Empagliflozin Protects against Diet-Induced NLRP-3 Inflammasome Activation and Lipid Accumulation

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ABSTRACT

The aim of this study was to evaluate the effects of chronic treatment with empagliflozin, a potent and selective sodium glucose cotransporter-2 inhibitor, in a murine model of diet-induced obesity and insulin resistance, focusing on drug effects on body weight reduction and nucleotide-binding domain, leucine-rich repeat containing protein (NLRP)-3 inflammasome activation, which have never been investigated to date. Male C57BL/6 mice were fed control or a high fat–high sugar (HFHS) diet for 4 months. Over the last 2 months, subsets of animals were treated with empagliflozin (1–10 mg/kg) added to the diet. Empagliflozin evoked body weight reduction (P < 0.001 for the highest dose) and positive effects on fasting glycemia and homeostasis model assessment of insulin resistance. In addition, the drug was able to reduce renal tubular damage and liver triglycerides level in a dose-dependent manner. Interestingly, empagliflozin also decreased cardiac lipid accumulation. Moreover, diet-induced activation of NLRP-3 in kidney and liver (not observed in the heart) was dose-dependently attenuated by empagliflozin. Our results clearly demonstrate the ability of empagliflozin to counteract the deleterious effects evoked by chronic exposure to HFHS diet. Most notably, empagliflozin treatment was associated with NLRP-3 inflammasome signaling modulation, suggesting that this inhibition may contribute to the drug therapeutic effects.

Introduction

In the past decade, compelling data have highlighted the association between metabolic disorders and inflammation (Hotamisligil, 2006; Shoelson et al., 2006). In particular, it has been demonstrated that abdominal obesity is associated with low-grade inflammation that leads to insulin resistance and metabolic disorders, thus supporting the recently emerged concept of type 2 diabetes mellitus as an inflammatory disease (Donath and Shoelson, 2011). One of the most accredited signaling pathways involved in the pathogenesis of obesity-induced insulin resistance is the nucleotide-binding domain, leucine-rich repeat containing protein (NLRP)-3 inflammasome (Davis et al., 2011; Vandannagasvar et al., 2011).

The NLRP-3 inflammasome is a large intracellular multi-meric protein danger-sensing platform that promotes the autocatalytic activation of the cysteine protease caspase-1 and mediates the proteolytic activation of proinflammatory cytokines, including pro-interleukin (IL)-1β (Lamkanfi, 2011).

We and others have previously demonstrated that the NLRP-3 inflammasome contributes to the metabolic abnormalities described in the pathogenesis of obesity-induced insulin resistance, and its pharmacological or genetic modulation exerts protective effects against adiposity increase and insulin resistance (Stienstra et al., 2011; Chiazza et al., 2015). In particular, a causal role for IL-1β in this scenario has been recognized, and its contribution to the impairment of insulin signaling and to the onset and/or maintenance of insulin resistance is well known (Larsen et al., 2007; Dinarello, 2009). Interestingly, the activation of NLRP-3 in type 2 diabetes mellitus pathogenesis is further supported by recent evidence that treatment with metformin, the most widely prescribed oral antihyperglycemic agent, triggers inhibition of caspase-1 cleavage and IL-1β maturation in monocyte-derived macrophages from diabetic patients (Lee et al., 2013).

Inhibitors of the sodium glucose cotransporter (SGLT)-2 are a novel class of antidiabetic drugs (Idris and Donnelly, 2009). SGLT-2 is located on the apical side of the proximal tubular cells and accounts for the majority (about 90%) of glucose reabsorption in the kidney (Wright, 2001). Inhibition of SGLT-2 activity increases glucose excretion, thus lowering blood glucose level (Bakris et al., 2009). Due to their insulin-independent mode of action, SGLT-2 inhibitors are an attractive pharmacological strategy, distinct from traditional oral antidiabetic therapies. Moreover, administration of these drugs is associated with additional beneficial effects, including body weight (BW) loss (Kurosaki and Ogasawara, 2013; Monami et al., 2014).

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ABBREVIATIONS: ACR, albumin-to-creatinine ratio; AUC, area under the curve; BW, body weight; ELISA, enzyme-linked immunosorbent assay; HFHS, high fat–high sugar; HOMA IR, homeostasis model assessment of insulin resistance; IL, interleukin; LDL, low-density lipoprotein; NLRP, nucleotide-binding domain, leucine-rich repeat containing protein; OGTT, oral glucose tolerance test; SGLT, sodium glucose cotransporter; TG, triglyceride.
Some studies have demonstrated that SGLT-2 inhibitors could counteract inflammation associated with diabetic conditions in kidney (Gembardt et al., 2014; Vallon et al., 2014) and liver organs (Tahara et al., 2013) of Akita or type 2 diabetic mice. However, to date, the effects of SGLT-2 inhibitors on NLRP-3 inflammasome activation have not been described.

The aim of this study was to evaluate the effects of empagliflozin, a potent and selective SGLT-2 inhibitor (Grempler et al., 2012), in a murine model of obesity and insulin resistance induced by a diet.

The research was focused on drug effects against BW increase and on the potential drug ability to affect NLRP-3 inflammasome activation in the target organs of obesity/diabetic diseases.

Materials and Methods

Animals and Experimental Procedures. Four-week-old male C57BL/6J mice (n = 120; provided by Charles River, Lecco, Italy) were housed in a controlled environment at 25 ± 2°C with alternating 12-hour light and dark cycles and fed normal diet during a 1-week adaptation period. The animals were then randomly (1:2) allocated in two experimental groups: mice fed a control diet or a high-fat–high sugar (HFHS) diet for 8 weeks. The HFHS diet (D12451) contained 45% kcal fat (lard and soybean oil), 20% protein (casein), and 35% carbohydrate (fructose 55% and glucose 45%; D12451, ssniff, Specialdiäten GmbH, Ferdinand-Gabriel-Weg, Germany). Animals were then randomly allocated in six groups (n = 20): control group (control); control group plus 10 mg/kg empagliflozin (C + E10); HFHS group (HFHS); HFHS plus 1 mg/kg empagliflozin (HFHS + E1); HFHS plus 3 mg/kg empagliflozin (HFHS + E3); and HFHS plus 10 mg/kg empagliflozin (HFHS + E10). Empagliflozin mixed in diet at different titrations was chosen to deliver a daily dosage of approximately 1, 3, and 10 mg/kg in regard to proven improvement of glycemia and feature of metabolic syndrome in diabetic rats (Thomas et al., 2012).

The animal protocol has been carried out in accordance with the European Directive 2010/63/EU as well as the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, and was approved by the local ethical committee.

Food Intake Determination. Every week 200/250 g foods were placed in every cage. Food intake was measured by dividing the food consumption for the number of animals present in the cage.

Oral Glucose Tolerance Test. The day before sacrifice, after a fasting period of 16 hours, oral glucose tolerance test (OGTT) was performed. Once before glucose administration (2 g/kg by oral gavage), and 15, 30, 60, 120, and 180 minutes afterward, blood samples were obtained from the saphenous vein puncture, and glucose concentrations were determined with a conventional Glucometer (Glucomen LX Plus; A. Menarini Diagnostics, Florence, Italy).

Biochemical Analysis. At the end of the study (week 16), after 16-h fasting period, the mice were anesthetized using isoflurane via an anesthesia machine (IsoFlo; Abbott Laboratories, Chicago, IL) and sacrificed by cardiac puncture/exsanguination. Glycemia was measured using the GlucoMen LX kit. Plasma lipid profiles were determined by measuring the content of triglycerides (TGs), total cholesterol, and high-density lipoprotein by using commercial reagent kits (Hospitex Diagnostics, Florence, Italy). Low-density lipoprotein (LDL) was determined by calculation [LDL = total cholesterol – (high-density lipoprotein + TG/5)]. Plasma insulin and tissue IL-1β levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine ELISA Kit; R&D Systems, Minneapolis, MN). Urinary albumin excretion was evaluated and expressed as albumin-to-creatinine ratio (ACR) in 18-hour urine collection (Mouse Albumin ELISA Quantitation Set, Bethyl Laboratories, Montgomery, TX). Creatinine Colorimetric Assay Kit, Cayman Chemical, Ann Arbor, MI).

Tissue Extracts. Kidney, liver, and cardiac apex extracts were prepared, as previously described (Benetti et al., 2013). Briefly, tissues were homogenized and centrifuged. Supernatants were removed, and the protein content was determined using a bicinchoninic acid protein assay following the manufacturer’s instructions (Pierce Biotechnology, Rockford, IL).

Western Blot Analysis. About 60 μg total proteins were loaded for Western blot experiments, as previously described (Collino et al., 2013). The membranes were stripped and incubated with β-actin/tubulin monoclonal antibody to assess gel-loading homogeneity.

Histopathological Examination. Sagittal sections of both kidneys and fragments from the left lateral and medial lobes of livers were fixed in 4% buffered formaldehyde solution overnight at 4°C. Dewaxed 5-μm sections were stained with H&E and examined under an Olympus Bx41 microscope (original magnification, 40×) with an AxioCamMR5 photographic attachment. Vacuolar degeneration of renal cortext proximal tubules was classified as light (<10% of tubules with vacuolar degeneration), moderate (10–20%), and severe (>20%). The level of macromolecular/microvesicular hepatic steatosis was evaluated according to the following grading: light (<30% hepatocytes with macrovesicles/microvesicles), moderate (31–60%), and severe (>60%).

Heart Oil Red Staining. Neutral lipids were assessed on frozen sections of cardiac apex (10 μm in thickness) by Oil Red O staining using an Olympus Bx41 microscope (original magnification, 40×) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany).

TG Level. Hepatic and cardiac TGs were extracted from total tissue homogenates of randomly selected animals and assayed using reagent kits, according to the manufacturer’s instructions (Triglyceride Quantification Kit; Abnova, Aachen, Germany).

Materials. Unless otherwise stated, all compounds were purchased from Sigma-Aldrich (St. Louis, MO). Polynvinylidene difluoride was from Millipore (Bedford, MA). Primary antibodies were from Epitomics-Abcam (Cambridge, UK), Adipogene (Liestal Switzerland), and Sigma-Aldrich for NLRP-3 (1:5000), caspase-1-1p-20 (1 μl/mil), and β-actin (1:10000), respectively. Secondary antibodies were from Cell Signaling Technology (Beverly, MA; 1:5000), and Luminol ECL from PerkinElmer (Waltham, MA).

Statistical Analysis. All values in both the text and figures are expressed as mean ± S.E.M. for n observations. One-way or two-way analysis of variance with Bonferroni’s post hoc test (when applicable) was performed using the GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA), and P values below 0.05 were considered as significant.

Results

Effect of Empagliflozin on the Diet-Induced BW Increase. Average BW of HFHS mice was higher than that recorded in the control group throughout the experiment (P < 0.001 versus HFHS, Fig. 1A).

At the end of the study (week 8), the BW were 25.17 ± 0.76 g (ΔBW = +13.54% versus week 8), and 34.59 ± 3.03 g (ΔBW = +22.58% versus week 8) for the control and HFHS groups, respectively.

In comparison with HFHS animals, a lower BW was recorded in empagliflozin-treated groups: 33.49 ± 3.39 g (ΔBW = +17.73% versus week 8), 33.92 ± 4.58 g (ΔBW = +18.01%), and 31.24 ± 2.46 g (ΔBW = +9.97%) for HFHS + E1, HFHS + E3, and HFHS + E10, respectively, albeit only the highest dose evoked statistically significant differences (P < 0.001 versus HFHS group). Notably, during the drug treatment (from week 8 to week 16) the average BW gain in mice treated with the highest dose of empagliflozin (10 mg/kg) was similar to that recorded in the control group (2.83 versus 3.1),
Empagliflozin Prevents NLRP-3 Inflammasome Activation

The homeostasis model assessment of insulin resistance (HOMA-IR) increased threefold in HFHS animals versus control animals \( (P < 0.001) \), and a significant reduction was evoked by empagliflozin at all the tested doses (Table 1).

Empagliflozin did not affect the diet-induced increase of plasma tot-cholesterol and LDL-cholesterol levels.

The fasting glucose, over time of experimentation, was highlighted in Fig. 2A. Chronic exposure to HFHS diet caused an increase in fasting glycemia since the first month of diet manipulation, reaching the maximum level after 2 months \( (P < 0.001 \text{ versus control}) \). Empagliflozin significantly improved glycemic control at each dose tested after the first month of drug administration (Fig. 2A).

In addition, HFHS diet caused a deterioration of glucose tolerance (OGTT, Fig. 2B) in comparison with control diet \( [\text{area under the curve (AUC)}_{\text{HFHS}} > \text{AUC}_{\text{control}}] \). As shown in Fig. 2, B and C, compared with HFHS group, 10 mg/kg empagliflozin exerted a significant improvement of glucose tolerance \( (\text{AUC}_{0–180\text{min}}, P < 0.05 \text{ versus HFHS, Fig. 2C}) \).

**Effect of Empagliflozin on Diet-Induced Renal Damage.** As shown in Fig. 3A, HFHS diet was associated with a severe vacuolar degeneration of the tubular epithelial cells of the S1–S2 of the contorted proximal tubules. Drug treatment attenuated damage severity in a dose-dependent manner (the effect of the highest dose is displayed in the picture).

Compared with control mice, HFHS animals showed a significant increase in the ACR, thus confirming renal dysfunction \( (D’Amico and Bazzi, 2003) \). Empagliflozin significantly counteracted this effect at all the tested doses (Fig. 3B).

**Effect of Empagliflozin on Renal NLRP-3 Inflammasome Activation.** As shown in Fig. 4, kidneys from HFHS mice showed both NLRP-3 overexpression (Fig. 4A) and caspase-1 activation, as indicated by the appearance of the p20 subunit of caspase-1 (Fig. 4B). The end product of NLRP-3 inflammasome activation, the mature cytokine IL-1\( \beta \), was significantly increased in the kidneys of HFHS mice, in comparison with control animals (Fig. 4C). Both caspase-1 activation and IL-1\( \beta \) production were decreased in kidneys from HFHS mice treated with empagliflozin. In particular, the effect was statistically significant for empagliflozin at doses of 3 mg/kg and 10 mg/kg.

**Effect of Empagliflozin on Diet-Induced Liver Damage.** HFHS feeding produced a severe mixed fatty change in terms of hepatocytes with micro- and macrovacuoles. They were detected predominantly in the peribiliary zones of the acini. When mice were treated with empagliflozin, the degree of steatosis was attenuated and the number of hepatocytes with lipid droplets was markedly reduced (higher dose effect represented in Fig. 5A). HFHS animals displayed a fourfold increase in hepatic TGs measured in liver homogenates, and this value was dose-dependently decreased by the drug (Fig. 5B).

**Effect of Empagliflozin on Hepatic Diet-Induced NLRP-3 Inflammasome Activation.** To further investigate the empagliflozin effect on liver, we evaluated whether hepatic steatosis was correlated with diet-induced NLRP-3 inflammasome activation and the ability of the drug to modulate this signaling pathway.

In comparison with HFHS animals, drug treatment was dose-dependently associated to a decrease of NLRP-3 expression (Fig. 6A), with a significant effect also on caspase-1 activation by the highest dose (Fig. 6B). The effect on inflammasome

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**Fig. 1.** Effects of diet manipulation and empagliflozin treatment on mice BW. BW (A) and food intake (B) of mice were measured every week during diet manipulation (1–16 weeks) and drug treatment (9–16 weeks). Values are means ± S.E.M. for \( n = 20 \) animals per group. At the end of week 1, weights of all HFHS groups are statistically different from control groups \( (P < 0.001). \# P < 0.05 \text{ versus HFHS, ###P < 0.001 versus HFHS, ***P < 0.001 versus Control.} \)

**Fig. 2.** Effects of diet manipulation and empagliflozin treatment on mice OGTT, AUC (area under the curve) was calculated for control and diet groups \( (\text{AUC}_{\text{HFHS}} > \text{AUC}_{\text{control}}) \). As shown in Fig. 2, B and C, compared with HFHS group, 10 mg/kg empagliflozin exerted a significant improvement of glucose tolerance \( (\text{AUC}_{0–180\text{min}}, P < 0.05 \text{ versus HFHS, Fig. 2C}) \).

**Fig. 3.** Effects of diet manipulation and empagliflozin treatment on mice renal damage (ACR). As shown in Fig. 3A, HFHS diet was associated with a significant increase in the ACR, thus confirming renal dysfunction \( (D’Amico and Bazzi, 2003) \). Empagliflozin significantly counteracted this effect at all the tested doses (Fig. 3B).

**Fig. 4.** Effects of diet manipulation and empagliflozin treatment on mice renal NLRP-3 inflammasome activation. As shown in Fig. 4, kidneys from HFHS mice showed both NLRP-3 overexpression (Fig. 4A) and caspase-1 activation, as indicated by the appearance of the p20 subunit of caspase-1 (Fig. 4B). The end product of NLRP-3 inflammasome activation, the mature cytokine IL-1\( \beta \), was significantly increased in the kidneys of HFHS mice, in comparison with control animals (Fig. 4C). Both caspase-1 activation and IL-1\( \beta \) production were decreased in kidneys from HFHS mice treated with empagliflozin. In particular, the effect was statistically significant for empagliflozin at doses of 3 mg/kg and 10 mg/kg.

**Fig. 5.** Effects of diet manipulation and empagliflozin treatment on mice hepatic damage. HFHS feeding produced a severe mixed fatty change in terms of hepatocytes with micro- and macrovacuoles. They were detected predominantly in the peribiliary zones of the acini. When mice were treated with empagliflozin, the degree of steatosis was attenuated and the number of hepatocytes with lipid droplets was markedly reduced (higher dose effect represented in Fig. 5A). HFHS animals displayed a fourfold increase in hepatic TGs measured in liver homogenates, and this value was dose-dependently decreased by the drug (Fig. 5B).

**Fig. 6.** Effects of diet manipulation and empagliflozin treatment on mice hepatic NLRP-3 inflammasome activation. To further investigate the empagliflozin effect on liver, we evaluated whether hepatic steatosis was correlated with diet-induced NLRP-3 inflammasome activation and the ability of the drug to modulate this signaling pathway.

In comparison with HFHS animals, drug treatment was dose-dependently associated to a decrease of NLRP-3 expression (Fig. 6A), with a significant effect also on caspase-1 activation by the highest dose (Fig. 6B). The effect on inflammasome
pathway resulted in a consequent dose-dependent reduction in IL-1β production (Fig. 6C). The effect was statistically significant at 10 mg/kg (P < 0.05 versus HFHS).

### Effect of Empagliflozin on Cardiac Lipid Accumulation and NLRP-3 Inflammasome.

Concurrent with the development of hepatic steatosis, HFHS animals displayed evident lipid deposition in the heart (Fig. 7A). As shown by Oil Red O staining (Fig. 7A), cytoplasmic micro- or macrovascular lipid droplets were detected in animal fed the HFHS diet. Empagliflozin administration attenuated fat accumulation, and this effect was confirmed by a significant diminution of TG content (Fig. 7B), already evident at 1 mg/kg.

In contrast to the effects of HFHS diet in kidney and liver, we did not detect NLRP-3 overexpression and caspase-1 activation in the heart (data not shown), as well as an increase of cardiac IL-1β production (Fig. 7C). In this condition, treatment with empagliflozin did not affect the cardiac NLRP-3 signaling pathway.

### Discussion

This study investigates, for the first time, the effects of the SGLT-2-selective inhibitor empagliflozin on the activation of NLRP-3 inflammasome in a murine model of diet-induced obesity and insulin resistance. To date, the effect of empagliflozin has been tested in diabetic models that exhibit obvious hyperglycemia and insulin resistance for genetic manipulation [Akyta mice (Vallon et al., 2014), ob/ob (Gembardt F et al., 2014), Zucker rat (Thomas et al., 2012)], and no studies have been performed in models in which the pathologic features (hyperglycemia, insulin resistance, and oversize) are due to an unhealthy life style (i.e., hypercaloric diet and sedentary life), closely resembling changes observed in human with type 2 diabetes (Panchal et al., 2011).

Drug administration started after 2 months of diet manipulation, when the detrimental effects of HFHS diet on metabolic parameters (e.g., BW, blood glucose level) were established. This therapeutic design was chosen to enhance validity and fidelity of our model, thus allowing a superior translatability.

According to literature (Liakos et al., 2014), empagliflozin treatment was associated with weight loss, especially at the dose of 10 mg/kg. At that dose, the BW gain of animals under HFHF diet was comparable to animals with chow diet.

In our study, no compensatory increase in food intake was observed in animals treated with empagliflozin, in contrast to results obtained in diet-induced obese rats treated with dapagliflozin (Devenny et al., 2012).

Empagliflozin showed a marked ability to decrease fasting glycemia and a modest, but still significant, effect on OGTT performed in fasted animals. In addition, empagliflozin significantly prevented diet-induced increase of HOMA IR index, a well-recognized marker of insulin resistance, thus supporting data indicating that SGLT-2 inhibition attenuates the hyperglycemia-induced decline in insulin sensitivity (Rossetti et al., 1987; Musso et al., 2012).

Drug treatment was also associated with a reduction of TG accumulation in liver and heart. Microscopic observations confirm that empagliflozin decreased morphologic alterations associated with steatosis in these two tissues. Such effect on liver TGs was previously reported with another SGLT-2 inhibitor, ipragliflozin, in a similar experimental model, thus suggesting that SGLT-2 inhibition could be useful to prevent the establishment of nonalcoholic fatty liver disease in type 2 diabetes (Tahara et al., 2013).

Interestingly, our study is the first one demonstrating that a SGLT-2 inhibitor counteracts the diet-induced cardiac fat accumulation. This observation could help to explain the improvement in cardiovascular injury and remodeling induced by the drug in ob/ob mice (Lin et al., 2014). Levelt et al. (2016) demonstrated that myocardial steatosis is a predictor of concentric left ventricle remodeling and a prominent and early feature of diabetic cardiomyopathy. Therefore, strategies aimed at reversing myocardial steatosis may potentially improve prognosis in patients with diabetes.

On this basis, the ability of empagliflozin to counteract cardiac fatty acid accumulation may contribute, at least in part, to prevent cardiovascular event in type 2 diabetic patients, as recently reported in the Empa-Reg Outcome study (Zinman et al., 2015).

Empagliflozin treatment caused a decrease in pathologic cell alterations in the kidneys of mice fed the HFHS diet. In

Effect of diet manipulation and empagliflozin treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control + E10</th>
<th>HFHS</th>
<th>HFHS + E1</th>
<th>HFHS + E3</th>
<th>HFHS + E10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caloric intake (KJ/die/mouse)*</td>
<td>37.16 ± 1.85</td>
<td>41.68 ± 3.51</td>
<td>47.78 ± 7.56***</td>
<td>49.01 ± 5.48***</td>
<td>50.1 ± 6.12***</td>
<td>48.15 ± 6.71***</td>
</tr>
<tr>
<td>Energy efficiency (g/MJ)*</td>
<td>1.40 ± 0.26</td>
<td>1.04 ± 0.40</td>
<td>2.23 ± 0.68***</td>
<td>1.83 ± 0.64</td>
<td>1.85 ± 0.99</td>
<td>1.65 ± 0.57***</td>
</tr>
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Anatomic Parameter

| Weight gain (g)* | 3.1 ± 0.55 | 2.42 ± 0.93 | 5.9 ± 1.81*** | 5.04 ± 1.75** | 5.18 ± 2.77*** | 2.83 ± 1.54*** |
| Liver weight (g) | 0.87 ± 0.09 | 0.85 ± 0.07 | 1.04 ± 0.16*** | 1.05 ± 0.14*** | 1.06 ± 0.13*** | 0.98 ± 0.08 |
| Kidney weight (g) | 0.26 ± 0.02 | 0.26 ± 0.02 | 0.30 ± 0.03*** | 0.32 ± 0.03*** | 0.32 ± 0.04*** | 0.32 ± 0.04*** |
| Heart weight (g) | 0.11 ± 0.01 | 0.11 ± 0.01 | 0.12 ± 0.02 | 0.13 ± 0.02 | 0.12 ± 0.02 | 0.12 ± 0.02 |
| Epididymal fat weight (g) | 0.43 ± 0.11 | 0.36 ± 0.09 | 1.72 ± 0.53*** | 1.55 ± 0.57*** | 1.65 ± 0.75*** | 1.31 ± 0.36**** |

Plasmatic Parameter

| Fasting glucose (mg/dl) | 113.30 ± 11.79 | 99.38 ± 24.04 | 171.3 ± 21.40*** | 148.6 ± 39.27** | 136.07 ± 25.22*** | 128.93 ± 21.93*** |
| Insulin (pg/ml) | 271.5 ± 105.3 | 230.2 ± 57.1 | 418.6 ± 131.90*** | 356.9 ± 96.7* | 422.5 ± 130.85* | 417.3 ± 109.25** |
| HOMA IR | 1.65 ± 1.03 | 1.27 ± 0.44 | 5.04 ± 1.45*** | 2.47 ± 1.06*** | 3.19 ± 0.74* | 2.85 ± 0.45*** |
| Total chol (mg/dl) | 102.32 ± 24.31 | 109.73 ± 5.34 | 138.86 ± 10.89* | 126.51 ± 31.51 | 117.82 ± 18.60 | 120.91 ± 17.54 |
| LDL chol (mg/dl) | 40.67 ± 2.84 | 42.18 ± 3.84 | 65.23 ± 5.80* | 54.87 ± 20.64 | 50.19 ± 13.31 | 52.27 ± 12.27 |
| HDL chol (mg/dl) | 43.68 ± 4.13 | 48.30 ± 4.28 | 53.26 ± 6.27 | 50.18 ± 8.20 | 49.64 ± 2.42 | 48.55 ± 11.94 |
| Triglycerides (mg/dl) | 86.2 ± 18.3 | 83.3 ± 9.5 | 108.5 ± 14.6 | 103.1 ± 8.9 | 91.3 ± 30.2 | 95.4 ± 11.3 |

Chol, cholesterol; HDL, high-density lipoprotein.

*Average from 8 to 16 weeks.

**P < 0.05, ***P < 0.01, ****P < 0.001 versus control; *P < 0.05, **P < 0.01 versus HFHS.
accordance with previous papers (Janigan and Santamaria, 1961; Trump and Janigan, 1962), we observed that a chronic exposure to a diet enriched in sugars caused swelling and vacuolization of the proximal tubular cells, thus producing a morphologic pattern known as osmotic nephrosis. Empagliflozin dose-dependently decreased the tubular damage, specifically the diet-induced tubular vacuolation, an early manifestation of tubular degeneration (Frazier et al., 2012). Morphologic improvement was associated with the beneficial effects on renal function, as shown by ability of the drug to prevent diet-induced ACR increase. The effects on tubular damage and ACR are most likely secondary to glycemic control (Vallon et al., 2014).

To better elucidate the molecular mechanism(s) underlying the beneficial effects associated with empagliflozin treatment, we focused our investigation on the NLRP-3 inflammasome, a protein complex involved in the development of obesity-induced insulin resistance. In keeping with our previous studies (Collino et al., 2013; Chiazza et al., 2015), in this work we confirm that hypercaloric diets induce NLRP-3 inflammasome activation in several organs, including liver and kidney. Interestingly, for the first time, this study demonstrates that animals treated with empagliflozin showed a decrease of NLRP-3 activation. The final point of this effect, namely the IL-1β production, was dose-dependently reduced in both kidney and liver, thus suggesting that drug beneficial effects are not limited to its target organ.

Fig. 2. Effects of diet manipulation and empagliflozin treatment on fasting glycemia (A) and OGTT [OGTT (B) and AUC (C)]. Blood glycemia was measured every month, whereas OGTT was performed at the end of the experimental protocol (week 16) in fasted animals. Values are means ± S.E.M. for n = 15 randomly selected animals per group. *P < 0.05, **P < 0.01, ***P < 0.001 versus control; #P < 0.05; ##P < 0.01; ###P < 0.001 versus HFHS.

Fig. 3. Effects of empagliflozin treatment on diet-induced kidney injury. Representative microphotographs (original magnification, 200×) of H&E-stained kidney sections from control and HFHS animals untreated or treated with 10 mg/kg empagliflozin. The arrowheads indicate the glomerulus, whereas stars highlight the S1–S2 segments of the proximal convoluted tubules (A). Urinary ACR measured in mice exposed to control or HFHS diet in the absence or presence of empagliflozin treatment (1–3–10 mg/kg). Values are means ± S.E.M. for n = 10 randomly selected animals per group. **P < 0.01 versus C; ##P < 0.05; ###P < 0.001 versus HFHS (B).
Renal NLRP-3 inflammasome inhibition has been suggested to counteract development of diabetic nephropathy and chronic kidney disease (Anders and Muruve, 2011; Chen et al., 2013; Wada and Makino, 2016), and its inhibition in the liver may exert further beneficial effects, such as reducing the progression of hepatic steatosis to more severe forms, including steatohepatitis (Wree et al., 2014). In the liver, we found an interesting correlation between the dose-dependent decrease of TGs and IL-1β induced by the drug. These effects could be related to the ability of IL-1β to induce hepatic TG accumulation. In keeping with this hypothesis, Negrin et al. (2014) have shown that IL-1β treatment increases hepatic fat accumulation in primary mouse hepatocytes and that pharmacological inhibition of IL-1 signaling attenuated obesity-induced hepatic steatosis. These data suggest a crucial role of NLRP-3 activation in the pathogenesis of liver steatosis. Taken together, these findings indicate that empagliflozin treatment may exert indirect effects related to NLRP-3 inflammasome activation, beyond the improvement of glycemic control, glucose tolerance, and insulin resistance.

As also metformin has been previously shown to attenuate inflammasome-mediated caspase-1 activation (Lee et al.,...
2013), we may speculate that the modulation of the NLRP-3/IL-1β pathway associated with glucose-lowering effect can significantly contribute to the beneficial therapeutic effects of antidiabetic drugs.

Although the NLRP-3 inflammasome is known to affect metabolic derangements in insulin-sensitive tissues, its role in the heart-suffering diet-induced pathogenic alterations has not been previously investigated. Our data show...
that myocardial steatosis observed in HFHS animals is not associated with local NLRP-3–induced IL-1β production. In contrast to other conditions, that is, ischemia/reperfusion injury (Mastrocola et al., 2016), infections (Wang et al., 2014), or diabetic cardiomyopathy (Luo et al., 2014), our study shows, for the first time to our knowledge, that this pathway is not activated in the cardiac tissue of animal fed HFHS diet. In addition, in patients with metabolic syndrome, Muniyappa et al. (2015) demonstrated that hepatic and peripheral index of insulin sensitivity were negatively related to hepatic fat content, but unrelated to myocardial fat accumulation, thus suggesting that the correlation between fat accumulation and insulin resistance is very strong in some tissues, such as liver, but not in the heart. This finding is consistent with the different response in NLRP-3 activation that we found between liver and heart.

Finally, we found that treatment with empagliflozin had not any effects on NLRP-3 signaling pathway in control animals. Despite the fact that we do not know whether it is possible to decrease the activity of NLRP-3 under basal conditions, this lack of effect could suggest that this drug does not exert a direct inhibition on NLRP-3 inflammasome.

In summary, empagliflozin attenuated in a dose-dependent manner the metabolic abnormalities induced by chronic exposure to a hypercaloric diet by abolishing the diet-induced BW increase, improving hyperglycemia, diminishing liver and cardiac steatosis, and decreasing the tissue injury associated with obesity and insulin resistance.

Interestingly, empagliflozin reduced in a dose-dependent manner the diet-induced activation of NLRP-3 inflammasome pathway in kidney and liver. Neither the diet manipulation nor the drug treatment affected myocardial NLRP-3 inflammasome pathway.

To our knowledge, this is the first paper that investigated the effect of a SGLT-2 inhibitor on diet-induced NLRP-3 inflammasome activation and myocardial fat accumulation, thus adding an original piece of evidence to the effects correlated with this new class of drugs.

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References


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