivity check index (QUICKI) as follows: $1/[\log \text{fasting insulin } (\mu\text{U/ml}) + \log \text{fasting glycemia } (\text{mg/dl})]$ (17). Thereafter, the animals were sacrificed and the submandibular glands procured. The right submandibular gland was immediately frozen in liquid nitrogen for Western blot analysis; the left submandibular gland was placed in buffered formalin for histopathological examination as described previously (16,18).

For Western blotting, the salivary gland tissue was pulverized and placed in $1\times$ RIPA Buffer (Temecula, CA) composed of 10 $\mu\text{l/ml}$ Protease Inhibitor Cocktail (Sigma-Aldrich), 20 mM of sodium orthovanadate, 10mM of sodium fluoride, 1mM of sodium pyrophosphate tetra-basic decahydrate. Protein concentration was measured using Bio-Rad DC protein assay kit. Standard protocols were used for Western blot analysis as described previously (19), utilizing primary antibodies against NF- κB , phospho NF- κB , VCAM-1 (rabbit polyclonal, Santa Cruz, Ca) and ICAM-1 (goat IgG, R&D Systems, Minneapolis, MN); secondary antibodies included goat anti-rabbit (Cell Signaling) and donkey anti-goat (Santa Cruz, CA); protein levels were corrected for β -actin and expressed as percent of the LZR group.

For caries detection, five hemi-mandibles of each group was dissected and clinically examined for existence of coronal cavities. The hemi-mandibles were then scanned in a micro- computerized tomography system (micro-CT; Skyscan 1172; Skyscan, Aartlesaar, Belgium) at an image pixel size of 30 μm . Reconstruction was done using a Skyscan Nrecon program. Data sets were loaded into Skyscan CT-analyzer software for measurement of mineral density of dentin and evaluation of radiological evidence of carious lesions; the average of three standardized regions of interest, one at each molar tooth, were measured as well as the average of two regions of interest in the a recognized coronal cavity (20-21).

Statistics

Data were analyzed by the analysis of variance (ANOVA). Duncan's post-hoc test was used to establish significance between mean values ($p<0.05$). All data are reported as means $\pm$ SEM.

Results

Effects of Obesity

The OZR gained more weight than their lean counterparts (Figure 1A). Salivary gland tissue weight was lower in the OZR than the LZR (Figure 1B). The OZR displayed marked elevation in plasma insulin concentration accompanied by a mild increase in plasma glucose level compared to the LZR group [(insulin: 584.5 ± 31.8 vs. 148.5 ± 10.1 pmol/L; $p<0.05$) and (glucose: 9.2 ± 0.4 vs. 7.3 ± 0.3 mmol/L; $p<0.05$)]. As a result, the QUICKI value, an index of insulin sensitivity, was significantly lower for the OZR than the LZR (Figure 1C).

Histologic evaluation of hematoxylin-eosin (H&E)-stained tissue revealed well-preserved lobular morphology with intact, uniform aggregates of parenchymal secretory units interspersed by evenly-spaced intercalated ducts in both LZR and OZR groups (Figure 2, panels A-B). Cytologic and nuclear details were uniform in shape and size and staining characteristics. Trichrome-stained tissue sections showed normal lobular architecture without intralobular fibrosis or collagen deposition (Figure 2, panels E-F). Surrounding some of the striated and excretory ducts, thin perilobular fibrous tissue is noted. Tissue sections stained with Oil-Red-O revealed, few scattered positive intra- and extra-cellular fatty droplet deposits in LZR; the OZR showed more fatty droplets per same unit area of examination (Figure 2, panels I-J).

Figure 3 shows expression profile of proinflammatory mediators in salivary tissue of experimental animals. The phospho-NF- κ B/total NF- κ B ratio was greater in the OZR than LZR (Figure 3A). Similarly, ICAM-1 level was greater in salivary tissue of the OZR than their lean counterparts (Figure 3B). However, VCAM-1 expression was not affected (Figure 3C).

On clinical examination, the LZR showed minimal or no caries (Figure 4A) while the untreated OZR group showed rampant caries ranging from simple class one cavity to badly decayed teeth involving the entire crown (Figure 4B). Micro CT shows no signs of coronal cavities in the LZR group (Figure 4E) as opposed to the OZR group with clear evidence of carious lesions (Figure 4F). Also, three dimensional reconstructs showed evidence of decay at various stages in OZR compared to LZR as evidenced by badly decayed teeth (Figure 4J vs. 4I).

Dentin mineral density (g/cm^3) was lower, albeit non-significantly, in the OZR group compared to the LZR group (0.56 ± 0.06 vs. 0.66 ± 0.05).

Effects of Cr(Pic) treatment

Treatment of OZR with the nutritional supplement, Cr(Pic)3, did not affect body weight or salivary gland weight compared to their untreated counterparts (Figure 1A-B). Also, plasma glucose concentration (9.7 ± 0.4 and 9.8 ± 0.3 mmol/L for OZR; 5 Cr and OZR; 10 Cr, respectively), plasma insulin concentration (508.9 ± 45.6 and 555.6 ± 69.0 pmol/L for OZR; 5 Cr and OZR; 10 Cr groups, respectively) or the QUICKI value were similar among the two Cr(Pic)3-treated OZR groups and the untreated OZR group (Figure 1C and above, under effects of obesity).

The Cr(Pic)3-treated OZR showed histological features generally resembling their untreated counterparts (Figure 2). Nonetheless, tissue sections from OZR; 10 Cr showed a benign expansion of the secretory acinar units, spacing out the ductal structures (Figure 2D), but with preserved lobular morphology and glandular microanatomy. The proliferative acinar units were uniform with respect to cellular and nuclear details.

A noted finding was the observation that dietary Cr(Pic)3 treatment of the OZR was associated with reversal of the upregulation of the phospho-NF- κ B/total NF- κ B ratio and ICAM-1 (Figure 3A-B). Also noteworthy was the finding that Cr(Pic)3-treated OZR groups showed carious lesions that were intermediate of those noted for LZR and untreated OZR (Figure 4). However, dentin mineral density (g/cm^3) was similar between the Cr(Pic)3-treated OZR [(OZR; 5 Cr: 0.61 ± 0.05) and (OZR; 10 Cr: 0.60 ± 0.10)] and their untreated counterparts (0.56 ± 0.06).

Discussion

Effects of Obesity

This study shows upregulation of a key proinflammatory transcription factor, NF- κ B, and increased expression of ICAM-1 in the submandibular gland of the OZR than LZR. However, histological features of the salivary tissue were generally similar between the OZR compared to the LZR control group. Importantly, compared to LZR, molar teeth of OZR displayed prominent carious lesions. To our knowledge, this is the first study that has examined the impact of obesity on salivary gland structure in the context of key inflammatory mediators and caries susceptibility.

Adipose tissue is now considered as a major endocrine organ producing a myriad of proinflammatory factors, among others. The association of obesity with inflammation was

first proposed by the seminal studies of Hotamisligi and colleagues which established tumor necrosis factor- α (TNF- α) as a key factor linking inflammation, obesity and insulin resistance (3). Subsequent studies demonstrated that binding of TNF- α with the TNF receptor results in mobilization of complex signaling pathways, one arm of which leads to serine phosphorylation of insulin receptor substrate-1, culminating in inhibition of insulin signaling (22). Another consequence of TNF- α signaling is activation of IKK β /NF- κ B pathways. Aside from being a major intracellular pathway regulating insulin resistance, upregulation of NF- κ B culminates in expression of proinflammatory adhesion molecules such as ICAM-1 and VCAM-1 (23). Thus obesity represents a proinflammatory state with elevations in plasma concentrations of a myriad of inflammatory mediators/markers such as IL-6, C-reactive protein and TNF- α (2,6,23). Interestingly, it is also believed that macrophages which reside within adipose tissue (and derived from blood monocytes) are a source of proinflammatory factors and that they could modulate the secretory capacity of adipocytes. Indeed, the mononuclear cells (derived from monocytes) of obese subjects have been shown to express increased amounts of proinflammatory cytokines and related factors (23). These cells also have increased binding capacity for NF- κ B and increased intracellular expression of p65, the major protein component of NF- κ B (23).

Consistent with our hypothesis that obesity upregulates inflammatory changes, salivary gland of OZR displayed upregulation of NF- κ B in association with increased expression of ICAM-1 compared to their lean counterparts. This finding is in general agreement with other reports indicating that OZR display increased expression of a number of proinflammatory markers in other tissues (e.g., cyclooxygenase-2 in renal microvasculature and whole kidney) (24,16). By contrast, VCAM-1 expression was similar between the OZR and the LZR which may relate to its determination in whole salivary tissue rather than in salivary tissue microvasculature. Nonetheless, despite promotion of an inflammatory state, histopathological examination of H&E-stained salivary gland tissue did not reveal differential changes between OZR and their lean counterparts.

Ectopic lipid accumulation is advanced as a potential mechanism for target organ dysfunction/damage in obesity/diabetes (i.e., lipotoxicity mechanism) (25). Interestingly, however, despite mild differences, Oil-Red-O staining did not reveal marked accumulation of fatty droplets within salivary gland tissue of OZR compared to their lean counterparts. In this context, recent studies have suggested that periorgan fat deposition, as occurs in OZR, can serve as a potential source for locally released chemokines/cytokines with detrimental effects on the involved organ. Indeed, an adverse effect of pericardiac fat deposition has been suggested to play a major pathogenic role in cardiac dysfunction of fat-fed rats (26). It remains to be established whether a similar mechanism contributes to abnormalities of salivary gland (e.g., encapsulated organs) in obesity.

The most noted finding of the study relates to rampant caries in the OZR than LZR. Clearly, caries development is the culmination of the interactions among host factors, microorganisms, substrate and tooth surface over time. Since LZR and OZR were provided with the same noncariogenic diet, it is unlikely that dietary-related factors contributed to the increased caries in the OZR. Further, to our knowledge, there is no information available regarding the status of oral microflora or salivary gland secretory products in OZR which precludes attributing their differential contributions to caries susceptibility in this animal model. Nonetheless, it is tempting to speculate that chronic mild-moderate upregulation of proinflammatory NF- κ B signaling on salivary gland could adversely impact salivary function thereby predisposing to increased caries susceptibility. In addition, the smaller salivary gland size could also adversely impact the contribution of salivary secretions to protection against caries. This contention is supported by several lines of evidence. First, the size of salivary gland is a determinant of the salivary flow and protein secretion rates under

baseline, unstimulated, condition (27). Second, a significant correlation exists between demineralization parameters (mineral loss, lesion depth and mineral distributions) in experimental root caries and unstimulated saliva flow rate (28). Indeed, dentin mineral density of molar teeth tended to be lower for OZR than LZR, an aspect that may also relate, in part, to the progression of carious lesion into dentin. Third, development of carious lesions is negatively related to the unstimulated saliva flow rate (29). In addition, the abnormal glycemic status of the OZR could also contribute to caries susceptibility. Indeed, previous studies suggest that the concentration of glucose in parotid saliva is elevated after glucose/food intake in individuals with glucose intolerance and diabetes mellitus (30). This is an important consideration since a direct relation has been established between salivary glucose concentration and decayed-missing-filled surface scores in type 1 diabetic children (31). Thus, while acknowledging differences in composition of human and rodent saliva (32), the obese Zucker rat may prove to be a valuable animal model for investigation of the impact and mechanisms of obesity/type 2 diabetes on oral health.

Effects of Cr(Pic)3 treatment

Another notable finding of this study relates to the lack of a beneficial influence of chronic Cr(Pic)3 treatment on body weight or insulin sensitivity of OZR; the chromium intake of animals in this study was several fold higher than those consumed by human subjects (14,16). Nonetheless, the treatment was associated with attenuated upregulation of both NF- κ B and ICAM-1 thereby suggesting an uncoupling between the effect of the formulation on glycemic control and inflammatory markers (33). In this context, it is noteworthy that a recent study shows downregulation of inflammatory markers such as IL-6 in plasma of Cr(Pic)3-treated rats (13). Interestingly, the Cr(Pic)3-induced attenuation of inflammatory markers was associated with reduced severity of carious lesions in OZR although the relevance and importance of this association remains to be established.

Conclusion—While the relation between changes in salivary gland inflammatory markers and presence of caries lesions in the OZR remains to be established, our collective observations are suggestive of a major adverse impact of obesity on oral health. Interestingly, the nutritional supplement, Cr(Pic)3, reversed upregulation of submandibular gland inflammatory markers in association with reduction in the severity of caries without influencing either body weight or glycemic control in OZR.

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Figure 1.

Bar graphs show body weight (A), submandibular gland tissue weight (B) and QUICKI values (C) of lean Zucker rats (LZR), obese Zucker rats (OZR) and OZR treated with chromium picolinate-enriched diets providing chromium at 5 and 10 mg/kg of diet (OZR; 5 Cr and OZR; 10 Cr, respectively). Data are average $\pm$ SEM of 7-9 animals/group.

*p<0.05 compared to other groups.

Figure 2.

Panels show representative hematoxylin-eosin stained (A-D; $\times 100$), Masson's trichrome-stained (E-H; $\times 100$) and Oil-Red O-stained (I-L, $\times 200$) sections of the submandibular glands from experimental groups. Arrows point to oil droplets in panels I-L.

Figure 3.

Bar graphs show the phospho-NF-κB/total NF-κB ratio (A), levels of ICAM-1 (B) and VCAM-1 (C) in the submandibular gland of experimental groups as described in Figure 1; the ICAM-1 and VCAM-1 values are normalized to β-actin and expressed as percent of the LZR group. Also shown are representative blots for each group.

*p<0.05 compared to other groups.

Figure 4.

Hemi-mandible of OZR (panel B) displaying prominent caries compared to that of LZR (panel A) or the OZR; 5 Cr (panel C) and OZR; 10 Cr (panel D). On the other hand, panels E-H show micro-CT images of experimental groups showing class I carious lesions (arrows). Finally, panels I-L show three dimensional reconstructs of hemi-mandibles of experimental groups showing badly decayed tooth in untreated OZR (panel J).