INTRODUCTION

Obesity, the epidemic of 21st century, is one of the main factors leading to heart failure. During the development of obesity, the share of circulating long chain fatty acids (LCFA) used in ATP generation in the heart rises from 50 – 70% (in physiological state) up to > 90% coinciding with a reduced glucose utilization (1). The cellular uptake of these energy substrates seems to be dependent on the expression of specific membrane proteins. The most important are fatty acid translocase/CD36 (FAT/CD36) and plasma membrane-associated fatty acid-binding protein (FABPpm) for LCFA (2, 3) as well as GLUT-1 and GLUT-4 for glucose (4). A growing evidence has also established an important role of these transporters’ distribution between cellular compartments on cardiac metabolic remodeling in response to elevated plasma fatty acid level (5). Studies on obese Zucker rats and diabetic mice revealed a permanent translocation of FAT/CD36 and FABPpm from intracellular pool to the plasma membrane as a major reason for cardiac LCFA oversupply (6-8). These conditions eventually lead to increased myocardial content of toxic lipids that contribute to deterioration of cardiac function, also termed as lipotoxic cardiomyopathy (5). Fatty acid overload coincides also with enhanced level of lipid peroxidation indicating oxidative stress induction (9). Interestingly, the elevated transport of LCFA into the cardiomyocytes may be, correspondingly, associated with relocation of FAT/CD36 to the mitochondrial membrane (10) and enhanced rate of fatty acid oxidation (FAO). Eventually however, FAO might be either insufficient to handle increased uptake or incomplete as a consequence of exceeded tricarboxylic acid cycle and oxidative phosphorylation capacities (11-13).

Inflammation is a significant component of myocardial dysfunction in obesity. The observations that interleukin-6 (IL-6) is implicated in lipid and glucose homeostasis as well as its connection with the pathogenesis of insulin resistance might suggest the involvement of this cytokine in metabolic disorders of the failing heart. In the present study we aimed to assess the effects of IL-6 ablation in mice fed with normal and high fat diet on the myocardial expression of glucose and fatty acid transporting proteins, and to evaluate the paralleled alterations in lipid content. We demonstrated that mice devoid of IL-6 exert reduced glucose transporter type 4 (GLUT-4) expression (~26%) and plasma membrane abundance (~43%), with no effect on glucose transporter type 1 (GLUT-1) content. Although there were no significant alterations in fatty acid translocase (FAT/CD36) and plasma membrane-associated fatty acid-binding protein (FABPpm) levels, we revealed a substantial decline in intramyocardial triacylglycerol level (~49%). Challenging of IL-6 knockout (KO) mice with high fat diet evoked an increase in FAT/CD36 expression (~19%) concomitantly with a trend for its reduced amount in plasma and mitochondrial membranes. Additionally, an increase in triacylglycerol level (+56%) was noticed, simultaneously with elevated content of saturated (+62%), monounsaturated (+69%) and polyunsaturated (+38%) fatty acids in this lipid fraction. The presented data reflect different roles of IL-6 in cardiomyocytes under selected conditions (i.e., normal and excessive lipid supply).

Key words: obesity, interleukin-6, fatty acid translocase, fatty acid-binding protein, high fat diet, lipids, heart, glucose transporter
performed according to the procedures previously described (10, 28, 29). Briefly, the thawed heart tissue was minced and incubated for 30 min in a high-salt solution (2 mol/l NaCl, 20 mmol/l HEPES pH 7.4, 5 mmol/l NaNO₃ and protease inhibitors) at 4°C. Next, the suspension was subjected to a series of centrifugation with increasing time and speed. After each step, the resulting pellet was resuspended in 300 µl TES-buffer (20 mmol/l Tris pH 7.4, 1 mmol/l EDTA, 250 mmol/l sucrose and protease inhibitors) and saved. The analysis of particular fractions with ATPase sodium/potassium pump (Na⁺/K⁺) and glucose transporter type 4 (GLUT-4) enabled the identification of the plasma membrane fraction.

Isolation of mitochondria

Mitochondria were isolated from mouse hearts by means of a trypsin digestion procedure (10, 30). In brief, ventricular tissue was minced, suspended in 10 ml of isolation medium (0.3 M sucrose, 10 mM sodium HEPES, pH 7.2, and 0.2 mM EDTA) and subjected to digestion by the addition of trypsin (1.25 mg) and 15 min incubation in 4°C. Next, samples were diluted with 10 ml of isolation buffer containing 1 mg/ml bovine serum albumin (BSA) and 6.5 mg of trypsin inhibitor. The suspension was mixed, and the supernatant was discarded. Afterwards, partially digested myocardial tissue was resuspended in 10 ml of isolation medium with 1 mg/ml BSA and homogenized with a glass homogenizer.

Subsequently, the homogenate was centrifuged for 10 min at 600 g, and resulting supernatant was centrifuged again (15 min, 8000 g). The pellet was twice resuspended in 10 ml of isolation buffer with 1 mg/ml BSA and centrifuged (15 min, 8000 g). The final washed pellet was resuspended in 500 µl of isolation buffer containing 1 mg/ml BSA.

Western blot analysis

The protein expression of FAT/CD36, FABPpm, GLUT-1 and GLUT-4 (30 µg) was determined in homogenate, plasma membrane and mitochondria of myocardium. Western blotting technique was applied to detect protein content as described previously (31-33). Briefly, bichromatic acid method with BSA serving as a protein standard was used to determine the total protein content in each sample. Next, the proteins were separated using 10% SDS-polyacrylamide gels, transferred to the nitrocellulose membrane and blocked in 7.5% BSA (GLUT-1) and 6.5 mg of trypsin inhibitor. The suspension was mixed, and the supernatant was discarded. Afterwards, partially digested myocardial tissue was resuspended in 10 ml of isolation medium with 1 mg/ml BSA and homogenized with a glass homogenizer.

Lipid analyses

TAG were analyzed by gas-liquid chromatography. The studied lipid fraction was extracted using the Folch et al. (34) method with modifications of van der Vusse et al. (35). Briefly, myocardium was extracted in chloroform-methanol (2:1, vol/vol). Next, TAG fraction was separated by thin-layer chromatography silica plates (Kieselgel 60, 0.22 mm, Merck, Darmstadt,
Germany). Subsequently, fatty acids and triheptadecanoate (Sigma Aldrich, St. Louis, MO), used as an internal standard, were transmethylated and dissolved in hexane. The fatty acid methyl esters (FAMEs) were identified and assessed quantitatively by gas liquid chromatography (Hewlett-Packard 5890 Series II gas chromatograph, HP-INNOWax capillary column) and flame ionization detector (Agilent Technologies, Santa Clara, CA). The total TAG content was estimated as the sum of particular fatty acid species. The value was expressed as nanomoles per gram of myocardium weight.

**Statistical analysis**

The analysis was conducted using Statistica 6.1 for Windows (StatSoft, Tulsa, USA). All results were presented as mean ± SEM. Statistical differences between groups were evaluated with Kruskal-Wallis ANOVA and Mann-Whitney tests. Any differences with P value less than 0.05 were considered statistically significant.

**RESULTS**

**Interleukin-6 genotyping**

Material from WT animals yielded DNA fragments ca. 1476 bp in size, whereas DNA fragments from IL-6 KO animals contained also a fragment of neomycin cassette and their size was about 2400 bp (Fig. 1).

**Myocardial expression of fatty acid, glucose transporters and intracellular lipids in interleukin-6 knockout and respective wild-type CB57BL/6J mice**

The total expression of FAT/CD36 tended to decline (–21%, P > 0.05) in IL-6 KO mice (Fig. 2), while FABPpm level in homogenate was slightly increased in animals devoid of IL-6 (+15%, P > 0.05) (Fig. 3). No difference was observed in plasmalemmal and mitochondrial expression of FAT/CD36 and FABPpm (Fig. 3). The expression of GLUT1 in homogenate remained unchanged, although its plasma membrane abundance tended to diminish (–22%, P > 0.05) (Fig. 4). We found a decrease in myocardial GLUT4 in IL-6 KO mice as compared to WT (–26% P < 0.05 in homogenate and –43% P < 0.01 in plasma membranes) (Fig. 5). Moreover, TAG content was significantly decreased in IL-6 KO mice (–49% P < 0.01 for total, –51% P < 0.01 for SFA, –58% P < 0.001 for MUFA and –39% P < 0.01 for PUFA) (Fig. 6).

**Effect of high fat diet on myocardial expression of fatty acid and glucose transporters, and intracellular lipids in wild-type CB57BL/6J mice**

We observed a tendency to increase in both plasmalemmal and mitochondrial expression of myocardial FAT/CD36 in WT mice fed normal as compared to high-fat diet (ns.) (Fig. 2). There was no difference in FABPpm expression in WT mice (homogenate, plasma membranes nor mitochondria) (Fig. 3). GLUT1 expression tended to be decreased both in homogenate and plasmalemmal fractions of WT mice fed normal as compared to high-fat diet (ns.) (Fig. 4). We observed a significant increase in FABPpm expression in plasma membranes and mitochondria of WT mice fed high-fat diet (Fig. 5). TAG content in homogenate and plasma membranes tended to decrease (–15% P > 0.05 and –20% P > 0.05), whereas in mitochondria it increased (+25% P < 0.05) (Fig. 6).

**Fig. 1.** IL-6 genotyping of wild-type (WT) - lanes 1-2 and IL-6 knockout (KO) - lanes 4-5 animals. DNA electrophoresis on agarose gel is shown. Lane 3 - DNA ladder.

**Fig. 2.** The effect of diet and IL-6–/– genotype on myocardial expression of FAT/CD36 in homogenate, plasma membranes and mitochondria. All fractions were prepared from left ventricle homogenates as described in Material and Methods. Data are based on 5 independent determinations for each group (mean ± SEM). 100% was assigned to the mean value in WT animals fed normal diet. Significant differences for IL-6 KO mice on high fat versus normal diet are shown as: * P < 0.05.

WT, wild type; IL6KO, IL-6 knockout; nd, normal diet; hfd, high fat diet. 
Fig. 3. The effect of diet and IL-6−/− genotype on myocardial expression of FABPpm in homogenate, plasma membranes and mitochondria. All fractions were prepared from left ventricle homogenates as described in Material and Methods. Data are based on 5 independent determinations for each group (mean ± SEM). 100% was assigned to the mean value in WT animals fed normal diet. WT, wild type; IL6KO, IL-6 knockout; nd, normal diet; hfd, high fat diet.

Fig. 4. The effect of diet and IL-6−/− genotype on myocardial expression of GLUT1 in homogenate and plasma membranes. The fractions were prepared from left ventricle homogenates as described in Material and Methods. Data are based on 5 independent determinations for each group (mean ± SEM). 100% was assigned to the mean value in WT animals fed normal diet. Significant differences for IL-6 KO mice on high fat versus normal diet are shown as: * P < 0.05, while for high fat diet fed WT versus IL-6 KO mice as ~ P < 0.01. WT, wild type; IL6KO, IL-6 knockout; nd, normal diet; hfd, high fat diet.

Fig. 5. The effect of diet and IL-6−/− genotype on myocardial expression of GLUT4 in homogenate and plasma membranes. The fractions were prepared from left ventricle homogenates as described in Material and Methods. Data are based on 5 independent determinations for each group (mean ± SEM). 100% was assigned to the mean value in WT animals fed normal diet. Significant differences for WT mice fed high fat versus normal diet are shown as: *P < 0.05; for IL-6 KO versus WT mice fed normal diet are shown as: ^P < 0.05; for IL-6 KO mice on high fat versus normal diet are shown as: *P < 0.05 and for high fat diet fed WT versus IL-6 KO mice as ~ P < 0.01. WT, wild type; IL6KO, IL-6 knockout; nd, normal diet; hfd, high fat diet.
and membrane fraction of WT animals fed high fat diet (ns.) (Fig. 4). The expression of GLUT4 was diminished in WT mice on high fat diet in plasma membrane fraction (~25% P < 0.05), but not in homogenate (Fig. 5).

There were no changes in the total content and composition of TAG (SFA, MUFA nor PUFA) in WT mice fed normal or high fat diet (Fig. 6).

Effect of high fat diet on myocardial expression of fatty acid, glucose transporters, and intracellular lipids in interleukin-6 knockout mice

We demonstrated increased expression of myocardial FAT/CD36 in homogenate of IL-6 KO mice fed high fat as compared to normal diet (+19% P < 0.05) (Fig. 2), whereas in plasma membrane and mitochondrial fractions FAT/CD36 expression tended to be decreased (~22%, ~10%, respectively, P > 0.05). There were no significant differences in FABPpm expression in IL-6 KO mice in homogenate and plasma membranes, although its level in mitochondrial fraction was slightly diminished (~19%, P > 0.05) (Fig. 3). GLUT1 expression in plasma membranes of cardiomyocytes was diminished in IL-6 KO mice fed high fat diet as compared to normal diet (~32%, P < 0.05) and wild type mice fed high fat diet (~26%, P < 0.01) (Fig. 4). GLUT4 expression was diminished in IL-6 KO mice on high fat diet in homogenate (~30% P < 0.05), but not in plasma membrane fraction (Fig. 5).

In IL-6 KO mice, unlike in WT animals, total TAG level rose significantly on high fat diet (~56%, P < 0.01), and so were particular fatty acid fractions (SFA +62%, P < 0.01; MUFA +69%, P < 0.01; PUFA +38%, P < 0.05) (Fig. 6).

DISCUSSION

The present study sought to determine the effects of IL-6 ablation in mice on several parameters of lipid and glucose myocardial metabolism. We revealed that IL-6 deficiency a) decreases GLUT-4 expression and plasma membrane abundance, b) does not significantly alter FAT/CD36 and FABPpm level, and c) declines TAG content in cardiomyocytes. When challenged with high fat diet these animals exhibited a) further reduced expression of glucose transporters, b) increased total expression of FAT/CD36 concomitantly with slightly diminished its plasmalemmal and mitochondrial content, and c) increased TAG level in both saturated and unsaturated fatty acid species.

Clinical data provide evidence that elevated LCFA supply is a profound cause of structural myocardial damage (i.e., ventricular fibration, inflammation and cardiomyocyte’s apoptosis) (36) as well as left ventricular hypertrophy and arrhythmias (37, 38). Further studies imply a strong association between excessive lipid accumulation and the development of insulin resistance (39, 40). Since FAT/CD36-related mechanisms account for ~50% of myocardial fatty acid incorporation (41), it may be a major culprit of lipotoxic cardiomyopathy in obesity. Previous data indicate enhanced FAT/CD36 protein expression in IL-6 KO mice, however it was not followed by changes in myocardial TAG deposition (16). On the contrary, we herein provide somewhat different observations in IL-6 KO mice fed standard diet, i.e., a slight decrease in FAT/CD36 content and a reduction in TAG amount. Such discrepancies may result from different age or sex of mice used in both experiments (i.e., female mice in the first study and male mice in our experiment). Furthermore, a reduction in TAG level might be also unexpected considering cumulative evidence towards downregulation of oxidation-promoting genes expression in mice devoid of IL-6. For instance, lack of functional IL-6 can abolish the activation of AMP-activated protein kinase (AMPK), a complex protein of well established role in glucose uptake and fatty acids oxidation (14). Moreover, IL-6 depleted mice have initially reduced level of peroxisome proliferator activated receptor α (PPARα), although high fat diet did not aggravate this effect (17, 42). PPARα and its coactivator PGC-1α act as profound regulators of oxidative metabolism, while the deletion of each protein is combined with a subsequent decrease in the myocardial
expression of fatty acid $\beta$-oxidation genes (41, 43). In line with this notion, the mRNA level of PGC-1$\alpha$ was diminished in IL-6 KO mice as compared to WT animals (17, 42), further resulting in decreased mitochondrial transcription factor (mTFA) expression (42). Moreover, a reduction in cytochrome $C$ content was demonstrated in IL-6 KO mice fed with high fat diet, despite no differences in the expression of COX IV and citrate synthase between IL-6 KO and WT mice on both diets (17). It might be presumed that genetic ablation of IL-6 under normal diet provision in our experimental model activates other regulatory factors that minimize the consequences of IL-6 depletion on lipid oxidation. IL-6 KO animals may also exhibit increased activity of other enzymes responsible for TAG degradation, i.e. triacylglycerol lipase (ATGL) and hormone-sensitive lipase (HSL) (44), while released fatty acids can be redirected into other lipid fractions. Interestingly, decreased TAG level in IL-6 KO mice is reminiscent of the effects demonstrated during tachycardia, where increased fatty acid incorporation into TAG is overwhelmed by the rate of TAG utilization (45). Nevertheless, the results of high fat diet treatment of IL-6 KO animals encompassed the increase in FAT/CD36 protein content, while Chen et al. also confirmed that on mRNA level (42). Moreover, we observed a tendency to a decrease in the mitochondrial content of FAT/CD36 and FABPpm proteins combined with a greater level of TAG accumulation in the cardiomyocytes of mice devoid of IL-6 fed high fat diet as compared to WT animals. Accordingly, histological examinations also confirmed a role for IL-6 depletion in diminishing the amount of myocardial lipid droplets in mice fed standard diet (17) and for the intensification of lipid storage in the model of HFD-induced obesity (42). These findings may suggest that the absence of IL-6 triggers an increase in cardiac susceptibility to lipid deposition and reduction in oxidative capacities under the condition of excessive lipid delivery. Additionally, the abovementioned results point out a differential metabolic response to IL-6 depletion between mice exposed to standard and high fat diets. In contrast, FABPpm expression remains unaltered in cardiomyocytes of IL-6 KO mice when challenged with excessive fatty acid level in a diet. It is in accordance with previous study (16), where no change in FABPpm level in basal conditions was noticed. We speculate that FABPpm regulation remains independent of IL-6 actions or this protein may have a minor role in fatty acid uptake in the heart regardless of the level of lipid delivery in a diet.

IL-6 KO mice when challenged with high fat diet exhibited increased TAG storage supporting a conclusion that mitochondrial $\beta$-oxidation is insufficient to cope with chronically enhanced level of circulating fatty acids. However, as TAG are one of neutral forms of lipids in cells, their role in lipid delivery in a diet remains independent of IL-6 actions or this protein may have a minor role in fatty acid uptake in the heart regardless of the level of lipid delivery in a diet.

In the lipid overload milieu, the cardiac flexible preference for substrate usage in oxidative metabolism is reduced, becoming enormously dependent on fatty acids (41, 54). We suggest that IL-6 deficiency combined with high fat diet may aggravate such metabolic switch through a reduction in GLUT-4 expression and its plasma membrane abundance, thereby implying a decline in glucose transport into cardiomyocytes. GLUT-1 seems to be less sensitive to IL-6 ablation, at least during standard diet feeding, whereas fatty acid oversupply diminishes its plasma membrane content. Taken together, these data may point out to the important role of IL-6 in carbohydrate uptake into cardiomyocytes in terms of both cardiac expression and cellular relocation of glucose transporters. The effect on glucose transporters may be also attributed to reduced PGC-1$\alpha$ expression since the coactivator has been implicated in the control of GLUT-4 expression and translocation to the plasma membrane (55).

In summary, the present study delivers a careful insight into lipid and glucose metabolism in the hearts of IL-6 KO mice fed with a high fat diet. Our data indicate a reduced GLUT-4 expression as well as a tendency towards decreased FAT/CD36 and FABPpm mitochondrial abundance in these animals, together with a greater level of myocardial TAG accumulation. These effects may serve as indicators of reduced $\beta$-oxidation upon IL-6 ablation in animals challenged with high fat diet, thereby emphasizing an important role of IL-6 level in obesity.

Conflict of interests: None declared.

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Received: June 3, 2018
Accepted: August 30, 2018

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