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Journal of Cellular Biochemistry 112:2616–2626 (2011)

Journal of Cellular Biochemistry

Apolipoprotein A-I Mimetic Peptide L-4F Prevents Myocardial and Coronary Dysfunction in Diabetic Mice

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ABSTRACT

Diabetes is a major health problem associated with adverse cardiovascular outcomes. The apolipoprotein A-I mimetic peptide L-4F is a putative anti-diabetic drug, has antioxidant and anti-inflammatory proprieties and improves endothelial function. In obese mice L-4F increases adiponectin levels, improving insulin sensitivity, and reducing visceral adiposity. We hypothesized that the pleiotropic actions of L-4F can prevent heart and coronary dysfunction in a mouse model of genetically induced Type II diabetes. We treated db/db mice with either L-4F or vehicle for 8 weeks. Trans-thoracic echocardiography was performed; thereafter, isolated hearts were subjected to ischemia/reperfusion (IR). Glucose, insulin, adiponectin, and pro-inflammatory cytokines (IL-1β, TNF-α, MCP-1) were measured in plasma and HO-1, pAMPK, peNOS, iNOS, adiponectin, and superoxide in cardiac tissue. In db/db mice L-4F decreased accumulation of subcutaneous and total fat, and increased insulin sensitivity and adiponectin levels while lowering inflammatory cytokines ($P < 0.05$). L-4F normalized in vivo left ventricular (LV) function of db/db mice, increasing ($P < 0.05$) fractional shortening and decreasing ($P < 0.05$) LV dimensions. In I/R experiments, L-4F prevented coronary microvascular resistance from increasing and LV function from deteriorating in the db/db mice. These changes were associated with increased cardiac expression of HO-1, pAMPK, peNOS, and adiponectin and decreased levels of superoxide and iNOS ($P < 0.01$). In the present study we showed that L-4F prevented myocardial and coronary functional abnormalities in db/db mice. These effects were associated with stimulation of HO-1 resulting in increased levels of anti-inflammatory, anti-oxidative, and vasodilatatory action through a mechanism involving increased levels of adiponectin, pAMPK, and peNOS. J. Cell. Biochem. 112: 2616–2626, 2011.

KEY WORDS: DIABETES; INFLAMMATION; OXIDATIVE STRESS; INSULIN SENSITIVITY; ADIPONECTIN; HEME OXYGENASE

Type II diabetes, insulin resistance, and obesity are clinical conditions with a growing prevalence in Western and developing countries [Wilson et al., 2005]. The higher risk of associated heart disease is mainly related to an increased prevalence of atherosclerosis, coronary syndromes, and heart failure, possibly mediated by a direct impairment of endothelial and myocardial function [Witteles and Fowler, 2006, 2008; Mottillo et al., 2010].

The mechanisms that produce myocardial dysfunction in these conditions are still incompletely understood. Besides the direct effects of impaired insulin pathways as well as hyperglycemia on myocardial metabolism, other relevant mechanisms include

Abbreviations Used: Apo A-I, apolipoprotein A-I; CO, carbon monoxide; HO, heme oxygenase; NO, nitric oxide.

The authors declare that there is no duality of interest associated with this manuscript.

C. Vecoli and J. Cao contributed equally to this work.

Grant sponsor: Scuola Superiore Sant'Anna; Grant sponsor: IFC-CNR; Grant sponsor: American Heart Association; Grant sponsor: National Institutes of Health; Grant numbers: HL55601, DK068134, HL34300, HL075265, R01HL091923.

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Received 8 March 2011; Accepted 11 May 2011 • DOI 10.1002/jcb.23188 • © 2011 Wiley-Liss, Inc.
Published online 19 May 2011 in Wiley Online Library (wileyonlinelibrary.com).

enhanced oxidative stress [Ceriello et al., 2002; Stevens, 2005], low oxygen availability secondary to coronary endothelial/vascular damage [Rask-Madsen and King 2007], and increased susceptibility to ischemia [Rytter et al., 1985; Hink et al., 2001; Cai et al., 2002]. Relevant contributing mechanisms involve the endocrine function of the adipose tissue and the inflammatory pathway [Berg and Scherer, 2005]. In fact, plasma adiponectin levels are reduced in obesity and Type II diabetes with negative effects on insulin sensitivity, endothelial function, myocardial protection, and modulation of the inflammatory response [Hotta et al., 2000; Kadowaki et al., 2006]. Moreover, diabetes and obesity are associated with a pro-inflammatory state, demonstrated by increased expression of inflammatory cytokines such as TNFα, IL-1, and IL-6 which in turn downregulate adiponectin and have direct negative effects on both vascular endothelium and myocardial function [Shibata et al., 2007].

4F compounds are apolipoprotein A-I (Apo A-I) mimetic peptides, synthesized from either D (D-4F) or L (L-4F) amino acids, able to bind oxidized or oxidizable lipids with higher affinity than native Apo A-I [Van Lenten et al., 2008]. These peptides enhance the ability of HDL-cholesterol to protect LDL-cholesterol against oxidation and to inhibit LDL-induced monocyte chemoattractant protein-1 (MCP-1) production by human endothelial cells, contributing to the reduction of atherosclerosis observed with 4F treatment in different animal models [Navab et al., 2006; Sherman et al., 2010]. Since HDL-cholesterol related mechanisms contribute to the pathogenesis of endothelial and myocardial dysfunction in diabetes [Van Linthout et al., 2010] 4F peptides are interesting molecules to test in this context. Previous studies on experimental models of obesity and diabetes have shown different favorable effects of 4F treatment including improvement of endothelial function, with restoration of the balance between nitric oxide (NO) and superoxide anions and enhancement of insulin sensitivity [Peterson et al., 2007; Van Lenten et al., 2009]. On the other hand, 4F peptides also induce heme oxygenase-1 (HO-1) expression in vascular and myocardial tissues together with marked increase in serum levels of adiponectin, decrease in inflammatory cytokines IL-1, IL-6, and TNF-α [Peterson et al., 2008] and improve vascular reactivity [Kruger et al., 2005] in different experimental models.

The aim of this study was to extend previous observations exploring the potential effect of 4F treatment in preventing the development of diabetic cardiomyopathy in a murine model of genetic type II diabetes. To this purpose, the cardiac effects of chronic treatment with L-4F were evaluated in db/db mice. These animals are characterized by obesity, hyperinsulinemia, hyperglycemia, and develop progressive left ventricular (LV) dysfunction resembling diabetic cardiomyopathy [Semeniuk et al., 2002; Carley and Severson, 2008]. We tested the hypothesis that L-4F treatment improves insulin sensitivity, reduces the inflammatory state, and induces protective HO-1 cardiac over-expression, eventually preventing both myocardial and coronary dysfunction.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University and conducted under the guidelines for the Care and Use of Laboratory Animals published by the Office of Science and Health Reports, National Institutes of Health.

ANIMAL TREATMENT

C57BL/6J control (n = 20) and db/db (n = 20) mice were purchased from Jackson Laboratory (Maine). Some control (n = 10) and some db/db (n = 10) mice were treated with either L-4F (i.e., Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2, synthesized from L-amino acids) or vehicle (ABCT: ammonium bicarbonate buffer at pH 7.4 containing 0.01% Tween-20). The powder of L-4F (i.e., Ac-D-W-F-K-A-F-Y-D-K-V-A-E-K-F-K-E-A-F-NH2, synthesized from L-amino acids), prepared as previously described by Navab et al. [2002], has been dissolved in ABCT buffer (ammonium bicarbonate 50 mM at pH 7.4 containing 0.01% Tween-20). Beginning at 7 weeks of age, L-4F or vehicle was injected intraperitoneally (i.p.), at a dose of 200 μg/100 g body weight daily for 8 weeks [Peterson et al., 2009]. Once a week during treatment, glucose monitoring was performed using a commercial Glucose Monitor Kit (Ascensia Contour Monitoring System, Bayer). The study was performed on four groups of animals: (1) wild-type (vehicle-treated) control, (2) control + L-4F, (3) db/db mice, and (4) db/db mice + L-4F. Blood was collected immediately before the animals were killed and plasma insulin, adiponectin (high molecular weight), IL-1β, MCP-1, and TNF-α were measured.

The HOMA index has been calculated as glucose (mg/dl) × insulin (μU/ml)/2,430 [Cacho et al., 2008].

ECHOCARDIOGRAPHY

In vivo cardiac morphology and function were assessed by transthoracic echocardiography (Acuson Sequoia C256, 13 MHz transducer; Siemens) in conscious mice as previously described [Takimoto et al., 2005]. M-Mode (mono-dimensional) echocardiogram allows simple, reproducible measurements of linear dimensions of cardiac structures (e.g., cavities, wall thickness) and their changes during the cardiac cycle.

From the M-mode echocardiogram image, the LV cavity diameter was measured at end-diastole (LVEDD) and end-systole (LVESD). Using the LVEDD and LVESD, we derived according to geometrical models, the fractional shortening (FS), that is, the percent change in LV dimensions due to contraction. We calculated FS (%) as [(LVEDD − LVESD)/LVEDD] × 100. All measurements were performed according to the guideline set by the American Echocardiography Society. For each mouse, three to five values for each measurement were obtained and averaged for evaluation.

ISOLATED HEART PREPARATION

At time of sacrifice, mice were anesthetized with pentobarbital i.p. injection, and heparinized via the left femoral vein (500 U im). The heart was rapidly excised and placed in cold perfusion medium. The isolated hearts were attached to the Langendorff apparatus and retrogradely perfused (at 37°C) as previously described by Sutherland et al. [2003]. The perfusion medium consisted of oxygenated Krebs–Henseleit buffer (NaCl 118.5; $NaHCO_3$ 25.0; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; glucose 11.0; $CaCl_2$ 1.4) and gassed with 95% O_2 and 5% CO_2 (pH 7.4). For measurement of LV developed

pressure (LVDevP) and end diastolic pressure (EDP) a custom-made latex balloon was inserted into the left ventricle of the heart through the mitral valve and connected to a Harvard pressure transducer. In each experiment the heart was paced at 450 bpm (Grass SD9 stimulator; Grass Instrument Co., Quincy, MA). EDP was set at ~5 mm Hg and kept stable during the first 10 min of perfusion. Data were acquired using a BioPac 100 System and analyzed with AcqKwnoledge software (BioPac System). LV pressure signal was continuously monitored. LV systolic pressure (LVSP), EDP, developed pressure (LVDevP = LVSP − EDP), dP/dt_{max}, and dP/dt_{min} were obtained. In all experiments, coronary flow was continuously monitored while collecting the cardiac effluent. Coronary resistance (CR) was defined as input pressure divided by coronary flow per gram of myocardial tissue (mm Hg·min·g/ mL).

Isolated hearts from the four groups of animals were studied under the following conditions: (1) baseline: the heart was studied for 30 min at control perfusion pressure (65 mm Hg); low pressure: after baseline, the input pressure was reduced to 30 mm Hg for 20 min (low pressure) and then restored to 65 mm Hg for 30 min (Reperfusion).

At the end of each experiment, the heart was rapidly frozen in liquid nitrogen.

SERUM INSULIN, ADIPONECTIN, IL-1β, MCP-1, AND TNF-α MEASUREMENT

Insulin, the high molecular weight (HMW) form of adiponectin, interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF) α were determined using ELISA assays (Millipore, Billerica, MA for insulin and Pierce Biotechnology, Inc., Woburn, MA for others).

WESTERN BLOT ANALYSIS OF CARDIAC TISSUE FOR HO-1 AND HO-2, ADIPONECTIN, eNOS, peNOS, iNOS, AMPK, AND pAMPK

Frozen hearts were pulverized under liquid nitrogen and placed in a homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% tergitol, pH 7.5), and protein levels were visualized by immunoblotting with antibodies against mice HO-1, HO-2 (Stressgen Biotechnologies Corp., Victoria, BC, Canada).

Antibodies against AMPK, pAMPK, and adiponectin were obtained from Cell Signaling Technology, Inc. (Beverly, MA). eNOS, iNOS, and peNOS from Santa Cruz Biotechnology (Santa Cruz, CA).

Briefly, 20 μg of heart tissue lysate supernatant was separated by 12% SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was performed as previously described [Abraham et al., 2003]. Chemiluminescence detection was performed with the Amersham ECL detection kit (Amersham, Piscataway, NJ), according to the manufacturer's instructions. The image was analyzed by densitometry. Densitometric analysis of developed blots was performed with the Quantity One program. Protein bands were quantified and values were normalized to those of α-actin. One out of three separate experiments with consistent results is shown.

MEASUREMENT OF HEART O_2^-

Control and diabetic hearts were placed in plastic scintillation minivials, containing 5 μM lucigenin for the detection of O_2^-, as previously described [Gupte et al., 2005]. Lucigenin chemiluminescence was measured in a liquid scintillation counter (LS6000IC, Beckman Instruments, San Diego, CA).

STATISTICAL ANALYSIS

Results are presented as mean ± standard error (SEM) for the number (n) of replicate determinations. Statistical significance between experimental groups and between different study conditions was determined by using a two-way ANOVA followed by the Fisher's exact test. $P < 0.05$ was considered significant.

RESULTS

EFFECTS OF L-4F ON BODY WEIGHT, GLUCOSE HOMEOSTASIS, AND CIRCULATING CYTOKINES

Figure 1 shows the time course of body weight (Fig. 1A) and glucose levels (Fig. 1B) during the 8 weeks of L-4F treatment. During this period food (and water) intake was not significantly decreased by L-4F treatment compared with control animals (data not shown). After 8 weeks, body weight was manifestly increased in db/db mice as compared with controls (53 ± 1.1 vs. 29.2 ± 1.16 g, $P < 0.0001$) but significantly less in L-4F treated diabetic mice (46.1 ± 1.8 g, $P < 0.0001$ vs. untreated db/db) in spite of similar food (and water) intake. L-4F did not significantly affect body weight in wild-type mice (27.3 ± 0.9 g controls + L-4F vs. 29.2 ± 1.16 g of controls). L-4F treatment significantly lowered both glucose and insulin levels in db/db mice ($P < 0.05$ and <0.01, respectively) while it did not alter either glycemia or insulinemia in control animals (Fig. 2, upper). L-4F treatment significantly reduced the HOMA index in db/db; the extent of such effect exceeded the one observed on body weight (Fig. 2, bottom).

Serum levels of adiponectin were decreased and inflammatory cytokines increased in db/db mice as compared with controls ($P < 0.01$). L4-F treatment increased circulating levels of adiponectin not only in db/db mice but also in controls above values measured in untreated animals. The over-expression of inflammatory cytokines in db/db was almost completely normalized by L-4F treatment (Fig. 3).

EFFECTS OF L-4F ON MYOCARDIAL AND CORONARY FUNCTION

At in vivo echocardiography, db/db mice showed evidence of cardiomyopathy as shown by significant increase in end-systolic (+30%) and end-diastolic LV dimensions (+37%) with reduced FS (−30%) as compared to control mice ($P < 0.05$) (Fig. 4). L-4F treatment prevented LV dysfunction in db/db mice which showed echocardiographic functional parameters similar to those of control mice (Fig. 4).

The ex vivo measurements obtained in the Langendorff preparations are summarized in Figure 5. At baseline, a depressed LV contractile function was confirmed in db/db mice by significantly lower values of LVDevP, dP/dt_{max} and dP/dt_{min} than in controls ($P < 0.05$). Moreover, in db/db hearts as compared with controls, myocardial contractile function was more depressed also during low-pressure ischemia and at reperfusion. L-4F treatment

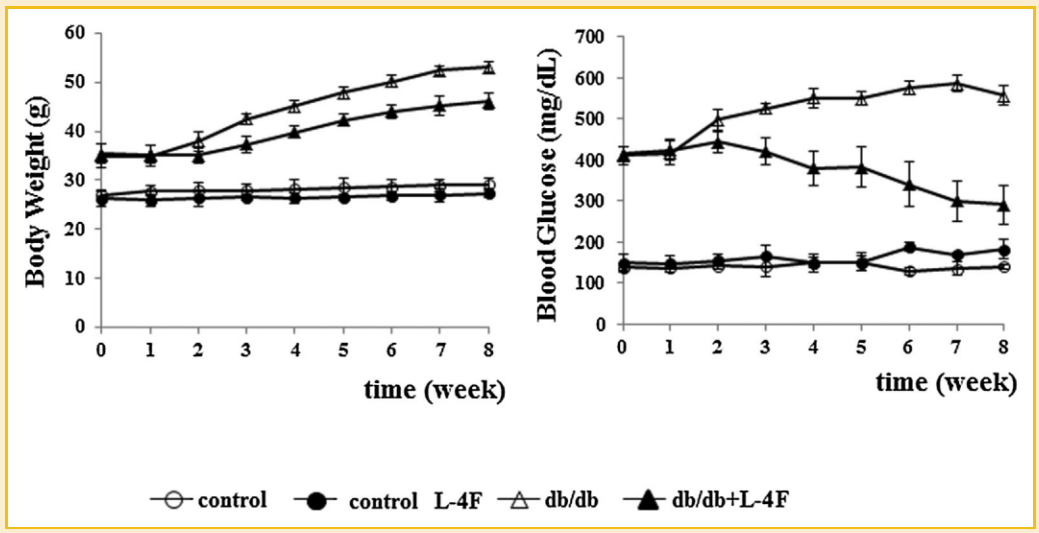

Fig. 1. Time course of body weight and glucose levels during the 8 weeks of L-4F or vehicle treatment in vehicle-treated wild-type mice, wild-type mice after L-4F treatment, db/db mice, and db/db mice after L-4F treatment mice.

improved all myocardial function parameters in db/db animals during all experimental conditions to values even exceeding the control ones. The db/db mice had higher CR than control animals after 20 min of perfusion at 65 mm Hg pressure (baseline condition) ($P < 0.001$). During low-pressure ischemia, CR increased in both groups (paradoxical vasoconstriction), more obviously in db/db mice where a sustained elevation in CR was also observed during reperfusion (Fig. 5). Treatment with L-4F significantly reduced CR both at baseline and during low-pressure ischemia in db/db mice as well as in controls ($P < 0.001$). Moreover, L4-F abolished the sustained coronary vasoconstriction that characterized db/db hearts at reperfusion.

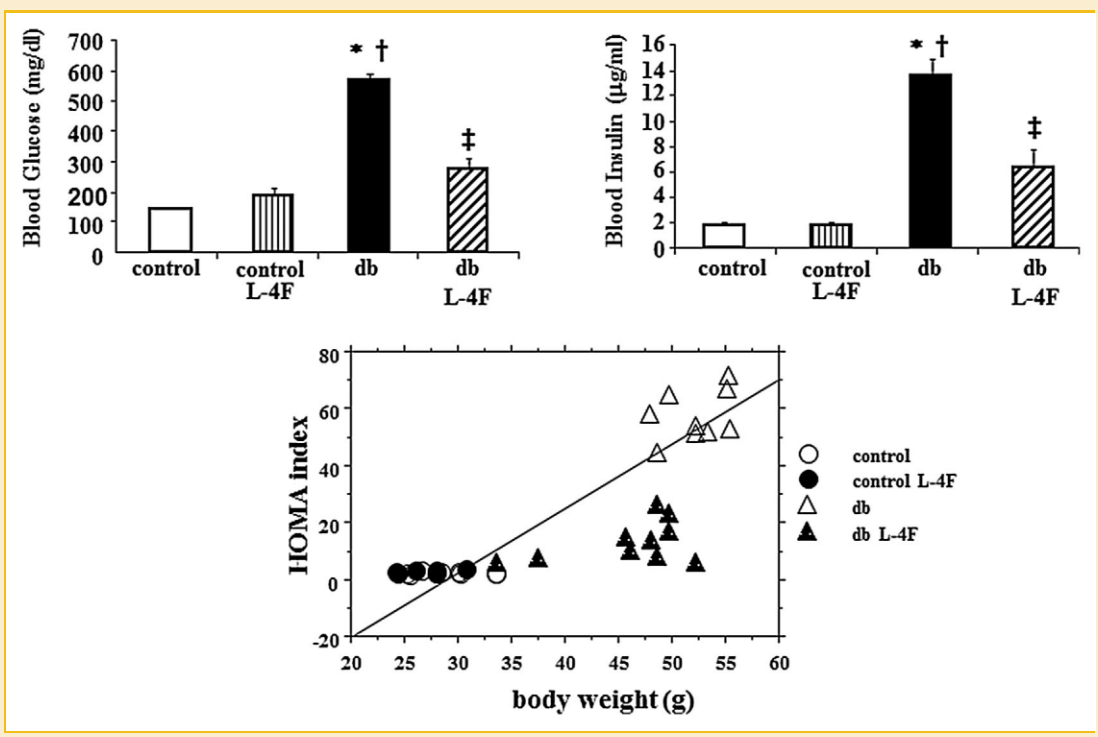

Fig. 2. Upper: Effect of L-4F on blood levels of glucose and insulin in vehicle-treated wild-type mice (control), wild-type mice after L-4F treatment (control L-4F), db/db mice (db), and db/db mice after L-4F treatment (db L-4F) mice. Bottom: Correlation between HOMA index and body weight in the four groups of animals. The results are means ± SEM; $^{*}P < 0.01$ versus control and control L-4F, $^{\dagger}P < 0.05$ versus db L-4F, $^{\ddagger}P < 0.05$ versus control and control L-4F.

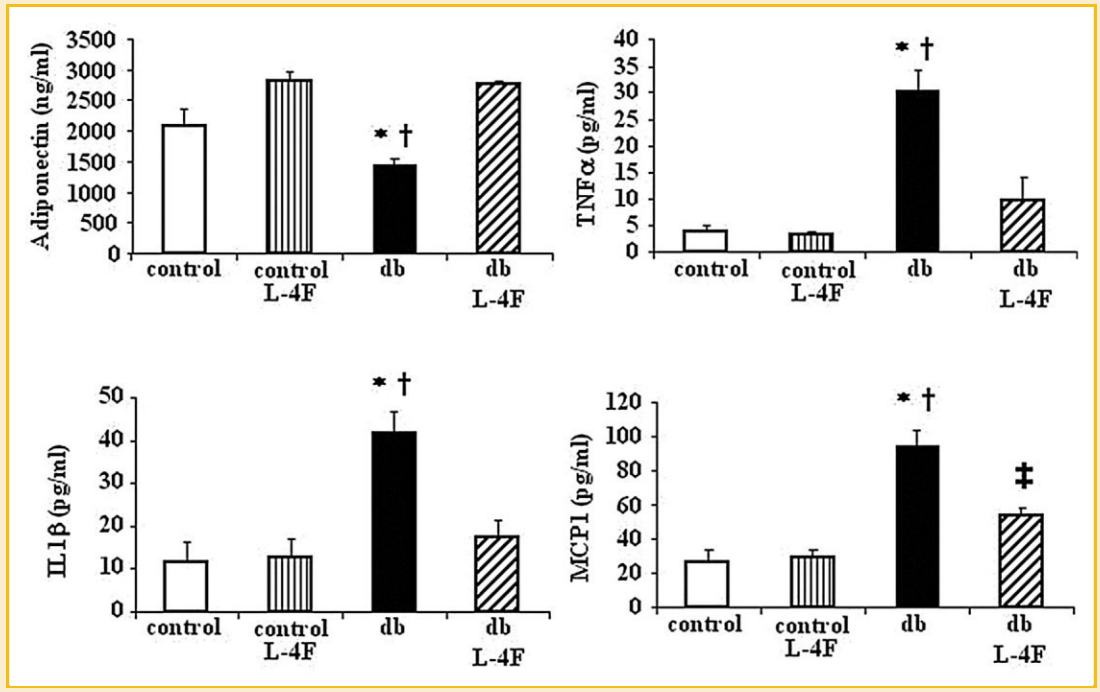

Fig. 3. Effect of L-4F on blood levels of adiponectin and cytokines levels (TNFα, IL1β, and MCP1) in vehicle-treated wild-type mice (control), wild-type mice after L-4F treatment (control L-4F), db/db mice (db), and db/db mice after L-4F treatment (db L-4F) mice. The results are means ± SEM; $^{*}P < 0.01$ versus control and control L-4F, $^{\dagger}P < 0.05$ versus db L-4F, $^{\ddagger}P < 0.05$ versus control and control L-4F.

EFFECTS OF L-4F ON MYOCARDIAL HO-1, NOS, AMPK EXPRESSION, SUPEROXIDE, AND ADIPONECTIN CONTENT

L-4F induced HO-1 over-expression in cardiac tissues of both control and db/db mice, while HO-2 levels did not change (Fig. 6).

Hearts from db/db mice, as compared with controls, showed increased superoxide levels (an index of oxidative stress) (Fig. 6) as well as increased expression of iNOS (Fig. 7); both alterations normalized with L-4F treatment. Conversely, adiponectin tissue content and eNOS expression were reduced in diabetic hearts; both abnormalities reversed to control values following L-4F. Similarly, heart tissue from db/db animals had reduced content of pAMPK and peNOS. L-4F increased tissue levels of both phosphorylated enzymes in db/db as well as in control mice, above values observed in untreated animals (Fig. 7).

DISCUSSION

The present study is the first to show that chronic treatment with the Apo A-I mimetic peptide L-4F is able to prevent the development of cardiomyopathy and coronary dysfunction in the murine model of genetically induced Type II diabetes.

In vivo echocardiography clearly showed enlarged hearts with reduced systolic function in db/db mice (FS ~70% of that in controls) but not in L-4F chronically treated animals. In vivo results were confirmed by ex vivo experiments. In fact, L-4F treatment was able to prevent diabetes-induced myocardial contractile dysfunction assessed in Langendorff perfused hearts where developed pressure is direct expression of inotropism not affected by pre- and after-load, nervous stimulation or cardio-active circulating substances. Isolated heart preparation also allowed measurement of coronary function. It was significantly impaired in db/db mice, as demonstrated by increased baseline CR and exacerbated vasoconstrictive response to low pressure ischemia and reperfusion, similarly to what was previously reported by our group in streptozotocin-induced diabetic rat hearts [L'Abbate et al., 2007]. The relevant new finding of our study was that L-4F treatment was able to prevent coronary vasoconstriction in diabetic animals. The coronary vasodilating effect of the drug was also evident in control mice so that both diabetic and control treated hearts had similar low levels of CR at rest and in response to ischemia.

The present study also documents that the remarkable effects of L-4F in preventing myocardial and coronary dysfunctions in db/db mice were associated with improved glycometabolic control as well as with the activation of protective cardiac molecular pathways. In fact, L-4F treatment significantly reduced circulating glucose and insulin levels in db/db mice and the HOMA index, an indicator of insulin sensitivity, was clearly improved. On the other hand, heart tissue from treated db/db animals showed increased levels of adiponectin, eNOS, pAMPK, and peNOS together with reduced superoxide content and suppression of the stress enzyme iNOS. These molecular changes were associated with over-expression of HO-1 and provide a framework for understanding the favorable effects of L-4F on myocardial and coronary phenotypes in diabetes.

Previous studies have shown that Apo A-I mimetic peptides such as D-4F and L-4F are able to improve systemic glycometabolic control in diabetes, enhancing insulin sensitivity [Peterson et al., 2007, 2009]. The effects of D-4F are similar to those of L-4F

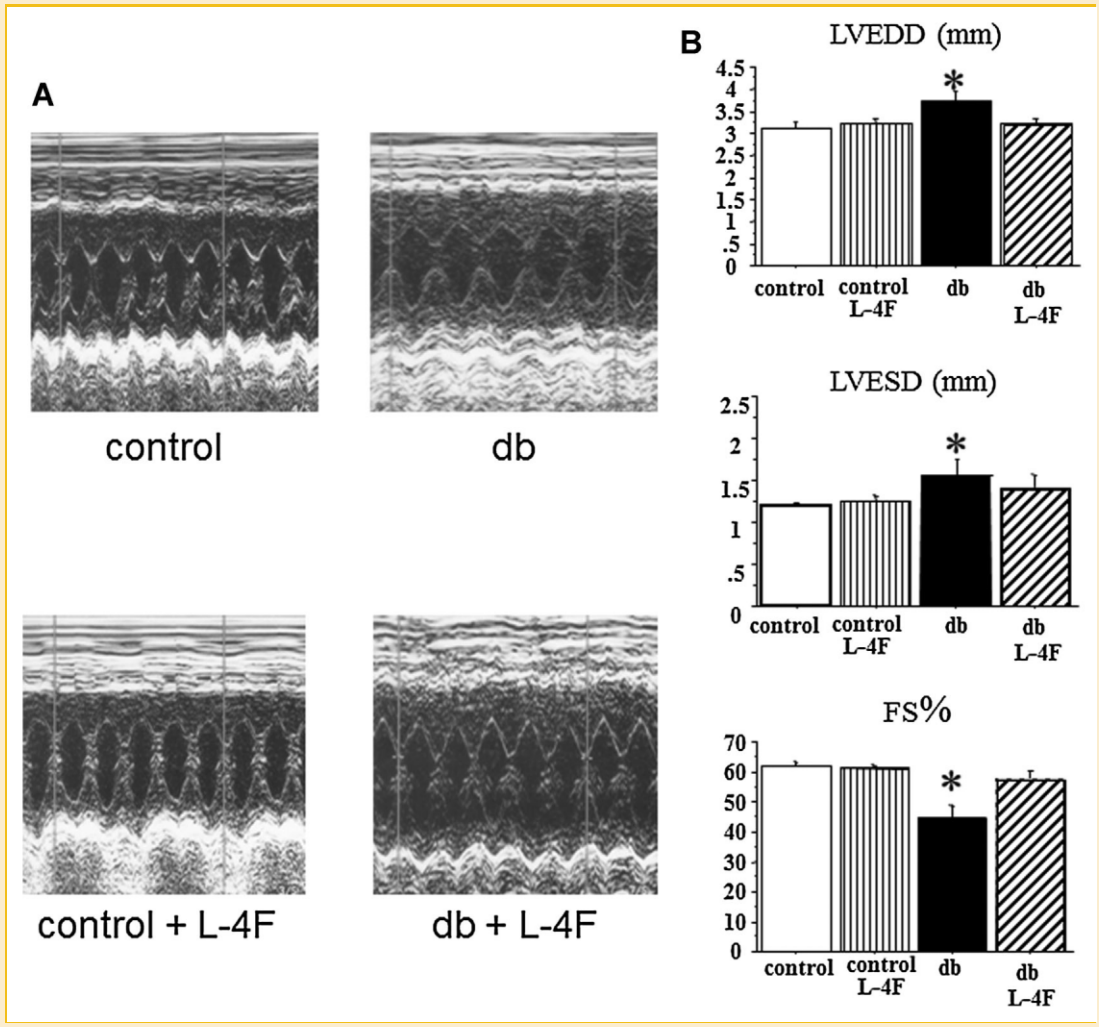

Fig. 4. Effect of L-4F on cardiac morpho-functional parameters. A: Representative M-mode echocardiograms. B: The LV chamber diameters were measured at the end of diastole (LVEDD) and systole (LVESD) and averaged from three to five beats. Percent fractional shortening (FS%) was calculated as follows: FS% = (LVEDD − LVESD)/LVEDD × 100. The results are means ± SEM. $^{*}P < 0.05$ versus other groups.

[Bloedon et al., 2008; Van Lenten et al., 2008; Navab et al., 2010]. However, in the D conformation, 4F is more resistant to metabolism as compared to 4F synthesized from L-amino acids, which is rapidly degraded after oral administration. In fact D-4F is synthesized from D-amino acids which are poorly degraded in mammals as discussed in previous reports [Garber et al., 1992]. Because of the above characteristic D-4F has prolonged tissue retention, particularly in liver and kidney. Conversely, L-4F is rapidly degraded in mammals. Here, we used L-4F given by i.p. injection thus bypassing the problem of degradation. Of importance, L-4F is the peptide used in recently clinical trial [Watson et al., 2011]. The exact mechanism by which L-4F exerts its insulin-sensitizing properties is not completely understood. Moreover, it is still unknown whether it acts through a specific receptor or binding the "original" receptor for Apo A-I. One of the possible mechanisms involves adipose tissue metabolism. Recently, Ruan et al. [2010] showed that administration of the L-4F peptide or overexpression of Apo A-I improved insulin sensitivity in obese mice, upregulating UCP1 (uncoupling proteins 1) in brown fat. They suggested that the consequent stimulation of lipid metabolism and enhancement of energy expenditure in fat tissue could account for the insulin-sensitizing properties of the drug [Ruan et al., 2010]. In the present study, L-4F treatment in db/db mice led to a smaller gain in body weight and to the reduction in body fat, despite no change in food intake, as previously reported also for ob/ob mice [Peterson et al., 2008]. However, the improvement in insulin sensitivity in db/db mice, as expressed by the HOMA index, seems to exceed the reduction in body weight and hence the metabolic effects of the drug. It is known that insulin acts centrally to decrease body weight and it has been suggested that the obesity state observed in obese mice is, in part, due to insulin resistance [Lazar, 2005]. Our results demonstrate that L-4F treatment improved insulin sensitivity according to significant reduction of circulating glucose and insulin levels in db/db mice and of the HOMA index. The ability of L-4F in reducing body fat has been previously investigated [Peterson et al., 2008, 2009]. The mechanism by which L-4F exerts its effect on body weight without altering food intake is unknown and further studies

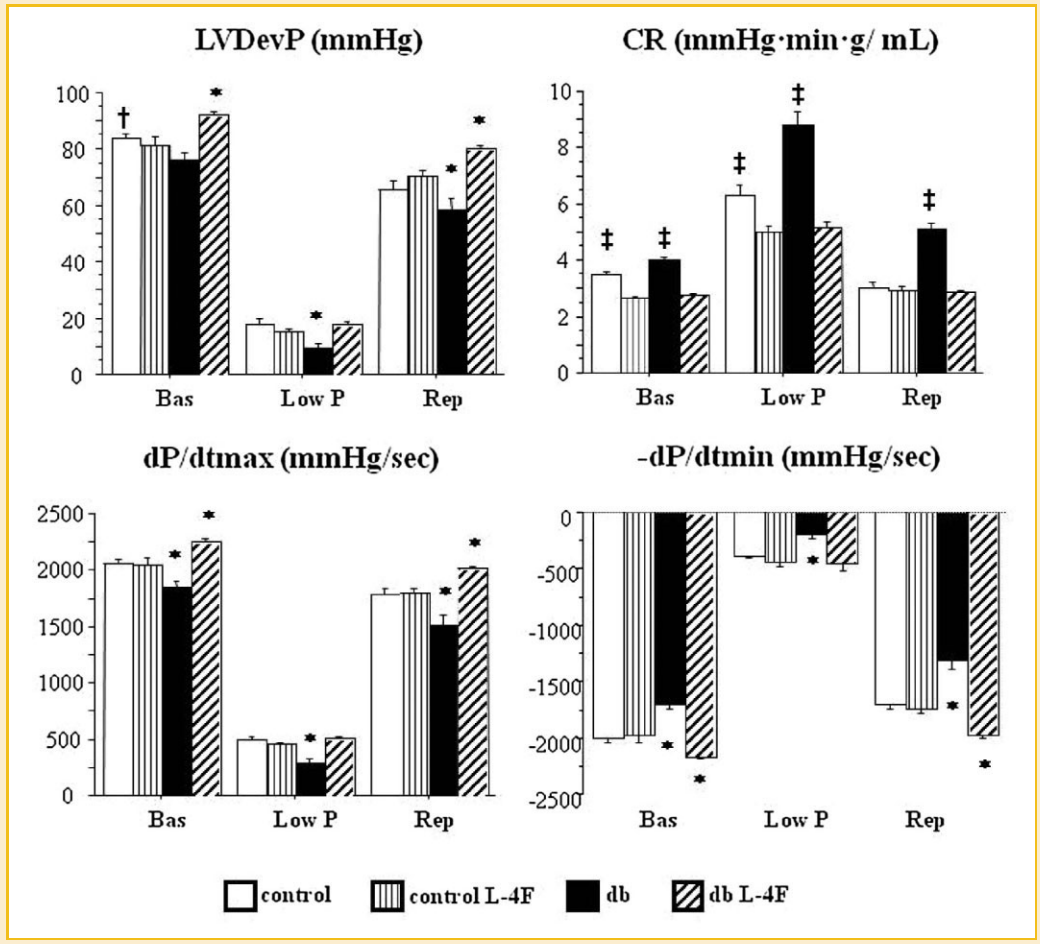

Fig. 5. Effect of L-4F on coronary resistance and LV function. Ex vivo hearts from vehicle-treated wild-type mice (control), wild-type mice after L-4F treatment (control L-4F), db/db mice (db), and db/db mice after L-4F treatment (db L-4F) were studied in Langendorff configuration with a protocol of ischemia/reperfusion. Coronary resistance (CR), left ventricular developed pressure (LVDevP), dP/dt_{max}, and dP/dt_{min} in each stage of ischemia/reperfusion, that is, baseline (bas), low pressure perfusion (Low P), and reperfusion (Rep) were monitored. The results are means ± SEM. $^{*}P < 0.05$ versus other groups, $^{\dagger}P < 0.001$ versus db mice, $^{\ddagger}P < 0.001$ versus other groups.

are needed. Nevertheless, Peterson et al. [2009] demonstrated that L-4F decreased adipogenesis and adiposity through a reprogramming of vascular tissue and adipocytes in a manner that resulted in the expression of a new phenotype that contains adipocytes of reduced cell size with restored insulin sensitivity. A similar favorable remodeling of adipose tissue and improvement in insulin sensitivity had been associated with over-expression of HO-1 in the adipocytes and increased serum adiponectin levels [Peterson et al., 2008]. Adiponectin, a cytokine secreted from adipose tissue has been shown to have insulin-sensitizing properties [Kadowaki et al., 2006; Shibata et al., 2007; Tao et al., 2007] in addition to anti-inflammatory and antioxidant effects. It is markedly reduced in diabetes [Kadowaki et al., 2006] and is stimulated by HO-1 inducing molecules [L'Abbate et al., 2007; Nicolai et al., 2009]. Indeed, in the present study, circulating levels of adiponectin were significantly depressed in db/db animals but maintained within control levels by chronic administration of L-4F. Moreover, an additional relevant effect of L-4F treatment in db/db mice was the blunting of systemic inflammation as demonstrated by maintenance of circulating cytokines, including MCP-1, within control levels. Although these results can be explained by multiple mechanisms elicited by L-4F treatment including quenching of oxidized lipids and improved ability of HDL to inhibit MCP-1 production by endothelial cells [Watson et al., 2011], the demonstrated increase in circulating adiponectin may have played a relevant role.

As a matter of fact, cardiac insulin resistance together with the effects of decreased adiponectin levels and increased inflammatory state are all recognized mechanisms leading to myocardial dysfunction in diabetes. Tao et al. [2007] showed that decreased levels of adiponectin mediated some of the deleterious effects of diabetes and hyperglycemia on myocardial function in adiponectin knockout mice [Tao et al., 2007]. Moreover, in clinical dilated cardiomyopathy an inverse relationship has been documented between circulating adiponectin levels and myocardial and coronary function [Giannessi et al., 2010]. On the other hand, an increasing amount of experimental and clinical evidence suggests that pro-inflammatory cytokines, in addition to neuro-hormonal activation, are involved in the pathogenesis of heart dysfunction [Deswal et al., 2001; Yndestad et al., 2007]. TNF-α is an important factor in the development and progression of heart failure and IL-1

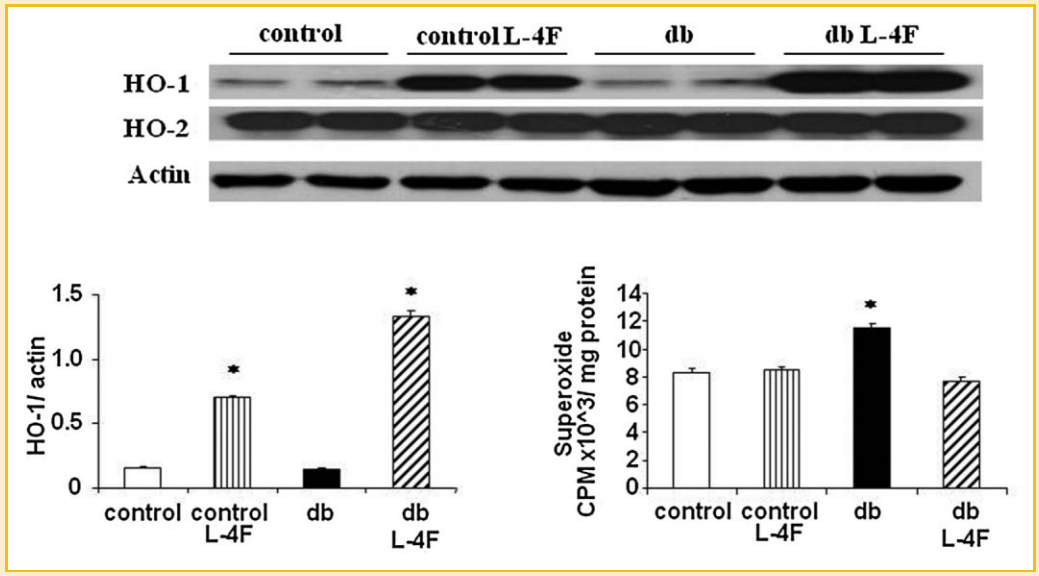

Fig. 6. Cardiac levels of HO-1 and superoxide. Above: Representative immunoblots of HO-1 and HO-2 protein are shown. Bottom: Cardiac HO-1/actin ratio and superoxide levels in hearts from vehicle-treated wild-type mice (control), wild-type mice after L-4F treatment (control + L-4F), db/db mice (db), and db/db mice after L-4F treatment (db L-4F) mice. The results are means ± SEM. $^{*}P < 0.05$ versus other groups.

in the pathogenesis of cardiac hypertrophy and failure. MCP-1 is involved in the development of atherosclerosis, myocardial remodeling, and experimental and clinical cardiac dysfunction [Yndestad et al., 2007]. Accordingly, the systemic effects of L-4F in improving insulin sensitivity, restoring glycometabolic control, normalizing the endocrine function of the adipose tissue and counteracting the inflammatory state, documented in the present study, may all have contributed to the prevention of progressive LV dysfunction and development of diabetic cardiomyopathy in db/db mice.

Although the described mechanisms may per se enhance myocardial function in diabetes [Stroedter et al., 1995; Iribarren et al., 2001], we also explored whether the beneficial effect of L-4F could also be related to direct action of the drug on cardiac tissue. On the basis of previous studies [L'Abbate et al., 2007], we specifically assessed the role of cardiac molecular signaling pathways related to HO-1 expression. Genetic or pharmacological over-expression of HO-1 in experimental models of the failing heart or diabetes was able to prevent adverse ventricular remodeling [Wang et al., 2010] and improve coronary function [L'Abbate et al., 2007], respectively. HO-1 is the isoform of HO which can be induced in the heart by stress. It catabolizes the heme group leading to production of bilirubin, a very powerful antioxidant, and carbon monoxide (CO), a gas molecule with vasodilating properties similar to NO. In the failing heart, HO-1 induction is an important cardioprotective adaptation that opposes LV remodeling, and this effect is mediated, at least in part, by CO-dependent inhibition of mitochondrial permeability transition and apoptosis [Wang et al., 2010]. In experimental models of diabetes, over-expression of HO-1 has been shown to improve coronary vascular function and adiponectin-dependent pathways [L'Abbate et al., 2007; Kusmic et al., 2010]. Pharmacological over-expression of HO-1 was also associated with normalization of reduced eNOS and increased iNOS in cardiac tissue [L'Abbate et al., 2007]. A link between HO-1 overexpression and enhanced AMPK and Akt phosphorylation through adiponectin has also been suggested [L'Abbate et al., 2007; Kusmic et al., 2010]. Indeed, stimulation of HO-1 induces adiponectin secretion from adipose tissue. In myocardium, adiponectin activates a cellular signaling cascade after binding to its specific receptors (AdipoR1 and AdipoR2) leading to AMPK/Akt phosphorylation [Kadowaki et al., 2006; Maruyama et al., 2010]. Moreover, pAMPK as well as pAkt utilizes eNOS as a substrate to enhance the levels of peNOS [Chen et al., 1999; Dimmeler et al., 1999]. In the present study, L-4F caused an evident overexpression of HO-1 in cardiac tissue of control as well as db/db animals. Accordingly, the abovementioned multiple effects of HO-1 over-expression might have variably contributed to the favorable changes in myocardial and coronary function observed in db/db mice. The known antioxidant properties of HO-1 are consistent with the normalization of the redox state in L-4F treated db/db mice as assessed by superoxide determination (markedly increased in the diabetic heart). Importantly, in healthy animals the treatment did not affect the redox balance. Moreover, L-4F treated diabetic animals showed normalized cardiac content of eNOS protein and reduced expression of iNOS, confirming previous results obtained following pharmacological HO-1 induction in a different experimental model of diabetes [L'Abbate et al., 2007]. Decreased levels of eNOS and augmented expression of iNOS, together with enhanced oxidative stress, have been implicated in the pathogenesis of abnormal coronary function and potential myocardial damage [Nagareddy et al., 2005]. The stress-induced NOS isoform, iNOS, is able to produce an abnormal amount of NO which, in an oxidative environment, reacts with superoxide, producing peroxynitrite and thus losing vasodilating properties. Peroxynitrite causes vasoconstriction and nitrosylation of cardiac

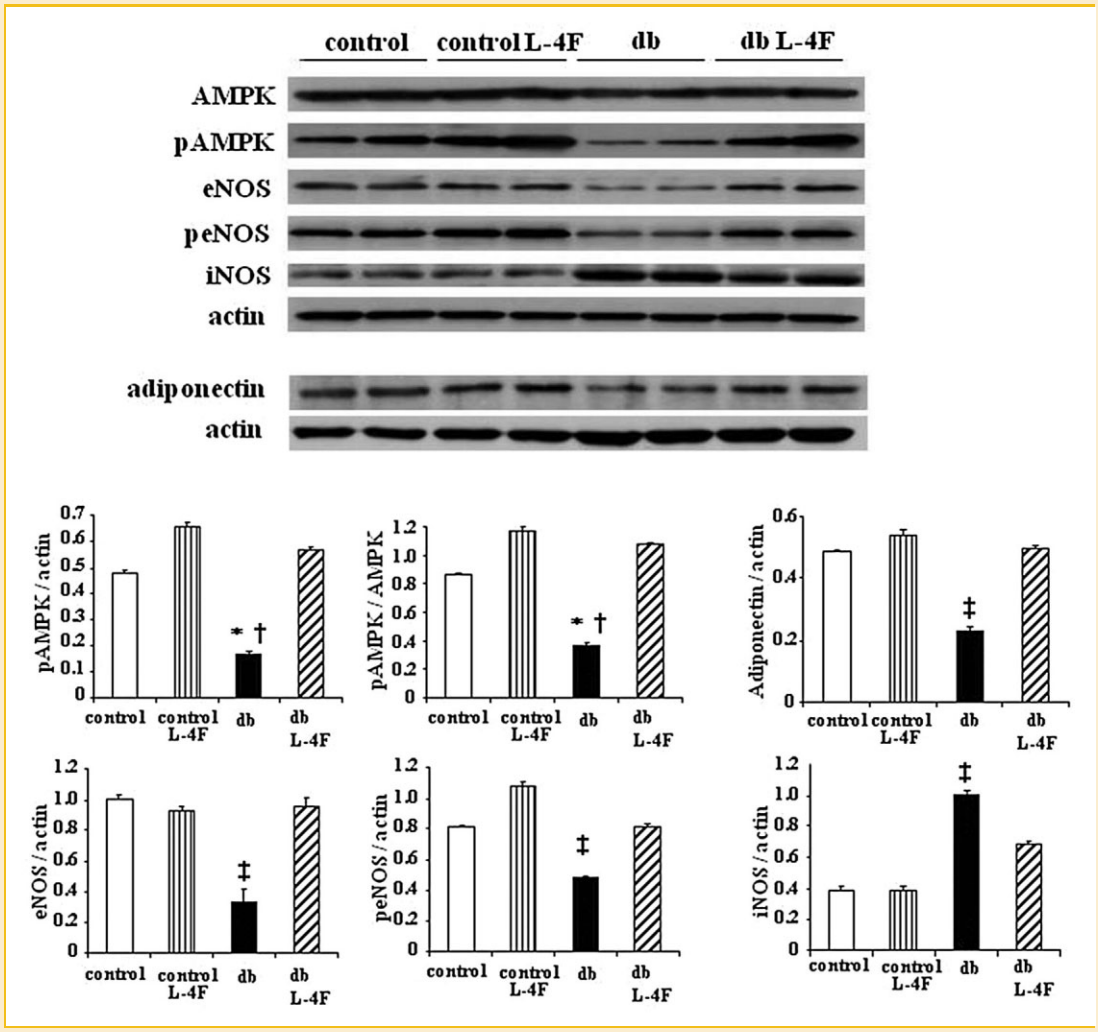

Fig. 7. Cardiac tissue homogenates were subjected to Western blotting for AMPK, pAMPK, eNOS, p-eNOS, iNOS, and adiponectin protein determination. The relative representative immunoblots (above) and the relative densitometry are shown in vehicle-treated wild-type mice (control), wild-type mice after L-4F treatment (control + L-4F), db/db mice (db), and db/db mice after L-4F treatment (db L-4F) mice. The results are means ± SEM. $^{*}P < 0.05$ versus control mice, $^{\dagger}P < 0.01$ versus L-4F treated animals, $^{\ddagger}P < 0.05$ versus other groups.

proteins with potential functional coronary and myocardial damage. Accordingly, L-4F treatment might have resulted in an increase of NO bioavailability and reduction of myocardial oxidative and nitrosative damage. Thus, according to the molecular signaling pathways elicited by L-4F treatment in the present study, increased levels of the vasoactive molecules NO and CO could have been responsible for the coronary vasodilation observed in both db/db and control treated hearts at rest and during ischemia. Of note is the abolishment of sustained coronary vasoconstriction at reperfusion in L-4F treated diabetic hearts. This effect is consistent with L-4F protection against ischemic-reperfusion damage characteristic of the diabetic state also observed in a different experimental model [Wang et al., 2010]. Finally, AMPK is also considered to be a ''fuel gauge'' or ''master switch'' for cellular energy levels. This is of particular interest in the setting of myocardial ischemia due to the very high energy demand and low energy reserve. It has been shown that AMPK plays a critical role in sustaining energy homeostasis and myocardial protection during ischemia, possibly by adapting cellular functions to both energy supply and utilization. Amplification of AMPK signaling appears beneficial during ischemia and reperfusion due to its ability to promote ATP generation and attenuate cardiomyocyte apoptosis [Russell et al., 2004].

Taken together, our results show the efficacy of Apo A-I mimetic peptides in the prevention of cardiac and coronary microvascular dysfunction in db/db mice L-4F resulted able to modulate different systemic and cardiac molecular pathways, eventually resulting in myocardial and coronary protection against diabetic and obesity noxae. Anyway, further studies are needed to establish whether this treatment would be able to improve cardiovascular function in different models of coronary and myocardial diseases associated (or not) with diabetes and/or insulin-resistance. Although these results cannot be extrapolated to the clinical setting, or to conditions with previously established diabetic cardiac alterations, L-4F appears to be a promising therapeutic agent for the prevention of cardiomy-

opathy and coronary dysfunction in diabetes and/or insulin-resistant states.

ACKNOWLEDGMENTS

We are indebted with Ms. Alison Frank for her invaluable help in editing the manuscript. This work was supported by Scuola Superiore Sant'Anna and IFC-CNR research grants and by the American Heart Association (SGD to NP) and National Institutes of Health grants (HL55601, DK068134 and HL34300 to NGA; HL075265 to NP; and R01HL091923 to NP).

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