ORIGINAL RESEARCH

AnneMarie Gagnon PhD¹
Hussein Abujrad MD²
Clara Irobi MD²
Heather A Lochnan MD^{1,2}
Alexander Sorisky MDCM^{1,2,3}

¹ Chronic Disease Program, Ottawa Hospital Research Institute

² Department of Medicine, University of Ottawa

³ Department of Biochemistry, Microbiology & Immunology, University of Ottawa

Serum fetuin-A levels following recombinant human thyroid-stimulating hormone stimulation

Abstract

Purpose: Fetuin-A is a hepatokine that is linked to lipid metabolism, obesity, insulin resistance, type 2 diabetes and cardiovascular disease. Elevated thyroid-stimulating hormone (TSH) levels are associated with metabolic and cardiovascular disturbances. Our aim was to determine if TSH can regulate fetuin-A levels.

Methods: Fetuin-A serum levels were examined in 26 subjects (19 women; previous thyroidectomy and radioactive iodine ablation) undergoing recombinant human TSH (rhTSH) stimulation to screen for thyroid cancer recurrence. Their age was 49 ± 10 years, and body mass index (BMI) was 28 ± 6 (both expressed as mean $\pm$ SD). The patients received two doses of rhTSH (0.9 mg), administered 24 hours apart on days 1 and 2, without discontinuation of ongoing L-thyroxine therapy. Morning blood samples were obtained on days 1 (prior to the first dose of rhTSH), 3 and 5.

Results: The baseline value of fetuin-A (mean $\pm$ SD) for all participants was 527 ± 186 mg/L. Values of fetuin-A did not change in response to rhTSH administration. The lack of response was not dependent on gender, age, baseline free thyroxine level or BMI.

Conclusion: Fetuin-A has been implicated in metabolic and inflammatory conditions, but there have been no reports on whether fetuin-A is influenced by TSH. Within the context of rhTSH administration for surveillance of thyroid cancer recurrence, there was no effect on serum levels of fetuin-A.

Manuscript submitted 21st May, 2013

Manuscript accepted 31st July, 2013

Clin Invest Med 2013; 36 (5): E264-E268.

Correspondence to:

Alexander Sorisky MD,
Ottawa Hospital Research Institute,
501 Smyth Road,
Ottawa, ON, K1H 8L6, Canada
Email: asorisky@ohri.ca

Subclinical hypothyroidism or mild thyroid gland failure results in a compensatory increase in the level of thyroid-stimulating hormone (TSH) that maintains normal thyroid hormone levels; however, this increase is associated with an elevated risk of cardiovascular disease (CVD) in longitudinal and cross-sectional population studies [1]. The pathophysiological mechanism responsible for this association is not known. Several metabolic and inflammatory CVD biomarker levels are raised in patients with subclinical hypothyroidism, including free fatty acids (FFA), interleukin 6 (IL-6), C-reactive protein and retinol binding protein 4 [2-4]. Adipocytes are capable of producing these pro-atherogenic molecules, and adipocytes also express TSH receptors [5]; therefore, these alterations may emanate from the action of high levels of TSH on extra-thyroidal cells such as adipocytes [6].

In vivo acute effects of high TSH levels can be investigated in the context of surveillance of thyroid cancer recurrence. Patients who have had thyroidectomies followed by radioablation for thyroid cancer are chronically treated with thyroid hormone. Some of them undergo recombinant human TSH (rhTSH) stimulation with subsequent measurement of thyroglobulin levels; a sensitive indicator of thyroid cancer recurrence. With this protocol, baseline bloodwork is obtained, followed by administration of rhTSH (0.9 mg i.m. x 2 daily doses) with serial blood sampling for thyroglobulin over 5 days. Using this particular experimental paradigm, rhTSH has been shown to increase serum levels of IL-6 and FFA *in vivo* [7, 8], and to increase IL-6, TNF α , lipoperoxide and leptin levels [9, 10].

Of these rhTSH-stimulated parameters, FFAs remain somewhat controversial with respect to their pro-inflammatory effect. FFAs were reported to bind and stimulate toll-like receptor 4 (TLR4) receptors, in monocytes/macrophages and adipocytes, and thus to activate inflammatory gene expression, [11]; however, subsequent studies did not find evidence of a direct ligand interaction between FFA and TLR4 [12]. Alternate mechanisms of FFA action have been invoked, such as reorganization of lipid rafts [13], or modulation of ceramide synthesis [14, 15], which may regulate the inflammatory state.

An entirely novel mechanism was recently proposed in which FFAs do engage TLR4 receptors, but only in the presence of fetuin-A. Fetuin-A is a hepatic glycoprotein, and it was shown, in mice, to play an adaptor role by binding FFAs and then interacting with TLR4 to stimulate pro-inflammatory signaling [16]. Fetuin-A levels are elevated in human obesity and diabetes, as are FFA levels [17]. Elevated fetuin-A, together with FFA, would be expected to induce a pro-inflammatory state. Since hepatocytes express TSH receptors [18], we hypothesized that rhTSH raises serum fetuin-A levels,

given the elevation in FFA we have previously reported caused by rhTSH [8].

Methods

Patients and blood sampling

Twenty-six participants (19 women and 7 men), who had previously undergone thyroidectomy followed by radioablative iodine therapy for thyroid cancer (approved by the Ottawa Hospital Research Ethics Board, protocol #2006558), were recruited for this study. Their age was 49 ± 10 years and their body mass index (BMI) was 28 ± 6 (both expressed as mean $\pm$ SD). None had evidence of metastatic disease and they were otherwise healthy. In the context of routine surveillance for thyroid cancer recurrence, the patients received two separate intramuscular doses of rhTSH (0.9 mg), administered 24 hours apart on days 1 and 2, without discontinuation of ongoing L-thyroxine therapy. Morning blood samples were obtained on days 1 (prior to the first dose of rhTSH), 3 and 5. Separate aliquots of blood from 19 of these patients had been used previously to assess FFA levels [8]. Fetuin-A was measured using the Human Fetuin-A Quantikine ELISA kit (R&D Systems), as per manufacturer's instructions. TSH and free thyroxine were measured in the clinical service laboratory by AutoIA (Abbott Dxl[®]).

Statistical analysis

Data were analyzed by analysis of variance, followed by post hoc Newman-Keul tests for multiple comparisons, or by unpaired t-test, with $P < 0.05$ taken as significant. Pearson's regression and correlation analysis were performed using Graph-PadInstat version 3.

Results

The baseline value of fetuin-A (mean $\pm$ SD) for all participants was 527 ± 186 mg/L. There was no overall correlation ($P = 0.26$) between baseline fetuin-A levels and age (see Fig. 1A). There was a trend of a positive correlation ($r = 0.35$, $P = 0.084$) between fetuin-A and BMI that just missed significance (see Fig. 1B). Fetuin-A values (mean $\pm$ SD) in those ($n = 16$) with a BMI < 30 were 486 ± 196 versus 618 ± 141 in those ($n = 9$) with a BMI ≥ 30 , indicative of the same trend of a higher fetuin-A level with a higher BMI ($P = 0.09$).

The baseline value of fetuin-A (mean $\pm$ SD) for the 19 females was 503 ± 200 mg/L and 593 ± 131 mg/L for the seven males. The difference in the mean values of fetuin-A between females and males was not significant. In the female group,

FIGURE 1. Correlation between circulating fetuin-A levels and age (A, C, E) or BMI (B, D, F) in all patients (A, B), women (C, D) or men (E, F) at baseline. Fetuin-A levels were quantified in serum samples collected at baseline, before rhTSH injection.

there was no correlation with age ($P=0.49$) or BMI ($P=0.13$) (Fig. 1C,D). In the male group, there was a trend toward a negative correlation between fetuin-A and age that did not quite reach significance ($r=-0.72$, $P=0.07$; Fig. 1E) and no correlation was observed with BMI ($P=0.63$; Fig. 1F).

The effect of rhTSH, administered on days 1 and 2, on fetuin-A levels was examined on days 3 and 5. Values of fetuin-A did not change in response to rhTSH administration (Fig. 2). There was no association of the fetuin-A response with age, gender, serum free thyroxine level or BMI. TSH levels (mU/L) rose from 0.5 ± 1 at baseline to >100 by day 3 and then decreased to 15 ± 7 by day 5.

Discussion

Fetuin-A has been implicated in a number of metabolic and inflammatory conditions, but there have been no reports on whether fetuin-A is influenced by TSH. Within the context of rhTSH administration to thyroid cancer patients for surveillance of recurrence, there was no effect on serum levels of fetuin-A.

Initial attention concerning fetuin-A arose from animal studies. In 1989, fetuin-A was cloned from rat liver and was characterized as an inhibitor of insulin resistance in muscle and liver [19]. Fetuin-A-null mice are protected from the obesity and insulin resistance that normally occurs with aging [20, 21]. Clinical studies have noted a positive correlation between

FIGURE 2. TSH does not affect circulating fetuin-A levels in thyroidectomized patients. Thyroidectomized patients were injected with rhTSH on days 1 and 2. Serum samples were collected on days 1 (prior to injection), 3 and 5, and fetuin-A levels were quantified by ELISA. Results are presented as raw data (A) and values normalized to day 1 level (B).

fetuin-A and a number of metabolic disturbances such as obesity, insulin resistance and hepatic steatosis [22]. A predictive role for type 2 diabetes or myocardial infarction was reported in longitudinal analyses [23-25]; however, the effect of TSH on fetuin-A has not been examined.

TSH has been shown to elicit pro-inflammatory factors when administered acutely in the context of surveillance for thyroid cancer recurrence. Increases in IL-6, TNF α , lipoperoxide, leptin and FFA have been observed [7-10]. The fact that TSH receptors are expressed in adipocytes could account for some of these responses [5,6]. Since hepatocytes also express TSH receptors [18], and since FFA may work in concert with fetuin-A, serum levels of fetuin-A, a hepatokine linked to insulin resistance and cardiovascular disease, were examined to see if they would be altered by rhTSH stimulation. Despite the increase in TSH levels, which peaked at day 3 and decreased only slightly at day 5, fetuin-A levels did not change. Since our study design was constrained by the time points of blood sample collection set out by the clinical rhTSH protocol, an early and transient increase in fetuin-A before day 3, or a delayed response beyond day 5, might have occurred unobserved in response to rhTSH.

Although our study is limited with respect to the number of subjects, it was of interest to consider whether these patients on thyroxine therapy would show any distinct patterns of baseline fetuin-A levels with respect to gender, age or BMI. There was no difference in fetuin-A levels between men and women, consistent with other studies [23, 25, 26]. No correlation between fetuin-A and age was observed in the overall group, but in men alone, there was a trend of a negative correlation although this was not statistically significant. In a large community study, the association of fetuin-A with metabolic syndrome risks was dependent on age [27]. The trend we observed between fetuin-A and BMI has been observed as a clear correlation in other studies, and weight loss reduces fetuin-A levels [26]. Overall, fetuin-A levels in our subjects appear to be similar to those in other studies.

In summary, our data show that rhTSH administration does not affect serum fetuin-A levels. It should be noted that this protocol acutely raises serum TSH to very high levels in the context of serum thyroxine levels, which remain in the upper normal or slightly elevated range as a result of ongoing thyroxine therapy for these patients who have undergone total thyroidectomy. In comparison, with subclinical hypothyroidism, the chronic elevation of TSH is milder in degree and serves to overcome the state of mild thyroid gland failure to maintain normal, but possibly suboptimal, thyroxine levels. It remains to be seen whether chronic exposure to elevated TSH levels in the context of subclinical hypothyroidism would alter fetuin-A levels.

Acknowledgments

This study was supported by Canadian Institutes of Health Research (CIHR) Operating Grant MOP-102585 to A.S.

References

1. Rodondi N, den Elzen WP, Bauer DC, Cappola AR, Razvi S, Walsh JP, Asvold BO, Iervasi G, Imaizumi M, Collet TH, Bremner A, Maisonneuve P, Sgarbi JA, Khaw KT, Vanderpump MP, Newman AB, Cornuz J, Franklyn JA, Westendorp RG, Vittinghoff E, Gussekloo J, Thyroid Studies Collaboration. Subclinical hypothyroidism and the risk of coronary heart disease and mortality. *JAMA* 2010; 304:1365-1374
2. Caraccio N, Natali A, Sironi A, Baldi S, Frascerra S, Dardano A, Monzani F, Ferrannini E. Muscle metabolism and exercise tolerance in subclinical hypothyroidism: a controlled trial of levothyroxine. *J Clin Endocrinol Metab* 2005; 90:4057-4062
3. Taddei S, Caraccio N, Virdis A, Dardano A, Versari D, Ghiadoni L, Ferrannini E, Salvetti A, Monzani F. Low-grade systemic inflammation causes endothelial dysfunction in patients with

- Hashimoto's thyroiditis. *J Clin Endocrinol Metab* 2006; 91:5076-5082
4. Choi SH, Lee YJ, Park YJ, Kim KW, Lee EJ, Lim S, Park DJ, Kim SE, Park KS, Jang HC, Cho BY. Retinol binding protein-4 elevation is associated with serum thyroid-stimulating hormone level independently of obesity in elderly subjects with normal glucose tolerance. *J Clin Endocrinol Metab* 2008; 93:2313-2318
 5. Latif R, Morshed SA, Zaidi M, Davies TF. The thyroid-stimulating hormone receptor: impact of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on multimerization, cleavage, and signaling. *Endocrinol Metab Clin North Am* 2009; 38:319-341
 6. Sorisky A, Antunes TT, Gagnon A. The Adipocyte as a novel TSH target. *Mini Rev Med Chem* 2008; 8:91-96
 7. Antunes TT, Gagnon A, Chen B, Pacini F, Smith TJ, Sorisky A. Interleukin-6 release from human abdominal adipose cells is regulated by thyroid-stimulating hormone: effect of adipocyte differentiation and anatomic depot. *Am J Physiol Endocrinol Metab* 2006; 290:E1140-E1144
 8. Gagnon A, Antunes TT, Ly T, Pongsuwan P, Gavin C, Lochnan HA, Sorisky A. TSH stimulates lipolysis in adipocytes in culture and raises serum free fatty acid levels in vivo. *Metabolism* 2010; 59:547-553
 9. Dardano A, Ghiadoni L, Plantinga Y, Caraccio N, Bemi A, Duranti E, Taddei S, Ferrannini E, Salvetti A, Monzani F. Recombinant human TSH reduces endothelium-dependent vasodilation in patients monitored for differentiated thyroid carcinoma. *J Clin Endocrinol Metab* 2006; 91:4175-4178
 10. Santini F, Galli G, Maffei M, Fierabracci P, Pelosini C, Marsili A, Giannetti M, Castagna MG, Checchi S, Molinaro E, Piaggi P, Pacini F, Elisei R, Vitti P, Pinchera A. Acute exogenous TSH administration stimulates leptin secretion in vivo. *Eur J Endocrinol* 2010; 163:63-67
 11. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 2006; 116:3015-3025
 12. Erridge C, Samani NJ. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. *Arterioscler Thromb Vasc Biol* 2009; 29:1944-1949
 13. Wong SW, Kwon MJ, Choi AM, Kim HP, Nakahira K, Hwang DH. Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. *J Biol Chem* 2009; 284:27384-27392
 14. Schwartz EA, Zhang WY, Karnik SK, Borwege S, Anand VR, Laine PS, Su Y, Reaven PD. Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. *Arterioscler Thromb Vasc Biol* 2010; 30:802-808
 15. Holland WL, Bikman BT, Wang L-P, Yuguang G, Sargent KM, Bulchand S, Knotts TA, Shui G, Clegg DJ, Wenk MR, Pagliassotti MJ, Scherer PE, Summers SA. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. *J Clin Invest* 2011; 121:1858-1870
 16. Pal D, Dasgupta S, Kundu RK, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med* 2012; 18:1279-1285
 17. Heinrichsdorff J, Olefsky JM. Fetuin-A: the missing link in lipid-induced inflammation. *Nat Med* 2012; 18:1182-1183
 18. Tian L, Song Y, Xing M, Zhang W, Ning G, Li X, Yu C, Qin C, Liu J, Tian X, Sun X, Fu R, Zhang L, Zhang X, Lu Y, Zou J, Wang L, Guan Q, Gao L, Zhao J. A novel role for thyroid-stimulating hormone: up-regulation of hepatic 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase expression through the cyclic adenosine monophosphate/protein kinase A/cyclic adenosine monophosphate-responsive element binding protein pathway. *Hepatology* 2010; 52:1401-1409
 19. Auberger P, Falquerho L, Contreras JO, Pages G, Le Cam G, Rossi B, Le Cam A. Characterization of a natural inhibitor of the insulin receptor tyrosine kinase: cDNA cloning, purification, and anti-mitogenic activity. *Cell* 1989; 58:631-640
 20. Mathews ST, Singh GP, Ranalletta M, Cintron VJ, Qiang X, Goustin AS, Jen KL, Charron MJ, Jahnen-Dechent W, Grunberger G. Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene. *Diabetes* 2002; 51:2450-2458
 21. Mathews ST, Rakhade S, Zhou X, Parker GC, Coscina DV, Grunberger G. Fetuin-null mice are protected against obesity and insulin resistance associated with aging. *Biochem Biophys Res Commun* 2006; 350:437-443
 22. Stefan N, Haring HU. The role of hepatokines in metabolism. *Nat Rev Endocrinol* 2013; 9:144-152
 23. Ix JH, Wassel CL, Kanaya AM, Vittinghoff E, Johnson KC, Koster A, Cauley JA, Harris TB, Cummings SR, Shlipak MG. Fetuin-A and incident diabetes mellitus in older persons. *JAMA* 2008; 300:182-188
 24. Stefan N, Haring HU, Schulze MB. Association of fetuin-A level and diabetes risk. *JAMA* 2008; 300:2247
 25. Weikert C, Stefan N, Schulze MB, Pischon T, Berger K, Joost HG, Haring HU, Boeing H, Fritsche A. Plasma fetuin-a levels and the risk of myocardial infarction and ischemic stroke. *Circulation* 2008; 118:2555-2562
 26. Brix JM, Stingl H, Hollerl F, Schernthaner GH, Kopp HP, Schernthaner G. Elevated fetuin-A concentrations in morbid obesity decrease after dramatic weight loss. *J Clin Endocrinol Metab* 2010; 95:4877-4881
 27. Xu Y, Xu M, Bi Y, Song A, Huang Y, Liu Y, Wu Y, Chen Y, Wang W, Li X, Ning G. Serum fetuin-A is correlated with metabolic syndrome in middle-aged and elderly Chinese. *Atherosclerosis* 2011; 216:180-186