Role of T Regulatory Lymphocytes in the Pathogenesis of High-Fructose Diet–Induced Metabolic Syndrome

Avshalom Leibowitz, Asia Rehman, Pierre Paradis, Ernesto L. Schiffrin

Abstract—We recently showed that T regulatory lymphocytes (Treg), which are immune suppressors of inflammatory responses, play a role blunting the development of hypertension-induced injury. Treg are unchanged or decreased in children with metabolic syndrome, and therefore, their role in metabolic syndrome remains unclear. We hypothesized that Treg number or function would be depressed in a high-fructose diet–induced metabolic syndrome-like model in rats. Sprague-Dawley rats were fed normal chow or a high-fructose diet for 5 weeks. The high-fructose diet–induced a 3.8-fold increase in plasma triglycerides and a 14% reduction in high-density lipoprotein cholesterol (P<0.001). The high-fructose diet increased reactive oxygen species in aorta and periaortic adipose tissue 2.8-fold (P<0.05), and reduced nicotinamide adenine dinucleotide phosphate oxidase activity 1.9-fold in aorta, and 2.5-fold in the heart (P<0.05). It also increased plasma nitric oxide metabolite levels 6.4-fold (P<0.001). Western blots showed that the high-fructose diet increased ≥2.3-folder vascular and in platelet endothelial cell adhesion molecule 1 in aorta (P<0.01). It did not affect monocyte/macrophage aortic infiltration but caused a 2.4-fold increase in collagen deposition in the aortic media (P<0.01). No change in plasma interleukin-10 was detected. The percentage of spleen CD4⁺CD25⁺ and Treg (CD4⁺CD25high) cells was unaltered by the high-fructose diet. However, cultured Treg from high-fructose diet–fed rats secreted 62% less interleukin-10 than control cells (P<0.05), suggesting a decreased Treg function, which could play a role in the development of cardiovascular complications of the metabolic syndrome. (Hypertension. 2013;61:1316-1321.) • Online Data Supplement

Key Words: inflammation ■ interleukin-10 ■ oxidative stress ■ vascular remodeling

Cardiovascular (CV) disease remains the major cause of mortality and morbidity in the Western world. Although multiple factors are responsible for these phenomena, the recent rise in the prevalence of metabolic syndrome suggests that it might be a major contributor. Among many reasons for this epidemic, the dramatic increase in carbohydrate consumption in the Western diet makes a significant contribution. Low-grade inflammation plays a role in the pathogenesis of CV disease, including hypertension and the metabolic syndrome. There is emerging evidence that the immune system, and particularly the adaptive immune response, may be involved in the triggering of these inflammatory processes. This was first suggested by Shao et al., who demonstrated that angiotensin (Ang) II infusion in rats caused recruitment of T lymphocytes in the kidney. Guzik et al. showed that Ang II–induced increase in blood pressure (BP), endothelial dysfunction, and superoxide production were reduced in recombination activating gene 1 knockout mice deficient in T and B lymphocytes and could be restored by adoptive transfer of T but not B cells. Recently, we showed that T regulatory cells (Treg), a T lymphocytes subset that typically produces interleukin (IL)-10 and transforming growth factor-β, which are crucial for the balance and homeostasis of the immune system and can suppress adaptive immune responses, were implicated in hypertension and vascular damage. We showed chromosome 2–dependent modulation of immune responses in genetic hypertension via Treg. In the Dahl salt-sensitive rats, vascular inflammation and BP elevation were associated with dysfunction of Treg inflammatory suppressor action. Consomic SSBN2 rats, which have the Dahl salt-sensitive rat genome and chromosome 2 from normotensive Brown Norway rats, had improved Treg function and decreased hypertension and vascular and systemic inflammatory mediators compared with salt-sensitive rats. We also showed that Ang II–induced hypertension and endothelial dysfunction and vascular remodeling, oxidative stress, and inflammation in mice were associated with a decrease in Treg number in the kidney. Adaptive transfer of Treg prevented these Ang II effects. More recently, we observed that Treg adoptive transfer prevented aldosterone-induced vascular damage in a BP-independent fashion. These data resembled previous data by Kvakan et al., who showed BP-independent protective effects of Treg adoptive transfer on cardiac hypertrophy and inflammation. Whether Treg play a role in the development of the metabolic syndrome has not been demonstrated.
Studies of Treg in peripheral blood of children with metabolic syndrome have provided conflicting results. The first study in which different Treg markers were evaluated showed a decrease in the percentage of CD4+CD25high lymphocytes but no change in the percentage of CD4+CD25highCD127low/− and CD4+CD25highFoxP3+ cells. In the second study, a decrease in the percentage of CD4+CD25highCD127lowFoxP3+ cells was observed in children with metabolic syndrome (2.5 versus 3.1%).

To determine the role of Treg in the metabolic syndrome, vascular oxidative stress and inflammation and the number and function of Treg were studied in high-fructose diet–fed Sprague-Dawley rats, a well-established model mimicking some features of the western diet–induced metabolic syndrome. These rats exhibit hypertension, insulin resistance, and abnormal lipid profile resembling the human metabolic syndrome better than a monogenic model. We hypothesized that vascular oxidative stress and inflammation and changes in the immune response, including Treg activity and response, participate in the mechanisms leading to the metabolic syndrome.

**Materials and Methods**

Additional materials and methods are described in the online-only Data Supplement.

**Experimental Design**

Eight-week-old Sprague-Dawley male rats (Harlan Laboratories, Indianapolis, IN) were fed a high-fructose diet (TD.89247, Harlan Laboratories) composed of 60% fructose, 21% protein, 5% fat, 8% cellulose, and standard vitamins and mineral mix or normal chow diet (control) for 5 weeks. Systolic BP (SBP) was measured by the tail-cuff method after 4 weeks on the diet. SBP was also determined by telemetry together with heart rate and animal activity every 5 minutes for 10 seconds for 2 consecutive days at baseline and at the end of every week during the 5 weeks of treatment. Three days before the end of the protocol, blood was collected from the saphenous vein on heparin for triglycerides, and high-density lipoprotein cholesterol was determined after 5 hours of fasting. At the end of the protocol, body weight was evaluated, and rats were anesthetized with 3% isoflurane in oxygen (depth of anesthesia confirmed by rear foot squeezing), and blood was collected by aorta puncture on EDTA for plasma nitric oxide metabolites (NOx) and IL-10 determinations (see online-only Data Supplement). The remaining tissues were frozen in liquid N2 and stored at −80°C for determination of reactive oxygen species, monocyte/macrophage infiltration, and media collagen fraction. The spleen was used to isolate Treg (CD4+CD25high) and CD4+CD25− cells for determination of Treg IL-10 secretion (see online-only Data Supplement). The remaining tissues were frozen in liquid N2 and stored at −80°C for determination of expression of vascular cell adhesion molecule 1 and platelet endothelial cell adhesion molecule 1. All experimental procedures were approved by the Animal Care Committee of the Lady Davis Institute for Medical Research, McGill University, and followed the recommendations of the Canadian Council of Animal Care.

**Data Analysis**

Results are presented as mean±SEM. Unpaired t test was performed, and P<0.05 was considered statistically significant.

**Results**

**High-Fructose Diet Induced Some Features of the Metabolic Syndrome in Rats**

The development of a metabolic-like syndrome with the high-fructose diet was confirmed by a 3.8-fold increase in plasma triglycerides and 14% reduction in high-density lipoprotein (Table). Weight of kidneys and liver increased. SBP measured by the tail-cuff method increased by 19 mm Hg after 4 weeks of high-fructose diet, but determination of telemetric BP did not demonstrate changes in SBP during day or night compared with control rats (Figure S2 in the online-only Data Supplement). Telemetry also showed that heart rate and animal activity were not altered. No change in body weight gain was detected in high-fructose diet–fed rats compared with control rats (Table), as previously reported. Plasma insulin (Table) and insulin C-peptide (Figure S3) and heart, lung, and spleen weights were unaffected by the diet (Table S1).

**Mesenteric Resistance Artery Endothelial Function and Vascular Structure**

Vasodilatory responses of resistance arteries to acetylcholine and media-to-lumen ratio and media cross-sectional area were unaltered by the high-fructose diet (Figure S4).

**Higher Oxidative Stress in the High-Fructose Diet–Fed Rats**

The high-fructose diet–induced metabolic-like syndrome was associated with increased reactive oxygen species production. Dihydroethidium fluorescence was 2.8-fold higher in aorta and perivascular adipose tissue of experimental rats compared with control rats (Figure 1A; Figure S5). The high-fructose diet also increased 1.9-fold reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in aorta as determined by the tail-cuff method (Figure 1B).

**Determination of reactive oxygen species production**

**Heart**

Dihydroethidium (DHE) staining demonstrating reactive oxygen species (ROS) production (A), reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in aorta (B) and heart (C) and plasma nitric oxide metabolites (NOx; D) were determined in rats fed a high-fructose diet (HFrD) or normal chow (Ctrl) for 5 weeks. Values are means±SEM. *P<0.05 and †P<0.001 vs Ctrl. n=5 for A, 5 to 6 for aorta in B, 8 for heart in B, and 7 for C.
and 2.5-fold in the heart (Figure 1B and 1C), and plasma NOx levels were 6.4-fold greater (Figure 1D).

Enhanced Vascular Inflammation in the High-Fructose Diet–Fed Rats

Expression of adhesion molecules vascular cell adhesion molecule 1 and platelet endothelial cell adhesion molecule 1 was increased 2.3- and 2.5-fold, respectively, in aorta in the high-fructose diet–fed rats compared with control rats (Figure 2A). Monocyte/macrophage infiltration occurred similarly in peri-aortic adipose tissue of both groups (Figure S6). Aortic collagen content, a consequence of inflammation, was 2.4-fold higher than in control rats (Figures 2B and S7).

Treg Function Is Impaired in the High-Fructose Diet–Fed Rats

The immunosuppressive action of Treg is mediated in part via secretion of IL-10. Plasma levels of IL-10, however, were unaltered by the high-fructose diet (Figure S8A). CD4+CD25− lymphocytes and Treg (CD4+CD25high lymphocytes) were isolated and quantified by fluorescence-activated cell sorting (Figure S1C). Purity of isolated cells was ≥99.5% for CD4+CD25− and ≥97.9% for Treg (Figure S1D and S1E). The high-fructose diet did not alter the percentage of CD4+CD25− cells and Treg (Figure S8B and S8C). However, cultured Treg originating from high-fructose diet–fed rats secreted 62% less IL-10 into the culture media than control rat cells (Figure 2C), suggesting decreased Treg function.

Discussion

Treg play a countervailing role on the development of vascular damage associated to BP rise10,12 or independently of BP in rodent models.11 Kvakan et al13 demonstrated that Treg protect from the development of Ang II–induced cardiac injury. In the present study, a high-fructose diet–induced metabolic-like syndrome and associated vascular oxidative stress and inflammation were accompanied by decreased Treg function, as suggested by reduced IL-10 secretion, which could play a role in the development of CV disease. This study supports previous findings in aldosterone–infused mice11 that showed that Treg play a protective role on the development of vascular injury independently of effects on BP.

In agreement with previous data, high-fructose diet–fed rats exhibited increased triglyceride and reduced high-density lipoprotein levels but no change in body weight or telemetrically measured BP compared with normal chow–fed rats.16,21,22 The lack of body weight gain in high-fructose diet–fed rats was not attributable to increases in activity as determined by telemetry. The development of features of the metabolic

### Table. Eight Weeks of High-Fructose Diet–Induced Metabolic Syndrome

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctrl</th>
<th>HFrD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>410±7</td>
<td>398±10</td>
</tr>
<tr>
<td>TL, cm</td>
<td>4.43±0.16</td>
<td>4.46±0.15</td>
</tr>
<tr>
<td>KW, g</td>
<td>2.21±0.06</td>
<td>2.52±0.12*</td>
</tr>
<tr>
<td>KW/BW, mg/g</td>
<td>5.37±0.08</td>
<td>6.28±0.19†</td>
</tr>
<tr>
<td>KW/TL, g/cm</td>
<td>0.50±0.02</td>
<td>0.57±0.02*</td>
</tr>
<tr>
<td>LIW, g</td>
<td>13.2±0.5</td>
<td>15.0±0.7*</td>
</tr>
<tr>
<td>LIW/BW, mg/g</td>
<td>32.1±0.7</td>
<td>37.6±0.9†</td>
</tr>
<tr>
<td>LIW/TL, g/cm</td>
<td>3.01±0.14</td>
<td>3.38±0.15*</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>134±5</td>
<td>153±4*</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>65±3</td>
<td>248±19†</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>66±2</td>
<td>57±2†</td>
</tr>
<tr>
<td>Insulin, µg/L</td>
<td>0.35±0.02</td>
<td>0.42±0.09</td>
</tr>
</tbody>
</table>

Body and tissue weights, tibia length (TL), systolic blood pressure (SBP), plasma triglycerides, and high-density lipoprotein (HDL) cholesterol were determined in rats fed a high-fructose diet (HFrD) or a normal chow (Ctrl) for 5 wk. Values are means±SEM, *P<0.05 and †P<0.001 vs Ctrl with n=9–10 for body and tissue weights and tibia length, 5 for SBP, and 15–20 for plasma triglycerides and HDL. BW indicates body weight; KW, weight of the kidneys; and LIW, weight of the liver.
syndrome was accompanied by increased liver weight suggesting steatosis,21 and increased kidney weight reflecting kidney injury25 as described in humans.

Oxidative stress plays a role in mechanisms leading to vascular injury and hypertension,24–27 and in the development of metabolic-like syndrome induced by a high-fructose diet. Increased NADPH oxidase activity and superoxide production occur in aorta and the heart of high-fructose diet–fed rats.18,28 Enhanced oxidative stress could be an early event because elevated superoxide production was detected in aorta 1 week after starting the high-fructose diet, and after 2 weeks in the heart.18 Oxidative stress is also present in another model of metabolic syndrome, the New Zealand Obese mice.29 The greater reactive oxygen species production in New Zealand Obese mice was not restricted to the vessel wall but was also present in perivascular fat. Similarly, high-fructose diet–fed rats had increased reactive oxygen species in the vessel wall and perivascular fat, as also found in hypertension and in other forms of vascular disease. Ang II and aldosterone infusion induce a similar phenotype.10,11 Increases in vascular and perivascular fat oxidative stress in the metabolic syndrome could thus contribute to the development of CV disease.

Oxidative stress participates in the development of vascular injury by reducing NO bioavailability through reaction of NO with superoxide to form the pro-oxidant metabolite peroxynitrite. NO is difficult to measure because it is rapidly oxidized into nitrite and nitrate (NOx) that can be determined as an index of NO formation. Despite the increase in oxidative stress, plasma NOx levels were elevated in rats fed a high-fructose diet for 5 weeks. Plasma NOx levels are increased in obese children with metabolic syndrome and in New Zealand Obese mice,29,30 whereas they are decreased in obese teenagers with metabolic syndrome.31 Other laboratories have observed that plasma NOx was increased in rats that were fed a high-fructose diet for 8 and 13 weeks.32,33 However, plasma NOx was decreased in another rat model treated with 10% fructose in drinking water for 6 weeks.34 Similarly, acetylcholine–induced renal NOx secretion was decreased in rats treated with 10% fructose in drinking water for 12 weeks.35 It is unclear why levels of NOx are different between these 2 models, but rats treated with 10% fructose in drinking water could present a more severe metabolic syndrome than those treated with 60% fructose in food. The increase in NO production could be an initial homeostatic mechanism to counteract the increased oxidative stress. With the progression of the metabolic syndrome, NO production could decrease, which would result in the development or aggravation of CV disease. In this study, these parameters were unaltered after 5 weeks of high-fructose diet, which suggests that this model is mimicking an early phase of the metabolic syndrome.

The increased oxidative stress in the metabolic syndrome and in Ang II and aldosterone–induced vascular injury with or without hypertension, respectively,10,11,36 is associated with low-grade inflammation.5,6 The metabolic syndrome is a risk factor for atherosclerosis,37 and it is well established that inflammation has a pivotal role in atherosclerosis.38 Inflammation is also relevant in the high-fructose diet model. The majority of the data in this area are derived from work focusing on the liver and adipose tissue,39 which have a cardinal role in lipid metabolism, especially in relation to triglycerides. Thus, fructose, a major source of triglycerides, directly affects these tissues.20 Our study demonstrates that a high-fructose diet can enhance vascular inflammation, which plays a role in vascular injury and ultimately in hypertension.5 Using Wistar rats fed 10% fructose in drinking water for 38 weeks, Tan et al40 showed increased expression of adhesion molecules vascular cell adhesion molecule 1 and intercellular adhesion molecule 1, as well as infiltration of monocyte/macrophages in the aortic wall. Our results have demonstrated increased expression of vascular cell adhesion molecule 1 and platelet endothelial cell adhesion molecule 1 in the aorta of rats fed a high-fructose diet for 5 weeks. However, although macrophage infiltration was detected in periaortic adipose tissue, it was unaltered by the diet. The difference in the levels of vascular inflammation could be attributed to the different length of the treatment protocol, which suggests that the increase in expression of adhesion molecules precedes the increase in monocyte/macrophage infiltration. A major by-product of inflammation is fibrosis, which is found in the heart, liver, and kidney in metabolic syndrome models, including the fructose–induced syndrome.41–43 Our study demonstrates that high-fructose diet–induced oxidative stress and inflammation lead to target organ damage in the form of collagen deposition and fibrosis, which also affects the vasculature.

Recently, several studies have revealed the relevance of the immune system in the pathogenesis of hypertension and vascular damage.5 Guzik et al8 demonstrated for the first time that lack of lymphocytes in mice blunts Ang II–induced BP rise and vascular injury, and that adoptive transfer of T cells restores Ang II effects. T lymphocyte populations include T effector lymphocytes (eg, Th1 and Th2) and Treg. T effector lymphocytes are proinflammatory, whereas Treg have suppressor properties and are anti-inflammatory. We demonstrated the importance of Treg in 3 different models of vascular injury: Dahl salt-sensitive rats, and Ang II– and aldosterone–infused mice.10–12 Data regarding a role of the adaptive immune response, especially T cells, in the pathogenesis of metabolic syndrome in high-fructose diet models are limited. Depending on the Treg markers used in the study considered, the percentage of Treg was unaltered or decreased in peripheral blood of children with metabolic syndrome.34,35 In this study, no change in the percentage of Treg was observed in the spleen of high-fructose diet–fed rats compared with control rats. However, cell culture demonstrated that Treg from high-fructose diet–fed rats secreted less IL-10 compared with control rats, which suggests a decrease in Treg function. The absence of change in plasma IL-10 might not reflect Treg function, because II-10 is secreted by several cell types. We noted that no change in CD4+ and CD4+CD25+ T cells was observed, similar to the observation of Luczyński et al in children.35 Decreased Treg function could be an early event in the development of metabolic syndrome.

SBP increased by 5 weeks of high-fructose diet when measured by the tail-cuff method but not by telemetry, similar to previous findings in which a high-fructose diet (66% fructose, 12% fat) for 8 weeks did not change the mean 24 hours telemetric SBP.44 However, these authors also observed that a high-fructose diet increased SBP measured with the tail-cuff
method. Increases in BP with the tail-cuff technique could be attributable to an elevated stress response by high-fructose-fed rats associated with contentment and cuff inflation. BP may also rise at specific periods during the day. A 40% sucrose diet (containing 20% fructose) for 4 months increased telemetric SBP in rats during the immediate postprandial period. Farah et al showed using telemetry that a high-fructose diet (60% fructose) for 8 weeks increased nighttime but not daytime SBP in mice. Finally, Brown et al demonstrated that in humans, fructose but not glucose ingestion acutely raised SBP. However, because no change in SBP was observed by telemetry during daytime or nighttime in the present study, it is also possible that this model simply mimics an early phase of the metabolic syndrome preceding the development of hypertension. A longer exposure to high-fructose diet might be necessary to observe a BP rise.

Limitations

The importance of Treg in vascular damage has been demonstrated by Treg adoptive transfer that prevented Ang II– and aldosterone–induced vascular injury and inflammation. It would have been interesting to use the same approach in the present study. However, 10 times more Treg per injection are required to treat rats compared with mice. Unfortunately, we failed to isolate enough Treg to perform adoptive transfer in rats. As well, whether the high-fructose diet contributes to the increase in plasma NOx could not be determined. Rats received food ad libitum, and food consumption was not measured. The nitrate content of the high-fructose diet is unknown. Grossly, high-fructose diet–fed rats ate less than control diet–fed rats. Bagul et al observed that a similar high-fructose diet (65% fructose, 5% fat) was associated with decreased food intake by 4% compared with rats fed a control diet. Ross et al showed that rats fed a high-fructose diet (60% fructose, 10% lipid) had a 7% higher food intake compared with control diet–fed rats. Altogether, these observations suggest that food consumption does not explain the 6.4-fold (540%) increase in plasma NOx. However, contribution of the high-fructose diet to the increase in plasma NOx cannot be unambiguously excluded.

Perspective

We demonstrate that oxidative stress and low-grade inflammation play a role in some features of a high-fructose diet–induced metabolic-like syndrome. These abnormalities are accompanied by Treg dysfunction as demonstrated by low levels of IL-10 secretion by cultured Treg. This study provides the novel information that beyond various metabolic and physiological effects, a high-fructose diet modulates the immune system response by affecting Treg function, which contributes to CV injury found in the metabolic syndrome. It will be important to determine whether in humans with metabolic syndrome, Treg function is altered as in the present rodent model.

Acknowledgments

We are grateful to André Turgeon, Marie-Eve Deschênes, and Véronique Michaud for their excellent technical support and animal care.

Sources of Funding

This work was funded by the Canadian Institutes of Health Research grants 82790 and 102606, a Canada Research Chair (CRC) on Hypertension and Vascular Research from the Canadian Institutes of Health Research/government of Canada CRC program, and by the Canada Fund for Innovation; all to Dr Schiffrin.

Disclosures

None.

References

Novelty and Significance

What Is New?

- Metabolic-like syndrome induced by high-fructose diet in rodents is associated with dysfunction of T regulatory lymphocytes (Treg).
- Reduced production of interleukin-10 by Treg is accompanied by oxidative stress and inflammation in the vascular wall and perivascular fat, vascular remodeling, and endothelial dysfunction.

What Is Relevant?

- This model of metabolic-like syndrome mimics an early phase of metabolic syndrome in humans. Treg dysfunction and interleukin-10 deficiency could participate in the mechanisms leading to cardiovascular complications in the human metabolic syndrome, and would need to be explored as a potential therapeutic target.

Summary

Oxidative stress and low-grade inflammation play a role in high-fructose diet–induced metabolic syndrome. These abnormalities are accompanied by Treg dysfunction as demonstrated by low levels of interleukin-10 secretion by cultured Treg. This study provides the novel information that beyond various metabolic and physiological effects, a high-fructose diet modulates the immune system response by affecting Treg function, which contributes to cardiovascular injury found in the metabolic syndrome.
Role of T Regulatory Lymphocytes in the Pathogenesis of High-Fructose Diet–Induced Metabolic Syndrome

Avshalom Leibowitz, Asia Rehman, Pierre Paradis and Ernesto L. Schiffrin

*Hypertension*. 2013;61:1316-1321; originally published online March 25, 2013; doi: 10.1161/HYPERTENSIONAHA.111.203521

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/61/6/1316

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/03/25/HYPERTENSIONAHA.111.203521.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Hypertension* is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Role of T regulatory lymphocytes in the pathogenesis of high-fructose diet-induced metabolic syndrome

Avshalom Leibowitz\textsuperscript{1}, Asia Rehman\textsuperscript{1}, Pierre Paradis\textsuperscript{1} and Ernesto L. Schiffrin\textsuperscript{1,2}
\textsuperscript{1}Lady Davis Institute for Medical Research, \textsuperscript{2}Department of Medicine, Sir Mortimer B. Davis-Jewish General Hospital, McGill University, Montreal, Quebec, Canada

Running title: High-fructose diet, metabolic syndrome and Treg

Address for correspondence and reprints:
Ernesto L. Schiffrin CM, MD, PhD, FRSC, FRCPC
Department of Medicine,
Sir Mortimer B. Davis-Jewish General Hospital, #B-127,
3755 Côte-Ste-Catherine Road,
Montréal, Québec, Canada H3T 1E2
Ph.: (514) 340-7538;
Fax: (514) 340-7539
Email: ernesto.schiffrin@mcgill.ca
Expanded materials and methods

Experimental design
Eight-week old Sprague-Dawley male rats (Harlan Laboratories, Indianapolis, IN) were fed normal chow diet (control) or high fructose diet for 5 weeks. The normal rodent chow (2018 Teklab, Harlan Laboratories, Madison, WI) was composed of 44% starch, 18.6% protein, 6.2% fat, 18.2% fibers and standard vitamins and mineral mix. The high-fructose diet (TD.89247, Harlan Laboratories) was composed of 60% fructose, 21% protein, 5% fat, 8% cellulose and standard vitamins and mineral mix. The rats were maintained in a temperature-controlled room (22 °C) and kept on a 14/10 h light–dark cycle. Food and water were available ad libitum. Blood pressure (BP) was measured by two methods, the tail-cuff technique and telemetry. Systolic BP by the tail-cuff method was measured as follows using a MC4000 BP analysis system (Hatteras Instruments, Cary, NC) after 4 weeks of high-fructose diet. In order to acclimatize the rats to blood pressure determination, 5 to 10 pre-measurements with cuff inflation were performed followed by 10 measurements. Artifactual blood pressure data caused by movement of the rats were removed and the average of at least 5 good determinations was used. BP was also determined by telemetry together with heart rate and animal activity in a separate group of rats. Five-week old male rats were anesthetized with isoflurane with 3% isoflurane mixed with O2 at 1 L/mL and depth of anesthesia confirmed by rear foot squeezing. Mouse PA-C10 telemetry transmitters (Data Sciences International, St. Paul, MN) were surgically instrumented in the abdominal aorta via the femoral artery as follows. An incision was made on the left rear leg, and the left femoral artery was isolated. The catheter of the telemetry transmitter was inserted inside the femoral artery to reach the abdominal aorta and attached to the blood vessel. The telemetry transmitter was placed in a subcutaneous pouch beside the abdominal cavity. The telemetry probe was turned on with a magnet, and the position of the catheter verified by detection of a pulsatile sound with an AM radio. The telemetry probe was turned off with the magnet, and the skin of the animal closed with sutures. The rat was placed into a cage inside a warmed recovery chamber until it regained righting reflexes. Rats were allowed to recover for 7 days. Thereafter, the BP, heart rate and activity were determined every 5 minutes for 10 seconds for 2 consecutive days at the baseline and at the end of every week during the five-week feeding with normal chow or high-fructose diet. The position of the catheter tip was confirmed at necropsy.

Three days before the end of the protocol, 0.5 mL of blood was collected from the saphenous vein on heparin for triglycerides and high-density lipoprotein (HDL) determination after 5 h of fasting fasting using a J&J Vitros 250 chemistry analyzer by the Diagnostic Research Support Services at the Comparative Medicine and Animal Resource Centre of McGill University. At the end of the protocol, body weight was measured. Rats were then anesthetized with 3 % isoflurane mixed with O2 at 1 L/min (depth of anesthesia confirmed by rear foot squeezing) and 3 mL of blood was collected by aorta puncture on EDTA for plasma nitric oxide (NO) metabolites (NOx) and interleukin (II)-10 determination. Heart, thoracic aorta, kidneys, liver, spleen and tibia were harvested and tissues were weighed and tibia length determined. Portions of the heart and aorta were kept in reactive oxygen species (ROS) assay buffer (RAB) (pH 7.4 containing [mmol/L]: 50 KH2PO4, 1 EGTA, 150 sucrose) for NADPH activity determination, embedded in VWR Clear Frozen Section Compound (VWR international, Edmonton, AL, Canada) for determination of ROS production and quantification of monocyte/macrophage infiltration by immunofluorescence or fixed in 4 % paraformaldehyde and embedded in paraffin for collagen deposition.
determination. The spleen was used to isolate Treg (CD4+CD25^{high}) and CD4^{+}CD25^{-} cells. The remaining tissues were frozen in liquid N\textsubscript{2} and stored at -80°C for determination of the expression of vascular (VCAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) by Western blot. All experimental procedures were approved by the Animal Care Committee of the Lady Davis Institute for Medical Research, McGill University, and followed the recommendations of the Canadian Council of Animal Care.

**Metabolic parameters**

Blood samples were centrifugation at 1,000 x g for 15 min at 4°C to remove blood cells followed by a centrifugation at 10,000 x g for 10 min at 4°C to remove platelets. Plasma samples were stored at -80°C until tested. Plasma triglycerides and HDL were measured using a J&J Vitros 250 chemistry analyzer by Diagnostic Research Support Services at the Comparative Medicine and Animal Resource Centre of McGill University.

**Small artery endothelial function and vascular remodeling**

Third-order branches of the mesenteric arterial tree (internal diameter between 180 and 250 µm) were dissected and mounted on a pressurized myograph as previously described.\textsuperscript{1} Vessels were equilibrated for 60 min at 45 mmHg of intraluminal pressure in warmed oxygenated (95% air–5% CO\textsubscript{2}) Krebs solution (pH 7.4) containing (mmol/l): 120 NaCl, 25 NaHCO\textsubscript{3}, 4.7 KCl, 1.18 KH\textsubscript{2}PO\textsubscript{4}, 1.18 MgSO\textsubscript{4}, 2.5 CaCl\textsubscript{2}, 0.026 EDTA and 5.5 glucose. Lumen diameter was measured with a computer-based video imaging system (Living Systems Instrumentation, Burlington, Virginia, USA). Endothelium-dependent relaxation was assessed by measuring vasodilatory responses of norepinephrine-precontracted vessels (5 x 10^{-5} mol/l) to cumulative increasing concentrations of acetylcholine (10^{-9}–10^{-4} mol/l). Media thickness and lumen diameter were measured at increasing intraluminal pressures from 3 to 140 mmHg. Media-to-lumen ratio and media cross-sectional area at 45 mmHg were calculated as described previously.\textsuperscript{2}

**Reactive oxygen species (ROS) production**

NADPH oxidase activity was measured in aorta and apex of the heart as previously described.\textsuperscript{1} Briefly, samples were incubated for 30 min in RAB and homogenized in ROS lysis buffer containing (mmol/L): 20 KH\textsubscript{2}PO\textsubscript{4} and 1 EGTA. A lucigenin-based chemiluminescence assay was used to determine NADPH oxidase activity using 100 µl of homogenate in an Orion II microplate luminometer (Berthold detection systems GmbH, Pforzheim, Germany). Background was determined over 10 s, 100 µl of lucigenin (12.5 µmol/L in RAB) were injected and light measured for 1 s every ~6 s for 3 min. Then 50 µL of NADPH (0.5 mmol/L in RAB) were injected and light was measured for an additional 3 min as for lucigenin. NADPH oxidase activity was calculated by subtracting the results of luminescence before from after NADPH addition. Activity was corrected for protein concentration and expressed as relative light units per mg protein. Vascular ROS production was assessed on 5 µm cryosections of aorta with the ROS-sensitive fluorescent dye dihydroethidium (DHE, 2 µmol/L) in dark conditions for 1 min at 37°C. Fluorescence was visualized and captured with a fluorescence microscope with a CY3 filter and quantified using ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA).
Lymphocyte isolation and Treg culture

CD4+CD25− lymphocytes and Treg (CD4+CD25high) were isolated from the spleen in two steps using magnetic bead negative selection with the Custom Rat EasySep® CD4+ Cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada) followed by fluorescence-activated cell sorting. Purity of sorted populations was ≥97.9 % (Supplemental Fig. S1). Sorted Treg were cultured on mouse anti-CD3 (G4.18, BD Biosciences)-coated 96-well plates (100,000 cells/well) in RPMI 1640+Glutamax (Invitrogen Corp.) containing 0.1 mmol/L non-essential aminoacids, 1 mM sodium pyruvate, 100 U/mL penicillin, 0.1 mg/ml streptomycin, 10 % heat inactivated fetal bovin serum and 0.02 mmol/L 2-mercaptoethanol at 37°C in a humidified incubator (5 % CO2-95 % air) for 48 h. Media was collected and stored at -80°C until IL-10 determination as above.

Plasma NO metabolites

Plasma NO metabolites (NOx), nitrite and nitrate, were measured with a colorimetric assay using the Griess reaction following conversion of nitrate to nitrite with cadmium as described previously.3 In brief, 50 µL of plasma sample were incubated with cadmium overnight at 4°C with gentle mixing. Fifty µL of 0.01% N-(1-naphtyl) ethylenediamine was added, and samples were incubated for 5-10 min. Fifty µL of 0.1 % p-aminobenzenesulfonamide was added, and samples were incubated another for 5-10 min. Nitrite was determined by measuring the absorbance at 548 nm. A solution of sodium nitrate was used to build the standard curve.

Plasma C peptide levels

Plasma insulin C peptide levels were measured by ultrasensitive ELISA kit (Mercodia AB, Uppsala, Sweden) according to the manufacturer’s protocol.

Immunofluorescence detection of macrophages.

Immunofluorescence microscopy was performed on 5 µm cryosections of aorta. Sections were air-dried for 30 min, fixed for 5 min in a mixture of acetone:methanol (1:1) at -20°C and washed with PBS containing 0.1 % Tween-20 (PBST) twice for 10 min. Sections were blocked with PBST containing 10% normal goat serum for 1 h at room temperature, and then incubated with a monoclonal mouse anti-rat CD68 (ED1, 1:100, AbD Serotec, Kidlington, UK) in blocking solution overnight at 4°C. The sections were washed 3 times with PBST and incubated with Alexa Fluor® 647 goat anti-mouse (1:200, Invitrogen Corp., Carlsbad, CA, USA) in the blocking solution for 1 h at room temperature and then washed 3 times with PBST and mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Images were captured using a fluorescent microscope with a CY5 filter.

Vascular collagen deposition

Sections (5 µm) of paraffin-embedded aorta were stained with Sirius red to determine aortic collagen type I and III, content and analyzed by an image analysis system (Northern Eclipse 5.0, EMPIX Imaging Inc, Mississauga, ON, Canada). Collagen type I and III depositions were evaluated in the entire aortic sections, using a 10X objective. Collagen fraction was defined as the ratio of the stained area to the total tissue area and expressed as a percentage.

Western blot analysis

Expression of vascular (VCAM-1) and platelet endothelial cell adhesion molecule 1 (PECAM-1) was determined by Western blot using goat anti-VCAM-1 (Santa Cruz Biotechnology, Santa
Cruz, CA, USA) and goat anti-PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signal was revealed by chemiluminescence (SuperSignal West Pico, Thermo Scientific, Rockford, IL, USA) with the Molecular Imager Chemidoc XRS system (Bio- Rad, Mississauga, ON, Canada), and quantified by densitometry using Image lab software (Bio-Rad). Membranes were subsequently stripped and probed with anti-β-actin antibody (Sigma-Aldrich, Oakville, ON, Canada) to verify equal loading.

**Lymphocyte isolation and Treg culture**

CD4⁺CD25⁻ cells and Treg (CD4⁺CD25high) were isolated from the spleen of control and high-fructose diet-fed rats (n = 7) in two steps. First, CD4⁺ enrichment was done by magnetic bead negative selection using the Custom Rat EasySep® CD4⁺ Cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada) following the manufacturer’s instructions. The CD4⁺ enriched population was stained with mouse anti-CD4-APC (OX-35, BD Biosciences, Mississauga, ON, Canada) and mouse anti-CD25-PE (OX-39). CD4⁺CD25⁻ cells and Treg were isolated using a fluorescence-activated cell sorter ARIA II (BD Biosciences). Cells were sorted directly into culture media (see below). Purity of sorted populations was assessed by running an aliquot of the cells on the sorter. Purity was ≥97.9 % (Supplemental Fig. S1). Sorted Treg were cultured on mouse anti-CD3 (G4.18, BD Biosciences)-coated 96-well plates (100,000 cells/well) in RPMI 1640+Glutamax (Invitrogen Corp.) containing 0.1 mmol/L non-essential aminoacids, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 % heat inactivated fetal bovine serum and 0.02 mmol/L 2-mercaptoethanol. The culture plates were maintained at 37°C in a humidified incubator (5 % CO₂-95 % air) during 48 h and media was collected and stored at -80°C until interleukin (IL)-10 determination.

**IL-10 levels measurement**

Blood samples were processed as above. IL-10 levels in plasma (on EDTA) and culture media were measured using micro-bead multiplex immunoassays on a Bio-Plex 200 (Bio-Rad Laboratories) in duplicate.
References


Supplemental Table S1. Heart, lung and spleen weight were not affected by 8 weeks of high-fructose diet induced metabolic syndrome

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctrl</th>
<th>High-fructose diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>HW (g)</td>
<td>1.18 ± 0.04</td>
<td>1.17 ± 0.03</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>2.88 ± 0.08</td>
<td>2.93 ± 0.05</td>
</tr>
<tr>
<td>HW/TL (g/cm)</td>
<td>0.27 ± 0.01</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>LuW (g)</td>
<td>1.48 ± 0.04</td>
<td>1.46 ± 0.08</td>
</tr>
<tr>
<td>LuW/BW (mg/g)</td>
<td>3.60 ± 0.07</td>
<td>3.65 ± 0.14</td>
</tr>
<tr>
<td>LuW/TL (g/cm)</td>
<td>0.34 ± 0.01</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>SW (g)</td>
<td>0.78 ± 0.03</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>SW/BW (mg/g)</td>
<td>1.90 ± 0.06</td>
<td>1.89 ± 0.07</td>
</tr>
<tr>
<td>SW/TL (g/cm)</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
</tbody>
</table>

Tissue weights were determined in rats fed a high-fructose diet or normal chow (Ctrl) for 5 weeks. Heart, lung and spleen weights, HW, LuW and SW; body weight, BW; tibia length, TL. Values are means ± SEM, with n= 10.
Supplemental Figure S1. CD4⁺CD25⁻ cells and Treg were isolated with high efficiency using a two steps procedure from spleen of rats fed a high-fructose diet (HFrD) or a normal chow (Ctrl) for 5 weeks. CD4⁺ cells were enriched from splenocytes using a magnetic beads negative selection. The numbers of splenocytes (A) and enriched CD4⁺ cells per spleen (B) were determined. A representative fluorescence-activated cell sorting analysis is shown in C. The population of lymphocytes was gated within pre-enriched CD4⁺ cells using the forward scatter (FSC) and side scatter (SSC). The CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were sorted from the lymphocytes with appropriate gating using anti-CD4-APC and anti-CD25-PE fluorescence signals. The percentage of sorted CD4⁺CD25⁻ (D) and CD4⁺CD25⁺ (E) cells was determined. Representative flow cytometry analysis of sorted CD4⁺CD25⁻ (D) and CD4⁺CD25⁺ (E) are shown.
Supplemental Figure S2. A high-fructose diet did not alter systolic blood pressure determined by telemetry. Mean 24 h, day and night systolic blood pressures, heart rate and activity were determined by telemetry in rats at baseline and at the end of every week during the five-week feeding with normal chow (Ctrl) or high-fructose diet (HFrD). Values are means ± SEM, n = 5.
Supplemental Figure S3. Plasma C-peptide levels were measured by ELISA in rats fed a high-fructose diet (HFrD) and normal chow (Ctrl) for 5 weeks. Values are means ± SEM, n = 6.
Supplemental Figure S4. A high-fructose diet (HFrD) did not alter mesenteric artery vasodilatory response to acetylcholine, media-to-lumen ratio and media cross-sectional area compared to control (Ctrl) rats. Values expressed are means ± SEM, n = 6-7
Supplemental Figure S5. A high-fructose diet increased oxidative stress in rats. Dihydroethidium (DHE) staining demonstrating reactive oxygen species (ROS) production was determined in rats fed a high-fructose diet (HFrD) or normal chow (Ctrl) for 5 weeks. Representative images of DHE-stained aortic sections and quantification are presented. Blue and green fluorescence represent nuclear DAPI staining and autofluorescence of elastin, respectively.
Supplemental Figure S6. Vascular Monocyte/macrophage infiltration. Monocyte/macrophage infiltration (red fluorescence, ED1 staining) in aorta and perivascular fat was determined in rats fed a high-fructose diet (HFrD) or normal chow (Ctrl) for 5 weeks. Representative Images of ED1⁺ cells in aortic adventitia and perivascular fat of Ctrl and the HFrD rats and ED1 density for each individual (open circle) and the means (close circle) for each group are presented. Blue and green fluorescence represent nuclear DAPI staining and autofluorescence of elastin, respectively. Values are means ± SEM, n = 5-8.
Supplemental Figure S7. High-fructose diet caused vascular fibrosis in rats. Collagen type I and III content in the media of descending thoracic aorta by Sirius red staining was determined in rats fed a high-fructose diet (HFrD) or normal chow (Ctrl) for 5 weeks. A. Representative images of Sirius red stained sections of aorta and quantification are shown. Values are means ± SEM, *P<0.01 vs. Ctrl, n = 6.
**Supplemental Figure S8.** Plasma interleukin (II)-10 (A) and percentage of spleen CD4^+CD25^- (B) and CD4^+CD25^{high} lymphocytes (C) were determined in rats fed a high-fructose diet (HFrD) or normal chow (Ctrl) for 5 weeks. Values are means ± SEM, n = 7.