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Activation of Retinoid Receptor-Mediated Signaling Ameliorates Diabetes-Induced Cardiac Dysfunction in Zucker Diabetic Rats

Rakeshwar S. Guleria,

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M Health Science Center; Central Texas Veterans Health Care System, Texas

Amar B. Singh,

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M Health Science Center; Central Texas Veterans Health Care System, Texas

Irina T. Nizamutdinova,

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M Health Science Center; Central Texas Veterans Health Care System, Texas

Tatiana Souslova,

Department of Pathology, Scott and White; Temple, Texas

Amin A. Mohammad,

Department of Pathology, Scott and White; Temple, Texas

Jonathan A. Kendall Jr.,

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M Health Science Center; Central Texas Veterans Health Care System, Texas

Kenneth M. Baker, and

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M Health Science Center; Central Texas Veterans Health Care System, Texas

Jing Pan*

Division of Molecular Cardiology, Department of Medicine, College of Medicine, Texas A&M Health Science Center; Central Texas Veterans Health Care System, Texas

Abstract

Diabetic cardiomyopathy (DCM) is a significant contributor to the morbidity and mortality associated with diabetes and metabolic syndrome. Retinoids, through activation of retinoic acid receptor (RAR) and retinoid X receptor (RXR), have been linked to control of glucose and lipid homeostasis, with effects on obesity and diabetes. However, the functional role of RAR and RXR in the development of DCM remains unclear. Zucker diabetic fatty (ZDF) and lean rats were treated with Am580 (RAR α agonist) or LGD1069 (RXR agonist) for 16 weeks, and cardiac

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*Address correspondence to: Jing Pan, MD, PhD, Division of Molecular Cardiology, Texas A & M Health Science Center, College of Medicine, 1901 South 1st Street, Bldg. 205, Temple, Texas 76704. Tel. 254-743-2461; Fax. 254-743-0165
jpan@medicine.tamhsc.edu.

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Disclosures

None declared

function and metabolic alterations were determined. Hyperglycemia, hyperlipidemia and insulin resistance were observed in ZDF rats. Diabetic cardiomyopathy was characterized in ZDF rats by increased oxidative stress, apoptosis, fibrosis, inflammation, activation of MAP kinases and NF- κ B signaling and diminished Akt phosphorylation, along with decreased glucose transport and increased cardiac lipid accumulation, and ultimately diastolic dysfunction. Am580 and LGD1069 attenuated diabetes-induced cardiac dysfunction and the pathological alterations, by improving glucose tolerance and insulin resistance; facilitating Akt activation and glucose utilization, and attenuating oxidative stress and interrelated MAP kinase and NF- κ B signaling pathways. Am580 inhibited body weight gain, attenuated the increased cardiac fatty acid uptake, β -oxidation and lipid accumulation in the hearts of ZDF rats. However, LGD1069 promoted body weight gain, hyperlipidemia and cardiac lipid accumulation. In conclusion, our data suggest that activation of RAR and RXR may have therapeutic potential in the treatment of diabetic cardiomyopathy. However, further studies are necessary to clarify the role of RAR and RXR in the regulation of lipid metabolism and homeostasis.

Keywords

retinoic acid; retinoid receptor; diabetic cardiomyopathy; cardiac remodeling; type 2 diabetes; Zucker diabetic fatty rats

1. INTRODUCTION

Diabetic cardiomyopathy (DCM), one of the most prevalent cardiovascular complications of diabetes, is characterized by both systolic and diastolic dysfunction, due to reduced contractility, prolonged relaxation and decreased compliance [1]. A growing body of clinical and experimental data suggest that cardiac insulin resistance and metabolic perturbations largely contribute to the development of DCM. The diminished glucose utilization and increased fatty acid oxidation in diabetic heart leads to lipid accumulation in myocardium [2]. Myocardial glucotoxicity and lipotoxicity triggers a series of maladaptive stimuli that result in increased oxidative stress, enhanced expression of renin-angiotensin system components, altered intracellular ion transients and calcium homeostasis, and activated apoptotic and inflammatory signaling pathways, such as mitogen-activated protein kinase (MAPK) and nuclear factor (NF)- κ B [3–5]. Elucidation of the molecular and metabolic mechanisms will provide a better understanding of the development of cardiac dysfunction associated with diabetes and the metabolic syndrome.

Retinoic acid-vitamin A metabolites, exerts a number of essential biological functions through activation of two classes of nuclear receptors, RAR (α , β and γ), which respond to all-*trans* retinoic acid (ATRA) and 9-*cis*-isomers of RA; and RXR (α , β and γ), which are activated by 9-*cis*-RA exclusively. Studies have shown that ATRA inhibits the development of type 1 diabetes [6]; and reduces body weight and adiposity through regulation of lipid metabolism in adipose tissue, liver and skeletal muscle of mice [7–9]. RXR agonists also have anti-diabetic effects in type 2 diabetic mouse models [10]. These data indicate the importance of RAR/RXR-mediated signaling in the regulation of glucose and lipid homeostasis. However, the role of RAR and RXR in regulation of cardiac glucose and lipid metabolism and its relationship with the development of DCM remains unclear. Recently, we reported that the expression and transcriptional activation of RAR α and RXR α are significantly suppressed in high glucose (HG)-treated cardiomyocytes and in the hearts of ZDF rats [11, 12]; and that silencing the expression of RAR α and RXR α in cardiomyocytes, further promoted HG-induced cell apoptosis. On the other hand, activation of RAR and RXR, by ATRA and 9-*cis* RA, protected cardiomyocytes from HG-induced apoptosis, through inhibition of oxidative stress-mediated activation of MAP kinases and NF- κ B-

mediated inflammatory signaling [11, 13]. These results led us to hypothesize that RAR and RXR-mediated signaling, through regulation of systemic and/or cardiac glucose and lipid homeostasis, has an important role in the development of DCM. ZDF rats were utilized to determine whether activation of RAR and RXR has beneficial effects on controlling functional, as well as morphological cardiac damage, through regulation of glucose/lipid metabolism and related signaling pathways in this representative rat model of the human metabolic syndrome.

2. RESEARCH DESIGN AND METHODS

2.1. Animals

Male ZDF rats and age matched Zucker lean rats (Charles River Laboratories) were housed in a temperature-controlled room under a 12/12 h light/dark cycle, with free access to water and the Purina 5008 diet. Animal use was approved by the Institutional Animal Care and Use Committee of the Texas A&M Health Science Center and conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Pub. No. 85-23, 1996).

2.2. Experimental protocol

ZDF and lean rats were randomized into 6 groups (8 rats/group) at the age of 9 weeks: (1) control lean rats; (2) lean rats treated with LGD1069 (20 mg/kg body weight/day, from LC Laboratories); (3) lean rats treated with Am580 (1 mg/kg body weight/day, Enzo Life Science); (4) control ZDF rats; (5) ZDF + LGD1069; (6) ZDF + Am580. Rats were given vehicle (corn oil), LGD1069 or Am580 daily, orally by gastric gavage, for 16 weeks. Body weight and fasting glucose levels were measured weekly. Before sacrificing, an oral glucose tolerance test (GTT) was performed. Additional information regarding short term treatment groups is provided in supplemental data.

2.3. Blood chemistry and metabolic analysis

Serum samples were collected from rats that were fasted overnight and insulin levels determined (Insulin ELISA Kit from Millipore). For the GTT, D-glucose (2 g/kg body weight, Sigma Aldrich) was orally administered after overnight fasting. Blood glucose was measured before, and after 30, 60 and 120 min of glucose uptake, using a commercially available glucometer (Bayer, IN). The area under the glucose curve (AUC) from 0–30, 0–60 and 0–120 min was calculated using the trapezoidal method [14]. Insulin sensitivity of individual animals was evaluated using the previously validated homeostasis model assessment (HOMA) index [15]. The HOMA-IR (HOMA-insulin resistance) is a method used to quantify insulin resistance and HOMA- β % to quantify β -cell function. The formula used was as follows: [HOMA-IR] = fasting serum glucose (mg/dl) $\times$ fasting serum insulin (mU/L)/405. [HOMA- β %] = fasting serum insulin $\times$ 360/(fasting serum glucose - 63). Plasma total cholesterol was measured using the polychromatic (452, 540, 700 nm) endpoint technique. The triglycerides (TG) and high-density lipoprotein (HDL) were measured using a bichromatic (510/700 nm; 600/700 nm) endpoint technique.

2.4. Histological analysis

Hearts were removed, weighed and separated into 2 halves along the anterior longitudinal mid-line. One half of the heart was fixed in formalin solution, embedded in paraffin and cut into sections 5 μ m thick for H&E, Masson's trichrome (Sigma Aldrich) and TUNEL staining (Promega) [16]. The other half of each heart was frozen in liquid nitrogen and sectioned (20 μ m) for Oil Red O staining (Sigma Aldrich), to identify lipid disposition [17] and for dihydroethidium (DHE) staining (Sigma Aldrich) to identify intracardiac superoxide production [16]. Interstitial and perivascular fibrosis were measured, using NIH Image J

software. Blue-stained areas and non-stained myocyte areas from each section were determined using color-based thresholding. The percentage area of total fibrosis was calculated as the summed blue-stained areas, divided by total ventricular area. The area of perivascular fibrosis was calculated as the ratio of the area of fibrosis surrounding the vessels, to the total vessel area.

2.5. Echocardiography

Transthoracic echocardiography was performed in anesthetized rats using HP sonos 5500 (Hewlett-Packard) with a 12-MHz imaging transducer. Left ventricle (LV) wall thickness, diameter, systolic and diastolic function were analyzed as described previously [18], by an experienced sonographer who was blinded to treatment.

2.6. Real time RT-PCR

Total RNA was extracted from left ventricles, using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Real time RT-PCR was performed to analyze mRNA expression for ANP, BNP, interleukin1 (IL-1 β), transforming growth factor β (TGF β), tumor necrosis factor- α (TNF- α), RAR α , RXR α , alcohol dehydrogenase (ADH) and retinaldehyde dehydrogenase (RALDH), glucose transporter 1 (GLUT1), GLUT4, aldolase A, hexokinase 2 (HK2), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), acetyl-CoA C-acyltransferase 1 (Acaa1), Acaa2, Acyl-CoA dehydrogenase (Acad), carnitine palmitoyltransferase 1 (CPT1) and fatty acid binding protein 3 (Fabp3) [11]. The mRNA expression levels were normalized to GAPDH mRNA. Data were shown as mean $\pm$ SEM. Primers used were purchased from Applied Biosystems.

2.7. Western blot analysis

Left ventricles were lysed in cell lysis buffer (Cell signaling Technology) containing protease inhibitor cocktail (Roche Diagnostics). Total protein was extracted and resolved on SDS-PAGE. Expression of Bcl2, Bax (Santa Cruz Biotechnology) and caspase 9, and phosphorylation of IKK α / β (Ser176/180), I κ B α (Ser32/36), p65 NF- κ B (Ser536), Akt (Ser473), GSK3 β (Ser9), ERK1/2 (Thr202/Tyr204), JNK (Thr183/Tyr185) and p38 MAPK (Thr180/Thr182) (Cell Signaling Technology) was determined by Western blot, as described previously [11, 13, 18].

2.8. Statistical analysis

Data are expressed as the mean $\pm$ SEM. Statistical significance between experimental groups was determined using one-way ANOVA, combined with the Tukey-Kramer Multiple Comparisons test. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Body weight changes

At 9 weeks of age, ZDF rats had a higher body weight (BW) than lean rats (292 ± 3 g vs 244 ± 5 g, Table 1, supplemental data). Lean control rats gained BW steadily from 9 to 25 weeks. Am580 and LGD1069 did not significantly affect the BW gain in lean rats. ZDF rats gained BW between 9 to 21 weeks, and there was no further gain from 21 to 25 weeks. Their BW was comparable to the lean rats at 21 weeks (380 ± 9 g in ZDF vs 390 ± 6 g in lean group) and lower at 25 weeks (381 ± 8 g in ZDF vs 405 ± 6 g in lean group, $p < 0.05$). The effect of RAR ligands on body weight was observed following 2 weeks of treatment. Rats receiving ATRA (all-trans RA) or Am580, for 2 weeks, significantly inhibited body weight gain in ZDF rats (Supplemental data, Fig. 1A). Am580 significantly inhibited body weight gain in ZDF rats, throughout all study periods (supplemental data, Table 1. 318 ± 27

in ZDF+Am vs 381 ± 8 g in ZDF, after 16 weeks of treatment, $p < 0.05$). However, rats receiving LGD1069 gained much more BW following treatment, and their BW was significantly higher than untreated ZDF rats, throughout the study (Supplemental data, Table 1 and Fig. 1A. 581 ± 29 g in ZDF+LGD vs 381 ± 8 g in ZDF, after 16 weeks of treatment; $p < 0.05$).

3.2. Am580 and LGD1069 improve glucose homeostasis and cardiac glucose metabolism

Fasting blood glucose levels were significantly elevated throughout the entire experimental period, in untreated-ZDF rats (Table 1, supplemental data), along with increased glucose tolerance (GTT), AUC-GTT and HOMA-IR index at the age of 25 weeks (Fig. 1A, B & D), suggesting insulin resistance in ZDF rats. Am580 and LGD1069, lowered the fasting glucose level and improved insulin resistance, as evidenced by the decreased GTT, AUC-GTT and HOMA-IR index. The beneficial effect of RAR and RXR ligands on glucose metabolism was observed as early as 1 to 2 weeks following treatment (Supplemental data, Fig. 1B & D), ATRA or LGD1069 significantly lowered the blood glucose level (after 2 weeks of treatment: 240 ± 21 mg/dl in ZDF+RA, 260 ± 12 mg/dL in ZDF+LGD vs 478 ± 12 mg/dL in ZDF; $p < 0.05$) and HOMA-IR index (supplemental data, Fig. 1D) after 2 weeks of treatment. Hyperinsulinemia was observed at age 11 weeks in ZDF rats (8.08 ± 0.75 ng/dL in ZDF vs 0.44 ± 0.03 ng/dL in lean, $p < 0.05$; supplemental data, Fig. 1C). At age 25 weeks, the serum insulin level in ZDF rats was decreased (compared to early phase) to a level comparable with lean rats (0.71 ± 0.07 ng/dL in ZDF vs 0.76 ± 0.09 ng/dL in lean, Fig. 1C). This observation was accompanied by a significantly increased HOMA-IR index and decreased HOMA- $\beta\%$ index, suggesting that impaired β cell function may contribute to the decreased insulin level and progression of the diabetic process. The observation was consistent with previous studies, which showed a decreased serum insulin level in aged ZDF rats [19, 20]. ATRA or LGD1069 treatment significantly lowered the increased insulin level in ZDF rats at the early stage (supplemental data, Fig. 1C), suggesting that insulin resistance was improved. At the age of 25 weeks, serum insulin was maintained at a higher level in Am580 and LGD1069 treated ZDF rats ($p < 0.05$, vs ZDF), along with a decreased HOMA-IR index and increased HOMA- $\beta\%$ index, (Fig. 1D & E), suggesting that Am580 and LGD1069 treatment has beneficial effects on improving insulin resistance and β cell function.

Cardiac glucose dysmetabolism was observed from the early stage of diabetes (age of 11 weeks) in ZDF rats, as evidenced by decreased phosphorylation of Akt and gene expression of GLUT4, suggesting that insulin signaling and glucose transport were impaired (supplemental data, Fig. 2A & B). However, no significant changes were observed in the gene expression of GLUT1 and HK2 at the early stage. Treatment with ATRA for 2 weeks, reversed the decreased Akt phosphorylation and promoted GLUT1 gene expression; but, had no significant effect on gene expression of GLUT4 and HK2. LGD1069 significantly promoted gene expression of GLUT1, GLUT4 and HK2; but, only slightly increased phosphorylation of Akt after 2 weeks of treatment (supplemental data, Fig. 2A & B). At age 25 weeks, glucose transport, uptake and glycolysis were further impaired, as demonstrated by a significantly decreased gene expression of GLUT1, GLUT4, HK2 and Aldolase A in ZDF rat hearts (Fig. 1F). In concordant with the impaired glucose transportation and metabolism, a decreased phosphorylation of Akt and GSK3 β was observed in ZDF rat hearts (Fig. 1G). Treatment with Am580 or LGD1069 for 16 weeks promoted phosphorylation of Akt and GSK3 β and reversed the downregulated gene expression of GLUT1, GLUT4, HK2 and Aldolase A, suggesting that activation of RAR and RXR improved cardiac glucose uptake and utilization, through activation of Akt-mediated insulin signaling.

3.3. Effect of Am580 and LGD1069 on lipid homeostasis and cardiac lipid metabolism

Significantly increased plasma cholesterol and triglyceride (TG) levels were observed in ZDF rats, which were further enhanced in LGD1069-treated ZDF rats (Fig. 2A). Am580 had no effect on the increased cholesterol and TG levels. No significant difference was observed in HDL levels between untreated-ZDF and lean rats; however, the level of HDL was increased in LGD1069 treated lean rats, and decreased in LGD1069-treated ZDF rats. No changes were observed in Am580 treated animals (Fig. 2A). With regard to cardiac lipid metabolism, we observed increased FA beta-oxidation at the early stage of diabetes (11 weeks of age), as shown by increased cardiac gene expression of CPT1 and Acad (supplemental data, Fig. 2C). However, gene expression of Fabp3 was unchanged at this stage. Both ATRA and LGD1069 inhibited the increased gene expression of CPT1 and Acad, in ZDF rat hearts, after 2 weeks of treatment. At age 25 weeks, gene expression of Fabp3 (a cytosolic protein involved in uptake and transport of FA from cell membrane to mitochondria); CPT1 (a rate-limiting step in mitochondrial FA oxidation); and Acad, Acaa1 and Acaa2 (enzymes that function to catalyze the initial and final steps in FA β -oxidation), were significantly increased in ZDF rat hearts ($p < 0.05$, vs lean), suggesting that cardiac FA uptake and beta-oxidation were increased significantly at the late stage of diabetes (Fig. 2B). However, no significant difference was observed in gene expression of PGC-1 α between groups. Am580 inhibited gene expression of Fabp3, CPT1, Acad, Acaa1 and Acaa2, to a level comparable to lean animals, following 16 weeks of treatment. However, LGD1069 further promoted gene expression of Acaa1, Acaa2 and Acad in ZDF rat hearts after 16 weeks of treatment. Though gene expression of Fabp3 and CPT1 was inhibited by LGD1069, the levels of Fabp3 and CPT1 were still significantly higher, compared to lean treated with or without LGD1069 ($p < 0.05$, vs ZDF+LGD1069). We further observed the effect of Am580 and LGD1069 on lipid accumulation in myocardium. As shown in Fig. 2C, Oil Red O staining showed increased lipid deposition in the myocardium of ZDF rats, which was inhibited by Am580. However, LGD1069 further promoted the lipid deposition in myocardium of ZDF rats; but, had no effect on lean rat hearts. These data suggested that activation of RAR α by Am580, inhibited FA uptake, beta-oxidation and lipid deposition in ZDF rat hearts; whereas activation of RXR, by LGD1069, promoted FA beta-oxidation and lipid accumulation in ZDF rat hearts.

3.4. Am580 and LGD1069 inhibit oxidative stress, apoptosis and activation of MAP kinases in ZDF rat hearts

Oxidative stress is a major feature linking diabetes-induced metabolic dysregulation to the development of cardiac dysfunction [21]. Increased ROS (reactive oxygen species) formation activates MAP kinase signaling pathways and promotes cardiomyocyte apoptosis [22, 23]. Significantly increased intracellular ROS generation (Fig. 3A & B) and decreased expression of SOD1 and SOD2 (Fig. 3C & D), along with increased phosphorylation of ERK, JNK1/2 and p38 (Fig. 3E & F) were observed in ZDF rat hearts. Increased apoptosis was also observed in ZDF rat hearts, as evidenced by decreased expression of Bcl2, a decreased Bcl2/Bax ratio and increased expression of Bax, caspase 9 and the percent of TUNEL-positive myocytes (23%) (Fig. 3G, H & I). Am580 and LGD1069 inhibited ROS generation and the activated MAP kinases and apoptotic signaling, as well as the percent of TUNEL-positive myocytes (10% and 2.3%, respectively, $p < 0.05$, vs ZDF). These data suggest that activation of RAR and RXR-mediated signaling inhibits cardiomyocyte apoptosis, by reducing oxidative stress and associated MAP kinase pathways.

3.5 Am580 and LGD1069 attenuate diabetes-induced myocardial inflammation and activation of NF- κ B signaling

Diabetic cardiomyopathy is associated with increased levels of the pro-inflammatory cytokines TNF- α and IL1- β [24]. The inflammatory response is a significant process in the

progression of heart failure in diabetic cardiomyopathy. NF- κ B is one of the most important regulators of pro-inflammatory gene expression [25]. There was a marked increase in the phosphorylation of IKK and I κ B α , as well as p65 NF- κ B, along with decreased expression of I κ B α in the myocardium of ZDF rats (Fig. 4A & B). In concordant with the activation of NF- κ B signaling, an increased gene expression of IL-1 β and TNF- α was also observed in hearts of ZDF rats (Fig. 4C). Am580 or LGD1069 treatment significantly attenuated the gene expression of IL-1 β and TNF- α and activation of NF- κ B signaling in hearts of ZDF rats, suggesting that activation of RAR and RXR has anti-inflammatory effects through regulation of the NF- κ B signaling pathway.

3.6. Am580 and LGD1069 inhibit cardiac fibrosis in ZDF rat hearts

Cardiac fibrosis leads to increased left ventricle (LV) stiffness and decreased ventricular wall compliance, resulting in both systolic and in particular diastolic dysfunction [26]. Studies have shown that oxidative stress induced activation of the MAP kinase pathway and gene expression of TGF β have an important role in regulation of cardiac fibroblast proliferation, differentiation and extracellular matrix protein synthesis [22, 27]. We consistently demonstrated markedly increased interstitial and perivascular fibrosis in ZDF rat hearts (Fig. 5A-C), which was accompanied with significantly increased gene expression of TGF β , and collagen type I, II and III (Fig. 5D & E). Both Am580 and LGD1069 significantly inhibited the increased perivascular and interstitial collagen deposition and gene expression in ZDF rat hearts. As we observed in Fig. 3, Am580 and LGD1069 significantly inhibited diabetes-induced oxidative stress and activation of MAP kinases in ZDF rat hearts, suggesting that activation of RAR and RXR-mediated signaling suppressed diabetes-induced cardiac fibrosis, through inhibition of ROS generation, gene expression of TGF β and activation of the MAP kinase pathway.

3.7. Effect of diabetes on cardiac RAR and RXR signaling

Our previous study implicated that the impaired RAR and RXR signaling has an important role in high glucose-induced cell apoptosis in cardiomyocytes [11, 12]. Thus, we determined whether chronic diabetes affect cardiac RAR and RXR signaling. As shown in Fig. 6 A and B, we observed significantly decreased gene and protein expression of RAR α and RXR α in ZDF rat hearts, which was prevented by Am580 or LGD1069. ADH and RALDH are two critical enzymes for retinoic acid synthesis from vitamin A [28, 29]. As shown in Fig. 6C & D, gene expression of ADH was significantly decreased in ZDF rat hearts, and the expression of RALDH was also decreased; but, did not reach statistical significance ($p > 0.05$, vs lean). The expression of ADH and RALDH was maintained at a level comparable to lean rats, in AM580 or LGD1069 treated ZDF rat hearts.

3.8. Effect of Am580 and LGD1069 on left ventricular structural and functional changes

There was no difference observed in heart rate between the groups (Table 1, supplemental data). Increased heart weight (HW), HW/BW ratio and HW/TL (tibia length) ratio were observed in untreated-ZDF rats ($p < 0.05$, vs lean control). Due to changes in body weight between groups, we used the HW/TL ratio, as an index, to compare the effect of Am580 and LGD1069 on cardiac hypertrophy. Am580 significantly inhibited the increased HW/TL in ZDF rats (29 ± 0.49 mg/mm in ZDF + Am vs 33.4 ± 0.86 mg/mm in ZDF; $p < 0.05$). A slightly decreased HW/TL ratio was observed in LGD1069 treated ZDF rats (31.5 ± 0.56 mg/mm); but, did not reach statistical significance, compared to ZDF rats ($p > 0.05$). A significantly increased thickness of the systolic posterior wall of the LV (LVPWs), along with decreased systolic inner diameter of the LV was observed in untreated ZDF rats at 25 weeks of age (Fig. 7A & B), representing a concentric cardiac hypertrophy, which was not observed in Am580 and LGD1069 treated ZDF rats. No difference was observed in the thickness of the septal wall in any group (data not shown). The increased HW/TL and

LVPWs were in line with the increased expression of the hypertrophic markers ANP and BNP (Fig. 7H). There was no significant decrease in LV ejection fraction (LVEF) and fractional shortening (FS) in ZDF rats (Fig. 7C & D), suggesting no significant systolic dysfunction in ZDF rats, at 25 weeks of age. However, a significantly decreased E/A ratio, and increased IVRT and DT were observed (Fig. 7E-G) in ZDF rats, at 21 and 25 weeks of age ($p < 0.05$, vs lean rats), indicating that diastolic function was impaired. Recent studies have shown that BNP is a useful marker for the severity of diastolic dysfunction in diabetic patients [30, 31]. Thus, the significant changes in BNP expression further confirmed the diastolic dysfunction in ZDF rats. Am580 or LGD1069 treatment significantly improved the diastolic dysfunction, and inhibited the gene expression of ANP and BNP in the hearts of ZDF rats, suggesting that activation of RAR and RXR mediated improved cardiac dysfunction that developed in ZDF rats.

4. DISCUSSION

To better understand the role of RAR and RXR in regulation of the development of DCM, the effects of RAR α (Am580) and RXR (LGD1069) agonists, on cardiac function and the involved mechanisms were determined in ZDF rats. Our study demonstrated that both Am580 and LGD1069 ameliorated diabetes-induced cardiac diastolic dysfunction and other pathological alterations. The beneficial effects of Am580 and LGD1069 may be related to the following mechanisms: 1) improvement of the impaired cardiac insulin metabolic signaling by reducing systemic hyperglycemia and insulin resistance; 2) promoting cardiac glucose uptake and utilization, and reducing glucotoxicity/lipotoxicity-induced oxidative stress; 3) restoring impaired RAR and RXR signaling and inhibiting the generation of ROS; 4) through inhibiting oxidative stress-induced activation of MAP kinases and NF- κ B signaling, ameliorating diabetes-induced cardiac inflammatory responses, apoptosis and fibrosis.

The majority of diabetic patients (90–95%) suffer from type 2 diabetes mellitus [32], which is typified by hyperglycemia, hyperinsulinemia, hyperlipidemia, insulin resistance. Decreased myocardial glucose transport, glycolysis and glucose oxidation and enhanced fatty acid (FA) metabolism have been reported in diabetes [33] and have an important role in the development of cardiomyopathy [11, 34, 35]. Hyperglycemia is a key initiator of diabetic cardiomyopathy. Hyperglycemia leads to an increase in oxidative stress, by exacerbating glucose oxidation and mitochondrial generation of ROS, which results in DNA damage and contributes to accelerated apoptosis [36]. In addition, hyperglycemia-induced activation of cardiac renin-angiotensin system (RAS) components, also serves as an important contributor to cardiomyocyte apoptosis and fibrosis [16, 37]. Hyperglycemia promotes hyperinsulinemia, which in turn leads to insulin resistance and impairment of insulin signaling, resulting in diminished glucose transport and utilization and impaired cardiac efficiency [38]. Hyperinsulinemia further facilitates circulating and cellular levels of free fatty acid (FFA), which leads to increased lipid accumulation and FA beta-oxidation in cardiomyocytes and ultimately to mitochondria dysfunction, resulting in myocardial contractile dysfunction and apoptosis [39, 40].

In the present study, hyperinsulinemia, hyperglycemia, hyperlipidemia and insulin resistance were observed in ZDF rats. Diabetic cardiomyopathy, as evidenced by diastolic dysfunction with hypertrophy, fibrosis, inflammation and apoptosis developed in ZDF rats. Divergent results have been published regarding cardiac structural and functional changes in ZDF rats, as reported with or without cardiac hypertrophy, and diastolic dysfunction alone or in combination with systolic dysfunction [41–43]. The discrepant results may be due to the difference in age during the study and the methods of analysis. We have analyzed the remodeling process from 9 to 25 weeks of age, by multiple methods. A number of factors

could contribute to the pathological effects we observed. First, hyperglycemia and hyperlipidemia-induced intracellular generation of ROS can act as signal transduction molecules to activate various signaling pathways, which ultimately lead to inflammation, cell apoptosis and fibrosis [44, 45]. We observed significantly increased oxidative stress (decreased SOD1, SOD2 and increased ROS generation), apoptosis, inflammatory responses and activation of MAP kinases and IKK/NF- κ B signaling pathways in ZDF rat hearts. We have previously shown that hyperglycemia-induced ROS and subsequent activation of MAP kinases and NF- κ B pathways had an important role in regulation of cell inflammatory responses and apoptosis in cardiomyocytes [11, 13]. Our *in vivo* data further confirmed that diabetes-induced cardiac oxidative stress had an important role in regulation of the development of DCM. Second, cardiac insulin resistance and impaired insulin signaling has been implicated in the development of DCM [46]. Insulin resistance reduces activation/phosphorylation of PI3K/Akt pathway, which is involved in regulation of genes that control glucose and lipid homeostasis and other cellular processes such as cellular hypertrophy, protein translation, nitric oxide generation, and apoptosis [47, 48]. We observed decreased phosphorylation of Akt/GSK3 β in ZDF rat hearts from the early stage of diabetes, along with cardiac glucose and lipid dysmetabolism and other structural and functional alterations, suggesting that impaired insulin signaling has an important role in diabetes-induced cardiac remodeling in ZDF rats. Third, impaired RAR/RXR signaling might also contribute to the pathogenesis of DCM. We have shown previously that silencing the expression of RAR α or RXR α , promoted cardiomyocyte apoptosis and activation of the JNK-mediated apoptotic pathway [11, 12], suggesting that impaired RAR and RXR signaling contributes to HG-induced cellular injury [11, 12]. In ZDF rat hearts, significantly decreased gene and protein expression of RAR α , RXR α , ADH and RALDH was observed, suggesting that cardiac RA signaling is impaired in diabetic myocardium. We have previously demonstrated that oxidative stress and activation of JNK has an important role in HG-induced impairment of RAR and RXR signaling in cultured cardiomyocytes [12]; thus, the inhibition of RAR and RXR signaling in ZDF rat hearts was likely linked with the increased oxidative stress and apoptosis. Furthermore, diabetes induced expression/activation of cardiac RAS components also serve as an important contributor to the development of DCM [49]. An increased or over-activated RAS is associated with cardiac insulin resistance, through regulation of PI3k/Akt pathways [50]; and contributes to cardiac hypertrophy, fibrosis and apoptosis through regulation of the MAP kinase pathway, promoting ROS generation [16, 51, 52]. Our observation that increased gene expression of angiotensinogen, AT $_1$ receptor and decreased expression of ACE2 in ZDF rat hearts (data not shown), suggests that the increased expression/activation of RAS components has an important role in the development of DCM in ZDF rats.

Notably, treatment with both Am580 and LGD1069 was capable of favorably modifying the pathologic and functional alterations induced by diabetes. The protective effects of Am580 and LGD1069 may be mediated by diverse mechanisms. It has been shown that both RAR and RXR ligands are involved in the regulation of systemic glucose metabolism, in type 1 and 2 diabetic animals [10, 53, 54]. Our findings were consistent with these previous studies and showed that RAR or RXR ligands significantly lowered blood glucose levels in ZDF rats, as early as 1 week of treatment. The glucose lowering mechanisms may include the following: 1) reduced insulin resistance, as evidenced by improved GTT, AUC-GTT and decreased HOMA-IR in Am580 or LGD1069 treated ZDF rats; 2) ameliorating the impaired beta cell function, as demonstrated by increased plasma insulin levels and HOMA- β %; 3) and by inhibiting the increased gluconeogenesis in the liver of ZDF rats (gene expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver of ZDF rats was inhibited by Am580 and LGD1069, data not shown). Am580 and LGD1069 not only improved systemic glucose homeostasis; but, also had a significant effect on cardiac glucose metabolism. Impaired Akt/GSK3 β insulin signaling and decreased gene expression of

GLUT1, GLUT4, aldolase A and hexokinase 2, in ZDF rat hearts, was improved by Am580 or LGD1069, indicating that activation of RAR and RXR signaling, rescued the impaired cardiac insulin signaling and promoted glucose transportation and utilization. Studies have shown that hyperglycemia significantly enhances free radical formation and mitochondrial generation of superoxide [21] and triggers various adaptive and maladaptive responses, which synergistically lead to development of heart failure [55]. We observed that diabetes-induced oxidative stress and apoptosis, along with activation of MAP kinases and NF- κ B pathways were inhibited by Am580 and LGD1069. Thus, it is likely that the beneficial effect of Am580 or LGD1069 on diabetic cardiomyopathy, is mediated at least partially, by reducing glucotoxicity-induced cardiac oxidative stress and activation of apoptotic signaling. We have previously demonstrated the lack of RAR and RXR signaling promoted expression of RAS components and cardiomyocyte apoptosis [11]. Am580 and LGD1069 can directly and/or indirectly (through inhibition of diabetes-induced oxidative stress and activation of JNK signaling) reverse the decreased expression of RAR and RXR, leading to protection of cardiomyocytes from angiotensin II and oxidative stress-induced cellular injury and ameliorate cardiac structural and functional alteration associated with diabetic conditions.

As reported in previous studies [56, 57], dyslipidemia (increased plasma TG and cholesterol level) was observed in ZDF rats, accompanied with increased cardiac lipid uptake, beta-oxidation (increased gene expression of Fabp3, CPT-1, Acaa1, Acaa2 and Acad) and intracellular lipid deposition. Though both lipid uptake and oxidation were increased in the myocardium of ZDF rats, the imbalance between FA delivery and cellular utilization, might be the major reason for intracellular lipid accumulation and subsequent lipotoxicity, including intracellular ROS generation, ceramide production, insulin resistance and impaired contractile function [58, 59]. We have observed that treatment with either Am580 or LGD1069 can ameliorate cardiac diastolic dysfunction in ZDF rats; however, Am580 and LGD1069 have different effects on obesity and lipid metabolism in ZDF rats. Previous studies have shown that ATRA suppresses hyperlipidemia, obesity and blocks adipogenesis, by enhancing FA oxidation and energy dissipation, through ATRA-induced activation of PPAR β/δ and RAR in adipocytes, liver and skeletal muscle [8, 9]. The role of ATRA in regulation of cardiac lipid metabolism has not been reported. We observed that ATRA suppressed the body weight gain and the increased cardiac FA beta-oxidation in ZDF rats, following 2 weeks of treatment. Am580 had a similar effect on inhibiting body weight gain, cardiac FA uptake and beta-oxidation in ZDF rats, following 16 weeks of treatment. These data suggest that ATRA and Am580 may alter substrate metabolism in diabetic heart, through rebalancing the utilization between glucose and FA, which further leads to the improvement in cardiac efficiency and function. Though Am580 had a favorable effect on cardiac lipid metabolism, it had no effect on the increased plasma cholesterol and TG levels. Am580, a selective agonist of RAR α , is not an activator of PPAR β/δ [60] and thus the mechanisms whereby Am580 regulates cardiac lipid metabolism and suppresses obesity, may be different than that of ATRA. Compared to ATRA and Am580, LGD1069 further promoted body weight gain and hyperlipidemia following 16 weeks of treatment. Previous studies have shown that RXR ligands, including LGD1069, decrease TG and increase HDL levels in db/db or ob/ob mice [10, 61]. The short treatment periods (1–2 weeks) and different species used in these experiments, likely provide an explanation for the observed differences. Our data are more favorable than the results observed in RXR antagonist treatment or ablation of RXR α in adipose tissues in animal models, which show that blocking RXR results in resistance to diet-induced obesity [62, 63]. Though LGD1069 had the additional effect of promoting gene expression of Acaa1, Acaa2 and Acad, suggesting further increased beta-oxidation and FA utilization, significantly increased intracardiac lipid deposition was observed in LGD1069 treated ZDF hearts. This might be due to the hyperlipidemia and imbalance between FA uptake and oxidation in response to LGD1069.

The beneficial effect of LGD1069 on heart function may primarily be related to improved insulin signaling and reduced glucotoxicity-induced oxidative stress. Chronic hyperlipidemia and cardiac lipid accumulation may attenuate the beneficial effect of LGD1069 on heart function in the late stage of diabetes. As we observed, the increased HW/TL ratio was not significantly inhibited in LGD1069 treated ZDF rats; however, the thickness of LVPWs by echocardiography and the gene expression of ANP and BNP were significantly inhibited in LGD1069 treated ZDF rats, along with improved heart function, suggesting that a compensatory hypertrophic response had developed at the period we observed. It has been described that RXR can form permissive heterodimers with PPARs, farnesoid-X-receptor and liver-X-receptors, and that these can be activated by both RXR-specific and partner specific ligands [64]. We have observed that PPAR α and LXR are activated by LGD1069 (data not shown), and that these receptors are also involved in regulation of lipid metabolism [65, 66]. The effect of LG1069 on lipid homeostasis we observed in the present study, may be regulated not only through activation of RXR, since we could not exclude the involvement of other heterodimer partners of RXR. Using transgenic tools to selectively manipulate RXR in the heart, will be necessary to understand the role of RXR in regulation of cardiac lipid metabolism.

In conclusion, our results suggest that activation of RAR or RXR ameliorated the development of DCM in ZDF rats, through improving insulin signaling and cardiac glucose metabolism, reducing glucotoxicity-induced oxidative stress, inflammation and apoptosis. In addition, RAR and RXR have a distinct role in the regulation of lipid homeostasis and cardiac lipid metabolism. Our data provide further evidence that impaired RAR and RXR signaling has an important role in diabetes-induced cardiomyopathy (Fig. 8). Thus, determining the molecular mechanisms of RAR and RXR in regulation of diabetic cardiomyopathy and the association with glucose and lipid metabolism, may be of significant benefit for future strategies in development of effective treatments of diabetic and possibly other cardiovascular complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Activation of RAR and RXR-mediated signaling ameliorated diabetic cardiomyopathy.
- RAR and RXR inhibit diabetes-induced activation of MAP kinases and NF- κ B.
- Activation of RAR and RXR improved cardiac insulin signaling and glucose metabolism.
- RAR and RXR differentially regulate lipid homeostasis and cardiac lipid metabolism.

Figure 1. Am580 and LGD1069 improve insulin resistance and cardiac glucose metabolism in ZDF rats

Plasma glucose was monitored during GTT in lean and ZDF rats. Glucose levels were measured at 0, 30, 60 and 120 min following glucose uptake (A) and AUC was calculated based on GTT results (B). Plasma insulin levels were determined at the end of the experiment (C), HOMA-IR (D) and HOMA-β% (E) were analyzed as described in the “Research Design and Methods” section. F. Cardiac gene expression of GLUT1, GLUT4, Aldolase A and HK2 was determined by real time RT-PCR. G. Total and phosphorylated Akt and GSK3β were determined by Western blot. Densitometric quantification of p-Akt

and p-GSK3 β was normalized to total Akt and GSK3 β . Values are expressed as the mean $\pm$ SEM (n=3). *, p<0.05, vs L group; #, p<0.05 vs Z group.

Figure 2. Effect of Am580 and LGD1069 on lipid metabolism in ZDF rats

A. Plasma cholesterol, TG and HDL levels were measured at the end of the experiments. **B.** Cardiac gene expression of PGC-1 α , CPT1, Fabp3, Acaa1, Acaa2 and Acad was determined by real time RT-PCR and normalized to GAPDH. Data (mean $\pm$ SEM, n=6) are expressed as a relative value compared to control. *, p<0.05, vs L group; #, p<0.05 vs Z group. **C.** Myocardial lipid deposition. Representative photomicrographs showing Oil Red O staining in sections isolated from hearts of Lean, Lean+LGD1069, Lean+Am580, ZDF, ZDF+LGD1069 and ZDF+Am580 rats.

Figure 3. Am580 and LGD1069 inhibit diabetes-induced oxidative stress and apoptosis in hearts of ZDF rats

A & B. Measurement of oxidative stress in heart sections was determined using red DHE staining. DHE fluorescence intensity was calculated from ten images per heart and three hearts per group. Values are expressed as the mean $\pm$ SEM. *, $p < 0.05$, vs L group; #, $p < 0.05$ vs Z group. Cardiac expression of SOD1, SOD2 (**C & D**), Bcl2, Bax and Caspase-9 (**G & H**) was determined by Western blot. Actin was used as a loading control. The intensity of the bands was analyzed by densitometry and the Bcl-2/Bax ratio calculated. Data are expressed as the mean $\pm$ SEM, from 3 independent experiments. *, $p < 0.05$, vs L group; #, $p < 0.05$ vs Z group. **E & F.** The phosphorylation of ERK1/2, p-38 and JNK was determined

by Western blot, using specific antibodies against p-ERK1/2, p-p-38 and p-JNK. Blots were reprobed for total ERK1/2, p-38 and JNK. **I.** TUNEL assay was performed as described in the “Research Design and Methods” section. TUNEL positive cell counting was expressed as the mean $\pm$ SEM. *, $p < 0.05$, vs L group; #, $p < 0.05$ vs Z group.

Figure 4. Effect of Am580 and LGD1069 on cardiac inflammatory responses and activation of NF-κB signaling

A. Left ventricles were isolated and lysates of myocardium prepared at the end of the experiments. The phosphorylation and expression of IKK, IκBα and NF-κB was determined by Western blot. Blots were reprobbed with anti-actin antibody to verify equal loading. **B.** The intensity of the phosphorylated proteins was analyzed by densitometry. Data are expressed as the mean ± SEM, from 3 independent experiments. *, $p < 0.05$, vs L group; #, $p < 0.05$ vs Z group. **C.** Gene expression of IL-1β and TNF-α was determined by real time RTPCR. *, $p < 0.05$, vs L group; #, $p < 0.05$ vs Z group. Data are expressed as the mean ± SEM (n=6).

Figure 5. Am580 and LGD1069 suppress diabetes-induced fibrosis in hearts of ZDF rats

A. Paraffin sections were obtained from heart specimens and stained with hematoxylin-eosin and Masson's trichrome at the end of the experiments. Photomicrographs were quantified with Image J software to measure the area of interstitial and perivascular fibrosis. Ten sections and 30 vessels were examined in each heart (**B & C**). **D-E.** Gene expression of TGF β, collagen type I, II and III was determined by real time RT-PCR. *, $p < 0.05$, vs L group; #, $p < 0.05$ vs Z group. Data are expressed as the mean $\pm$ SEM (n=6).

Figure 6. Retinoic acid signaling in diabetic hearts in ZDF rats

Cardiac gene expression of RARα, RXRα (A), ADH (C) and RALDH (D) was determined by real time RT-PCR. The mRNA levels were normalized to GAPDH. Data (mean ± SEM, n=6) are expressed as a relative value compared to control. *, p<0.05, vs L group; #, p<0.05 vs Z group. B. Nuclear protein was extracted from left ventricles and protein expression of RARα and RXRα determined by Western blot. Equal loading was determined using anti-histone antibody. Densitometric quantification of RARα and RXRα protein bands was normalized to histone.

Figure 7. Am580 and LGD1069 improve diabetes-induced cardiac dysfunction in ZDF rats

A-G. Echocardiographic studies were performed and analyzed before (0 week) and after treatment with Am580 and LGD1069 for 8 and 16 weeks in Zucker lean (L) and ZDF (Z) rats (n=8). **H.** Total RNA was extracted from left ventricles at the end of the studies. Gene expression of ANP and BNP was determined by real time RT-PCR. The mRNA levels were normalized to GAPDH. Data (mean $\pm$ SEM, n=6) are expressed as a relative value compared to control. *, p<0.05, vs L group; #, p<0.05 vs Z group. LL (Lean+LGD1069); LA (Lean +Am580); ZL (ZDF+LGD1069); ZA (ZDF+Am580).

Figure 8. Proposed scheme for RA signaling-mediated amelioration of diabetes-induced cardiac diastolic dysfunction

Diabetes mellitus induce insulin resistance, glucose and lipid dysmetabolism, which promote the development of DCM (solid line). Oxidative stress and activation of JNK, results in impairment of RAR and RXR, further facilitating cell apoptosis and contributing to the development of DCM. Am580 and LGD1069 ameliorate the development of DCM, through the indicated signaling mechanisms, as shown in the diagram (broken line). Am, Am580; LGD, LGD1069; IR, insulin receptor; RAS, renin-angiotensin system; DCM, diabetic cardiomyopathy.