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Brief Report

Beneficial paracrine effects of adipocytes from obese rats on cultured endothelial cells

Nathan T. Jenkins [a,*], Jaume Padilla [b,c,d], M. Harold Laughlin [e,f]

[a] Department of Kinesiology, University of Georgia, 115M Ramsey Center, 330 River Road, Athens, GA 30602-6554, USA
[b] Nutrition and Exercise Physiology, University of Missouri, Columbia, MO, USA
[c] Child Health, University of Missouri, Columbia, MO, USA
[d] Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO, USA
[e] Biomedical Sciences, University of Missouri, Columbia, MO, USA
[f] Medical Pharmacology and Physiology, University of Missouri, Columbia, MO, USA

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ABSTRACT

The purpose was to test the hypothesis that adipocytes from obese rats would exert pro-atherogenic paracrine effects on cultured endothelial cells compared to adipocytes from lean rats, and that the adverse obesity-associated paracrine effects of adipocytes would be more pronounced in visceral than subcutaneous adipose tissue. Epididymal and subcutaneous adipose tissues were harvested from 32-wk old obese Otsuka Long Evans Tokushima Fatty (OLETF) and lean Long Evans Tokushima Otsuka (LETO) rats. Cultured rat aortic endothelial cells were treated for 24 h with media conditioned with LETO subcutaneous adipocytes (LSA), OLETF subcutaneous adipocytes (OSA), LETO epididymal adipocytes (LEA), and OLETF epididymal adipocytes (OEA). The amount of key adipokines secreted by ATs was measured in the supernatant fluid with ELISA and mRNA levels of a number of pro- and anti-atherogenic genes were assessed in treated endothelial cells via quantitative real-time PCR. Compared to adipocytes from other depots, secretion of leptin and TNFα was highest and vaspin secretion was lowest from OEA. However, endothelial cells treated with OEA conditioned media exhibited lower expression of several pro-atherogenic genes. These data suggest that adipocytes isolated from obese visceral adipose tissue secrete some as-yet unidentified factor(s) that confers a beneficial effect on transcription of pro- and anti-atherogenic genes of endothelial cells.

Introduction

The mechanisms responsible for obesity-associated cardiovascular disorders are not fully understood but one proposed mechanism relates to the increasingly-appreciated endocrine functions of adipose tissue. Adipose tissue secretes a number of cytokines involved in inflammatory processes [e.g. leptin and tumor necrosis factor (TNF)-α], which may contribute to the development of endothelial dysfunction, atherosclerosis, and other cardiovascular abnormalities [8]. However, there is also now substantial evidence that obesity is not always associated with adverse cardiovascular outcomes. Rather, there exists an "obesity paradox" in that obesity may be associated with better outcomes in certain circumstances. For instance, obese patients with established cardiovascular diseases including hypertension, heart failure, coronary artery disease, and peripheral arterial disease have a more favorable short- and long-term prognosis than normal weight individuals [1,9,10].

Recently, we reported increased secretion of pro-inflammatory cytokines (e.g. leptin) and reduced secretion of anti-inflammatory cytokines (e.g. IL-10) from whole explants of visceral adipose tissue of obese, hyperphagic Otsuka Long Evans Tokushima Fatty (OLETF) rats [4]. Based on these findings, our hypothesis at the outset of the present study was that adipocytes from obese rats would exert pro-atherogenic paracrine effects on cultured endothelial cells compared to adipocytes from lean rats. Moreover, as visceral adipose tissue is thought to be a major source of pro-inflammatory cytokines

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DXA, dual x-ray absorptiometry; ELISA, enzyme linked immunoassay; eNOS, endothelial nitric oxide synthase; EPI, epididymal; ET-1, endothelin-1; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LEA, LETO epididymal adipocytes; LETO, Long Evans Tokushima Otsuka; LOX-1, lectin-like oxidized LDL receptor-1; LSA, LETO subcutaneous adipocytes; MCP-1, monocyte chemoattractant protein-1; NOX, NADPH oxidase; OEA, OLETF epididymal adipocytes; OLETF, Otsuka Long Evans Tokushima Fatty; OSA, OLETF subcutaneous adipocytes; RP, retroperitoneal; SOD, superoxide dismutase; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1.

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* Corresponding author. Tel.: +1 706 542 0631.
E-mail address: jenkinsn@uga.edu (N.T. Jenkins).

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in obesity, we also hypothesized that the adverse paracrine effects of adipocytes would be more pronounced in visceral than subcutaneous adipose tissue.

Methods

The present study was approved by the Animal Care and Use Committee at the University of Missouri. Obese OLETF ($n = 10$) and lean Long Evans Tokushima Otsuka (LETO; $n = 5$) rats were obtained at 4 weeks of age (Japan SLC, Inc. 3371-8, Kotoh-Cho, Hamamatsu, Shizuoka, Japan) and housed in temperature-controlled (21 °C) animal quarters with a 12 h light/dark cycle. The OLETF rat, characterized by a mutated cholecystokinin-1 receptor which results in a hyperphagic phenotype, is an established model of obesity, insulin resistance, and type 2 diabetes [7]. All groups were provided with *ad libitum* access to standard rodent chow (Formulab 5008, Purina Mills, St. Louis, MO). At 30–32 weeks of age, animals were anesthetized (100 ml/kg intraperitoneal pentobarbital injection), body composition was assessed by dual x-ray absorptiometry (DXA; Hologic), and epididymal and inguinal subcutaneous adipose tissues were harvested as described [4,13]. Rats were then euthanized by exsanguination in full compliance with the American Veterinary Medical Association Guidelines on Euthanasia.

Adipocytes were disassociated using a collagenase digestion protocol. Briefly, 1 g adipose tissue was placed in physiological saline solution containing 2 mg/ml collagenase type 1 and the tissue was minced into ~1 mm^3 pieces. Following 1-h incubation at 37 °C in a shaking water bath, tissue digests were centrifuged at 100*g* and the adipocyte supernatant fraction was harvested. Adipocytes were resuspended in phenol red- and serum-free culture medium (DMEM) containing 1% penicillin/streptomycin on 12-well culture plates and stored in a humidified incubator (37 °C, 5% CO_2 atmosphere). Media conditioned with LETO subcutaneous adipocytes (LSA), OLETF subcutaneous adipocytes (OSA), LETO epididymal adipocytes (LEA), and OLETF epididymal adipocytes (OEA) were harvested after 24 h and stored for future analysis. Conditioned media were assayed for concentrations of leptin and TNFα using a multiplex ELISA (Millipore Milliplex) as described [4,13] and vaspin using a commercially available ELISA kit (Raybiotech). Leptin and TNFα assays were performed on individual conditioned media samples from each rat, whereas the vaspin assay was performed after samples had already been pooled for cell culture experiments. Cultured rat aortic endothelial cells (passage 3) were grown to ~90% confluence in Rat Endothelial Cell Growth Medium (Cell Applications, San Diego, CA) and treated with pooled samples of LSA-, OSA-, LEA-, and OSA-conditioned media (20%) for 24 h. Total RNA was isolated and endothelial samples ($n = 6$ experimental replicates per condition) were processed for gene expression analysis via quantitative real-time PCR, as described [12,13].

Statistical analyses were performed using SPSS 19.0 (IBM). All data were assessed for assumptions of normality and equality of variances. Descriptive characteristics were analyzed using unpaired Student *t*-tests. Effects of conditioned media were analyzed using a 2 factor (group × depot) ANOVA with Fisher's least significant difference (LSD) post hoc analysis. Statistical significance was accepted at $P \leq 0.05$.

Results

As expected, OLETF animals had greater body weights, % body fat, and visceral fat pad masses than LETO rats (Fig. 1A). The adipocytes from OLETF animals secreted greater amounts of leptin from both subcutaneous and epididymal fat pads compared to LETO, with the most pronounced effect being in the epididymal depot (Fig. 1B). TNFα secretion was greater from OLETF epididymal

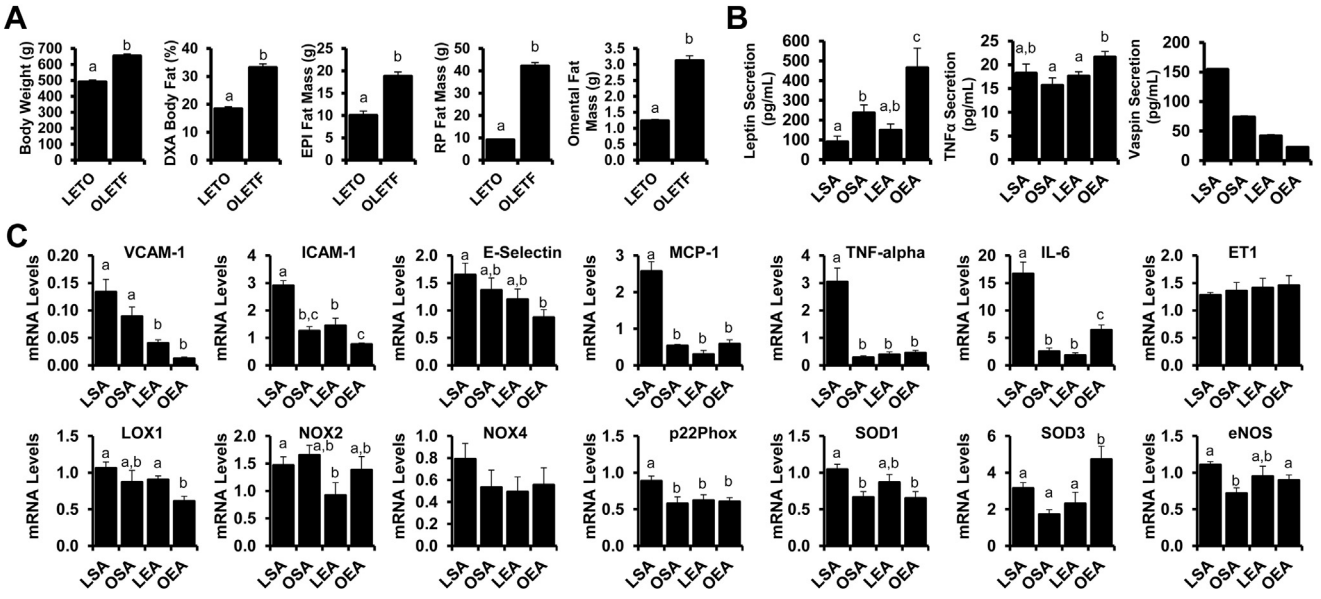

Figure 1. (A) Body weight, % body fat, and fat pad weights in OLETF and LETO rats. DXA, dual x-ray absorptiometry; EPI, epididymal; RP, retroperitoneal. Data were analyzed by independent samples *t*-test. Values are means ± SEM. Data with different letters are significantly different from each other. (B) Leptin, tumor necrosis factor (TNF)α, and vaspin secretion from LETO subcutaneous adipocytes (LSA), OLETF subcutaneous adipocytes (OSA), LETO epididymal adipocytes (LEA), and OLETF epididymal adipocytes (OEA). Leptin and TNFα data were analyzed using analysis of variance with least significant differences post hoc tests. Values are means ± SEM. Vaspin was assayed on pooled conditioned media samples. Therefore, the error bars represent the standard error of four technical replicates from each pooled sample. As such, statistical analysis was not performed on the vaspin data. (C) mRNA levels of pro- and atherogenic genes in cultured rat aortic endothelial cells treated with LSA, OSA, LEA, and OEA for 24 h. VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1, IL-6, interleukin-6; ET-1, endothelin-1; LOX-1, lectin-like oxidized LDL receptor-1, NOX, NADPH oxidase; SOD, superoxide dismutase; eNOS, endothelial nitric oxide synthase. 18S was used as the reference gene, and normalized gene expression was quantified using the $2^{\Delta Ct}$ method, whereby $\Delta Ct = 18S\ Ct - \text{gene of interest Ct}$. Data were normalized to $2^{\Delta Ct}$ values in untreated control endothelial cells (set to 1), and are expressed as mean ± SEM. Data were analyzed by analysis of variance with least significant differences post hoc tests. Values with unlike letters are statistically different from each other.

adipocytes than that from OSA and LEA (Fig. 1B), while vaspin secretion was greatest from LSA and least from OEA (Fig. 1B). Endothelial cells treated with OEA conditioned media exhibited lower expression of several pro-atherogenic mRNAs [vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM-)1, E-selectin, TNFα, $p22^{phox}$] and greater levels of the anti-oxidant gene superoxide dismutase (SOD)3 compared to conditioned media from LSA, OSA, or LEA (Fig. 1C). Additionally, conditioned media from OSA, but not OEA, exhibited reduced eNOS compared to LSA (Fig. 1C). Because these results were so contrary to our hypothesis, we repeated the conditioned media endothelial cell culture experiments and observed identical results.

Discussion

Our finding that OEA conditioned medium reduced the expression of pro-atherogenic genes in cultured endothelial cells compared to the other adipocyte conditioned media examined suggests that adipocytes from visceral adipose tissue of obese rats secrete some as-yet unidentified factor(s) that confers a beneficial effect on endothelial cells at the mRNA level. Although these results were contrary to our initial hypothesis, based on our earlier findings of increased pro-inflammatory cytokine secretion from adipose tissue in OLETF rats (e.g. leptin [4]), the present data might provide initial experimental evidence pointing to a biological explanation for the obesity paradox. Previously, some ideas regarding a physiological basis for the obesity paradox have been proposed, e.g. chronic intermittent hypoxia caused by obstructive sleep apnea has been theorized to enhance cardio-protective mechanisms and partially account for reduced cardiovascular mortality among obese cardiovascular disease patients [11]. It has also been posited that cardiovascular diseases characterized by an obesity paradox, e.g. heart failure, might be associated with altered adipokine secretion patterns [1]. Our current *in vitro* data support the existence of a beneficial adipocyte-derived signal to the endothelium, but future research is required to confirm this hypothesis *in vivo* and identify the factors involved.

It has been proposed that the obese adipose tissue has to become pathologically inflamed in order to promote obesity-associated diseases [2]. In support of this concept, classification of obese patients on the basis of the presence or absence of macrophage crown-like structures in subcutaneous abdominal adipose tissue biopsy samples revealed that patients without crown-like structures had similar levels of cardiometabolic risk factors and brachial artery flow-mediated dilation compared to lean subjects [3]. Although we did not assess crown like structures in the present study, in our previous study we did not find evidence of crown like structures in OLETF rats [4]. Thus, it is possible that our results reflect the lack of substantial adipose tissue inflammation in the OLETF rat model, and stimulate the hypothesis that expanded adipocytes secrete some unknown factor(s) responsible for the induction of beneficial gene expression in endothelial cells.

We assessed concentrations of the leptin and TNFα in conditioned media samples, to provide insight into factors that may explain our unexpected observation of beneficial effects of OEA condition media on endothelial cell gene expression, as these adipokines have well-established pro-atherogenic effects on endothelial cells [8]. Our finding that leptin and TNFα secretion were highest from OEA suggests that these cytokines are unlikely to have been responsible for the attenuated expression of pro-atherogenic genes observed in the OEA conditioned medium-treated endothelial cells. In addition, we assessed concentrations of vaspin in light of evidence that this adipocyte-specific anti-inflammatory cytokine exerts powerful beneficial effects on endothelial cells. For example, vaspin has been shown to be anti-apoptotic [6] and capable of enhancing nitric oxide bioavailability [5]. Results indicated that vaspin concentrations were lowest in OEA-conditioned media samples, indicating that vaspin also was probably not responsible for our observation of attenuated pro-atherogenic effects of OEA conditioned media.

Limitations

The methods we used to generate adipocyte conditioned media warrant mentioning. We processed 1 g of each fat depot to obtain adipocytes. However, we did not control for potential differences in recovery during purification or cell death during culturing. The primary contributing factor to the potential problem of differences among depots in cell recovery would be differences in adipocyte size (for example, isolation of adipocytes from an obese animal might yield fewer adipocytes). We have previously documented that while adipocytes from OELTF are indeed significantly larger than those from LETO, the magnitude of the difference is relatively small (i.e. ~100 μm in LETO vs. 110 μm in OLETF) [4]. Thus, we do not expect that this 10 μm difference created a large difference in number of adipocytes per gram. Regarding the possibility of cell death during isolation or culturing, the same methods were applied uniformly to all tissue samples. Thus, any contamination of dead adipocytes would be expected to be uniform across all samples. Nevertheless, we acknowledge that adipocyte numbers and cell death were not measured directly in the present study, and this may have implications for the interpretation of differences in endothelial cell readout. Finally, it is also acknowledged that we only assessed gene expression at the mRNA level, not the protein level. Future investigations are warranted to confirm "paradoxical" paracrine effects of visceral adipocytes on endothelial cell function.

Overall, our study provides initial experimental evidence that visceral adipocytes secrete some factor(s) that stimulate a reduction in the expression of a number of pro-atherogenic genes in cultured endothelial cells. However, we did not identify the adipocyte-derived molecule(s) responsible for our observations. We speculate that it was not a single factor acting in isolation, but rather multiple factors and their complex interactions that produced favorable effects of OEA on endothelial gene expression in our experiments. This multi-factorial, complex paradigm seems likely to characterize the biological underpinnings of the obesity paradox in humans.

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References

[1] Arena R, Lavie CJ. The obesity paradox and outcome in heart failure: is excess bodyweight truly protective? Future Cardiol 2010;6:1–6.
[2] Bays HE. Adiposopathy is "sick fat" a cardiovascular disease? J Am Coll Cardiol 2011;57:2461–73.
[3] Farb MG, Bigornia S, Mott M, Tanriverdi K, Morin KM, Freedman JE, et al. Reduced adipose tissue inflammation represents an intermediate cardiometabolic phenotype in obesity. J Am Coll Cardiol 2011;58:232–7.
[4] Jenkins NT, Padilla J, Arce-Esquivel AA, Bayless DS, Martin JS, Leidy HJ, et al. Effects of endurance exercise training, metformin, and their combination on adipose tissue leptin and IL-10 secretion in OLETF rats. J Appl Physiol 2012;113:1873–83.
[5] Jung CH, Lee WJ, Hwang JY, Lee MJ, Seol SM, Kim YM, et al. Vaspin increases nitric oxide bioavailability through the reduction of asymmetric dimethylarginine in vascular endothelial cells. PLoS One 2012;7:e52346.

[6] Jung CH, Lee WJ, Hwang JY, Seol SM, Kim YM, Lee YL, et al. Vaspin protects vascular endothelial cells against free fatty acid-induced apoptosis through a phosphatidylinositol 3-kinase/Akt pathway. Biochem Biophys Res Commun 2011;413:264–9.
[7] Kawano K, Hirashima T, Mori S, Saitoh Y, Kurosumi M, Natori T. Spontaneous long-term hyperglycemic rat with diabetic complications. Otsuka Long-Evans Tokushima Fatty (OLETF) strain. Diabetes 1992;41:1422–8.
[8] Lau DC, Dhillon B, Yan H, Szmitko PE, Verma S. Adipokines: molecular links between obesity and atherosclerosis. Am J Physiol Heart Circ Physiol 2005;288:H2031–41.
[9] Lavie CJ, Milani RV, Ventura HO. Obesity and cardiovascular disease: risk factor, paradox, and impact of weight loss. J Am Coll Cardiol 2009;53:1925–32.
[10] Lavie CJ, Milani RV, Ventura HO. Obesity and the "obesity paradox" in cardiovascular diseases. Clin Pharmacol Ther 2011;90:23–5.
[11] Ozeke O, Ozer C, Gungor M, Celenk MK, Dincer H, Ilicin G. Chronic intermittent hypoxia caused by obstructive sleep apnea may play an important role in explaining the morbidity-mortality paradox of obesity. Med Hypotheses 2011;76:61–3.
[12] Padilla J, Jenkins NT, Roberts MD, Arce-Esquivel AA, Martin JS, Laughlin MH, et al. Differential changes in vascular mRNA levels between rat iliac and renal arteries produced by cessation of voluntary running. Exp Physiol 2013;98:337–47.
[13] Padilla J, Jenkins NT, Vieira-Potter VJ, Laughlin MH. Divergent phenotype of rat thoracic and abdominal perivascular adipose tissues. Am J Physiol Regul Integr Comp Physiol 2013;304:R543–52.