The role of epicardial adipose tissue in the pathogenesis of myocardial dysfunction

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„Das schönste Glück des denkenden Menschen ist, das Erforschliche erforscht zu haben und das Unerforschliche zu verehren.“

-Johann Wolfgang von Goethe-

Zusammenfassung

Es wird vermutet, dass Veränderungen im epikardialen Fettgewebe (EAT) einen Risikofaktor für die Entstehung von kardiovaskulären Erkrankungen bei Patienten mit Typ 2 Diabetes mellitus (DM2) und dem metabolischen Syndrom darstellen. Beim EAT handelt es sich um ein viszerales Fettdepot, welches faszienfrei auf dem Myokard aufliegt. Daher können Faktoren, die von diesem Fettgewebe freigesetzt werden, direkt auf das Myokard wirken. Ziel dieser Arbeit ist es, die Bedeutung des EAT für die Entstehung von Herzerkrankungen zu definieren, indem seine direkte Auswirkung auf die Funktion, den Stoffwechsel und die Struktur des Herzens untersucht wird. Da humanes EAT limitiert ist und meist von multimorbiden Patienten stammt, wird hier zunächst auf ein Tiermodell zurückgegriffen. Sowohl bei Ratten als auch bei Mäusen ist die Menge an EAT allerdings sehr gering, daher wurden hier Meerschweinchen verwendet, wo gezeigt werden konnte, dass die Masse an EAT mit dem Alter zunimmt. Dazu wurde EAT und subkutanes Fettgewebe (SAT) von Meerschweinchen gesammelt, die mit einer Hoch-Fett Diät (HFD) gefüttert wurden und sowohl eine Insulinresistenz als auch eine verminderte linksventrikuläre Funktion des Herzens, im Vergleich zu Meerschweinchen die mit einer Standarddiät gefüttert wurden, entwickelt haben. Zusätzlich wurden humane Fettbiopsien von EAT, SAT und perikardialem Fettgewebe (PAT) von Patienten mit und ohne DM2 entnommen. Um die Freisetzung und Wirkung von Faktoren, die aus dem Fettgewebe ausgeschüttet werden, zu testen, wurden hier konditionierte Medien mit Hilfe der Fettexplantat-Technologie generiert. Rattenkardiomyozyten, die mit konditionierten Medien (EAT) von DM2-Patienten oder HFD-gefütterten Tieren behandelt wurden, zeigen eine verminderte Akt-Ser473-Phosphorylierung im Vergleich zu Zellen, die mit konditionierten Medien von SAT bzw. mit den entsprechenden Kontrollen inkubiert wurden. Kontraktionsmessungen zeigen zudem, dass konditionierte Medien (EAT) die Sarkomer-verkürzung und den Kalziumtransienten im Vergleich zu SAT vermindern und gleichzeitig kontraktile Parameter in Kardiomyozyten verschlechtern. Dies konnte sowohl bei den HFD-gefütterten Meerschweinchenproben als auch bei den Proben der DM2-Patienten beobachtet

werden. Zusätzlich zur verminderten Kontraktilität konnte eine verringerte Proteinexpression von SERCA2a in Kardiomyozyten beobachtet werden, die mit konditioniertem Medium (EAT) der DM2-Patienten oder HFD-gefütterten Tiere behandelt wurden. Daher wird vermutet, dass ein verminderter Kalziumtransient durch eine verminderte Expression von SERCA2a in diesen so behandelten Kardiomyozyten zu erklären ist.

Mit Hilfe dieser Studie konnte zum ersten Mal gezeigt werden, dass das Sekretionsprofil des EAT sich von anderen Fettdepots unterscheidet, und dass sowohl HFD als auch DM2 zu Veränderungen in der Freisetzung der Faktoren aus dem EAT führen können. Analysen der Medien zeigten, dass ein Teil der schädlichen Effekte möglicherweise darauf zurückzuführen ist, dass es vermehrt zu einer Akkumulation von Activin A im konditionierten Medium vom EAT, sowohl bei DM2-Patienten als auch von HFD-gefütterten Meerschweinchen, gekommen ist. Bei Activin A handelt es sich um ein Zytokin, welches zur TGF-ß Familie zählt und eine Rolle bei einer Vielzahl von Prozessen wie etwa der Apoptose spielt. Im Einklang mit früheren Studien, die zeigten, dass Activin A die Expression von Genen erhöht, die eine wichtige Rolle bei myokardialen Umbauprozessen spielen, konnte in dieser Studie gezeigt werden, dass Kardiomyozyten, die mit rekombinantem Activin A behandelt wurden, eine Verminderung in der kontraktilen Funktion aufwiesen. Dementsprechend konnte durch die Verwendung des TGF-ß-Rezeptor-Inhibitors SB431542 der durch konditioniertes Medium hervorgerufene kardiosuppressive Effekt zum Teil wieder aufgehoben werden. Dadurch, dass dieser Effekt allerdings nicht vollkommen reversibel war, kann davon ausgegangen werden, dass neben Activin A noch weitere Mediatoren vom Fettgewebe freigesetzt werden, die diesen kardiosuppressiven Effekt hervorrufen bzw. dazu beitragen. Darüberhinaus konnten die beiden potenziellen Faktoren Follistatin-like 1 (Fstl1), ein physiologischer Antagonist von Activin A, und Omentin identifiziert werden, die vermindert aus dem EAT von DM2-Patienten freigesetzt werden. Durch die Zugabe dieser Faktoren zu Kardiomyozyten, die mit konditionierten Medien behandelt wurden, konnte der durch konditioniertes Medium hervorgerufene kardiosuppressive Effekt wieder aufgehoben werden.

Zusammenfassend lässt sich sagen, dass es bei DM2 sowie bei einer HFD zu einer depot-spezifischen Veränderung in der Freisetzung von Zytokinen kommt. Diese Fettgewebs-freigesetzten Faktoren wiederum können in isolierten Kardiomyozyten zu einer Insulinresistenz

und zu einer Einschränkung in der kontraktilen Funktion führen. Hierbei hat sich verstärkt gezeigt, dass EAT von DM2-Patienten und HFD-gefütterten Tieren im Vergleich zu den jeweiligen Kontrollen die Funktion dieser Zellen deutlicher beeinträchtigt. Diese Daten lassen vermuten, dass sekretierte Faktoren aus dem EAT mit dem Myokard interagieren und somit das EAT eine Rolle bei der Pathogenese von diabetischer Kardiomyopathie spielen könnte.

Summary

It is assumed that epicardial adipose tissue (EAT) dysfunction can be a risk factor for the development of cardiovascular disease in patients with type 2 diabetes mellitus (DM2) and the metabolic syndrome. EAT is a visceral thoracic fat depot around the heart, which is not separated by a fascial boundary from the underlying tissues. Consequently, secretory products from EAT may directly affect the function of the myocardium and coronary vessels. The aim of the study was to define the role of EAT in the pathogenesis of cardiac disease by assessing its direct effect on parameters of cardiac function, metabolism and structure. As EAT is limited and mostly from multimorbid patients, an animal model was established first. Because the amount of EAT is scarce in rat and mouse, we used guinea pigs in which the amount of EAT increases with age. EAT and subcutaneous adipose tissue (SAT) were collected from guinea pigs fed a high-fat diet (HFD) which develop insulin resistance and decreased left ventricular as compared to standard fed animals. In subsequent studies, human adipose tissue biopsies were collected from EAT, SAT and pericardial adipose tissue (PAT) from patients with and without DM2 undergoing coronary artery bypass surgery. To analyze the secretion of adipose tissue-derived factors and the effect of these factors on cardiomyocytes, conditioned media were prepared using explant technology. Isolated primary rat cardiomyocytes exposed to conditioned media generated from EAT of HFD-fed guinea pigs or DM2-patients showed an decreased insulin-mediated phosphorylation of Akt-Ser473 compared to conditioned media from other fat depots and corresponding controls. Furthermore, we observed that, compared to control adipocyte media, exposure of primary cardiomyocytes to conditioned media generated from EAT of HFD-fed guinea pigs and DM2-patients markedly impaired contractile function, as illustrated by reductions in sarcomere shortening and cytosolic Ca^{2+}-fluxes. The reductions in sarcomere shortening and cytosolic Ca^{2+}-fluxes were paralleled by a decreased protein expression of SERCA2a in cardiomyocytes. Therefore, it seems likely that the reduction of Ca^{2+} may account for the reduction transients in SERCA2a expression in cardiomyocytes treated with conditioned media, generated from EAT of DM2-patients or HFD-fed guinea pigs.

These studies show for the first time that the adipokine secretory profile of EAT is distinct from that of other fat depots, and that HFD and DM2 induce alterations in the factors secreted by EAT compared to the corresponding controls. Profiling of the conditioned media subsequently demonstrated that part of the detrimental effects could be ascribed to accumulation of activin A in conditioned media from EAT of both DM2-patients and HFD-fed guinea pigs. Activin A belongs to the transforming growth factor (TGF)-ß family and plays a role in cell proliferation, apoptosis and cell differentiation. In line with previous reports, demonstrating that activin A increases the expression of genes involved in myocardial remodelling, the present study shows that incubation of cardiomyocytes with recombinant activin A, at a concentration comparable to that found in conditioned media from EAT of DM2-patients, reduced cardiomyocyte contractile function. Accordingly, the cardiosuppressive effects of conditioned media from EAT of DM2-patients could be partially reversed by pharmacological inhibition of the activin A receptor by using SB431542. Although these findings highlight the involvement of activin A in the induction of cardiomyocyte dysfunction by conditioned media from EAT of DM2-patients, they also indicate an involvement of as yet unknown secreted factors because the cardiosuppressive effect of conditioned media could only partially reversed by SB431542. In subsequent studies, we have characterized the role of two potential candidate factors, i.e. follistatin-like protein 1 (Fstl1), a physiological antagonist of activin A, and omentin. Both factors were reduced in EAT from DM2-patients, and addition of Fstl1 or omentin could reverse the induction of insulin resistance and contractile dysfunction induced by adipose tissue-derived factors in cardiomyocytes.

In conclusion, conditioned media generated from EAT of DM2-patients induce myocardial dysfunction and insulin resistance in cardiomyocytes. We demonstrated that the secretory profile of EAT is distinct from that of other fat depots, and that HFD and DM2 induce alterations in the secretory profile of EAT. Most notably, the release of activin A is enhanced by EAT from DM2-subjects and HFD-fed animals, and exposure of cardiomyocytes to activin A causes cardiomyocyte dysfunction. Conversely, omentin and Fstl1 are reduced in EAT from DM2-subjects and have been identified as cardioprotective factors. Therefore, this work shows that DM2- and HFD-related alterations in the secretory profile of EAT can contribute to the development of cardiac dysfunction in patients with DM2 and the metabolic syndrome.

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List of Abbreviations

ALK	activin receptor-like kinase
AMH	anti-Müllerian hormone
BMP	bone morphogenic protein
BMI	body mass index
CAD	coronary artery disease
CD14	cluster of differentiation 14
CD62E	CD62 antigen-like family member E
CM	conditioned medium
CVD	cardiovascular disease
DCM	diabetic cardiomyopathy
DM1/2	diabetes mellitus type 1/2
EAT	epicardial adipose tissue
eNOS	endothelial oxide synthase
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FABP	fatty acid binding protein
FSTlI	Follistatin-like protein 1
GAD	glutamic acid decarboxylase
GDF15	growth differentiation factor 15
GLUT	glucose transporter
GSK	glycogen synthase kinase
HFD	high fat diet
HOMA	homeostatic model assessment

ICA	islet cell antigen
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
IGT	impaired glucose tolerance
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LADA	latent auto-immune diabetes with onset in adults
LPS	lipopolysaacharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein 1
MIP	macrophage inflammatory protein
MMIF	macrophage migration inhibitory factor
MODI	maturity onset diabetes
mTORC	mammalian target of rapamycin complex
NGF	nerve growth factor
NF-kB	nuclear factor-k B
NIDDM	non-insulin-dependent diabetes mellitus
PAI-1	plasminogen activator inhibitor 1
PAT	pericardial adipose tissue
PDK1	PI3K-dependent serine/threonine kinase 1
PI	phophoinositol
PI3K	phosphatidylinositol 3-kinase

PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKA/B/C	protein kinase A/B/C
PPAR	peroxisome proliferator-activated receptor
PRDM 16	PR-domain missing 16
PTEN	phosphatase and tensin homolog
PVAT	perivascular adipose tissue
RAS	rennin angiotensin system
ROS	reactive oxygen species
R-Smad	receptor activated Smad
SAPK	stress-activated protein kinase
SAT	subcutaneous adipose tissue
SERCA	sarco/endoplasmic reticulum Ca^{2+} -ATPase
sPLA-IIA	secretory type II phospholipase A2
Smad	mother against decapentaplegic
SOCS3	suppressor of cytokine signalling 3
TAK	TGF-ß activated kinase
TGFβ	transforming growth factor β
TIMP 1	tissue inhibitor of metalloproteinase 1
TNF	tumor necrosis factor
UCP-1	uncoupling protein-1
VEGF	vascular endothelial growth factor

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Introduction and

Outline of the thesis

Chapter 1

1 General Introduction

1.1 Type 2 diabetes mellitus, insulin resistance and cardiovascular diseases

1.1.1 Pathophysiology of type 2 diabetes mellitus and the metabolic syndrome

Type 2 diabetes mellitus (DM2), which is also known as non-insulin-dependent diabetes mellitus (NIDDM), is a chronic disease, characterized by relative insulin deficiency and insulin resistance in peripheral tissues.[1] Moreover, DM2 is directly associated with hyperglycemia, that results from defects in insulin secretion and action, and inadequate suppression of glucagon production.[2] These metabolic defects result in insufficient uptake, storage, and disposal of ingested glucose and enhanced production of glucose in the liver.[3] The second main form of diabetes is type 1 diabetes mellitus (DM1), which is formerly known as insulin-dependent diabetes mellitus (IDDM). DM1 is characterized by a total lack of insulin secretion because of the destruction of the insulin-producing ß-cells in the pancreas, typically due to an auto-immune reaction frequently caused by autoantibodies, where the ß-cells are attacked by lymphocytes.[4] Some known predictors of IDDM are autoantibodies against islet cell antigens such as glutamic acid decarboxylase (GAD), or islet cell antibodies (ICA). The etiology of DM1 is still unknown, but it has been suggested that it is triggered by environmental factors in genetically susceptible subjects.[5] In DM1, the ß-cells are attacked by the body's defence system and it has been assumed that viral infections, such as Coxsackie B4 enterovirus, or cytokines, like Interleukin (IL)-1 alone or in combination with Interferon (IFN)-γ and tumor necrosis factor (TNF) α, leads to

ß-cell apoptosis.[6,7] Data regarding the genetic influence, which contribute to the development of diabetes, are limited, but up to now at least 20 different susceptibility genes have been identified linked to DM1.[8] In particular, multiple current studies prove that two different loci have emerged with consistent evidence of association with DM1, namely the human leukocyte antigen (HLA) region on chromosome 6 and the insulin gene region on chromosome 11.[9] Interestingly, about 10 - 20 % of patients develop diabetes because of MODY (maturity-onset diabetes of the young), a monogenic disorders with autosomal dominant inheritance, or because of LADA (latent autoimmune diabetes of the adult), an auto-immune disorder with a lack some DM2-symptoms such as obesity.[10,11] Zimmet *et al.* introduced the term LADA to describe this subgroup of adult phenotypic DM2-patients positive for the autoantibody GAD and who present clinically without ketoacidosis and weight loss.[12]

Worldwide, diabetes continues to be a major health problem and in general, it is assumed that diabetes is the fourth or fifth leading cause of death in most developed countries. Because of changes in human environment, human behaviour and lifestyle, the number of people diagnosed with diabetes increased rapidly in the past two decades. Interestingly, relative to DM1, more than 90 % of all diabetes cases accounts for DM2, whereas only 5-10 % accounts for DM1.[13,14] In 2010, according to the International Diabetes Federation (IDF) Atlas, more than 285 million individuals (6.6 % of adults aged from 20 to 79 years) suffer from diabetes, of whom about 80 % reside in developing countries. For 2030, IGF estimated that more than 380 million will be diagnosed with diabetes and more than 473 million with impaired glucose tolerance (IGT).[15] India and South East Asia, for instance, are two of the regions in which the number of people with diabetes increases immensely from 46.9 million in 2007 to 119.5 million individuals by the year 2025. In Africa, Eastern Mediterranean and Middle East, there is also a high prevalence of diabetes (Africa 10.4 million, Eastern Mediterranean and Middle East 24.5 million in 2007), which will still increase by over 80 % until 2025. By the year 2025, the adult diabetic population in South and Central America (16.2 million) is expected to increase over 102 % to 32.7 million individuals. Moreover, in North America (28.3 million) and Western Pacific (67 million) the prevalence of diabetes is also enhanced and by the year 2025, the number of diabetic patients will be increased over 40 % up to 40.5 million individuals in North America and 99.4 million individuals in the Western pacific region. From a percentage point of view, in

Europe the number of people who suffer from diabetes (53.2 million in 2007) will not increase as much as for the other regions. Here, it will be expected that the amount of diabetics rise to about 20 % from 53.2 million to 64.1 million. However, in Germany, the country with the largest population in Europe, data regarding the prevalence of diabetes are limited. For the year 2025 a diabetes prevalence of 13.3 % was predicted for Germany but more precise data are rare. A study of diabetic patients from south Germany (aged from 35-59 years) called KORA F4 revealed that disturbed glucose regulation is a major health problem in the younger and middle aged population in South Germany and that about 40 % of the population 55 to 74 years in the Augsburg region had disturbed glucose tolerance or diabetes.[16,17] Despite the large number of people diagnosed with diabetes, approximately 1.5 million individuals of diabetes cases in Germany were undetected in the age group 55–74 years.

Early symptoms of diabetes are hyperglycemia, and loss of glucose in the urine. However, chronic hyperglycemia results in long-term micro- and macrovascular complications, which reduce life expectancy up to 8 years.[18] Microvascular complications include diabetic nephropathy, retinopathy, neuropathy and macrovascular complications such as cardiovascular disease, in particular ischemic heart disease, but also peripheral vascular and cerebrovascular disease for instance stroke.[19,20] But one of the most important characteristics in DM2 is the insulin resistance in liver, skeletal muscle and adipose tissue.

DM2 often occurs together with the metabolic syndrome. Patients with the metabolic syndrome have an increased risk of developing cardiovascular disease.[21,22] In general, the metabolic syndrome, previously also described as syndrome x or the insulin resistance syndrome, is defined as a group of conditions that can increase the risk of heart attack, stroke, and diabetes.[23] According to the IDF and AHA/NHLBI, 20-25 % of the world's adults have metabolic syndrome and it usually diagnosed if patients displays three of the following characteristics listed in Table 1.1.[24,25]

Table 1.1 Criteria for clinical diagnosis of the metabolic syndrome

Measure	**Categorical cut points**
Elevated waist circumstances	Population- and country-specific definitions
Raised blood pressure	(systolic >130/ diastolic >85 mm Hg)
Raised triglyceride levels	(>150 mg/dL)
Reduced high-density lipoprotein cholesterol	(<40 mg/dL ♂, < 50 mg/dL ♀)
Elevated fasting plasma glucose	(>100 mg/dL)

Moreover, it is well known that an increased number of evident metabolic syndrome risk factors contributes to an increased risk of cardiovascular mortality.[26]

1.1.2 Diabetic cardiomyopathy

Patients with diabetes have high morbidity and mortality rates that have been ascribed to cardiac dysfunction resulting from diabetic cardiomyopathy.[27] Diabetic cardiomyopathy was first described by Rubler *et al.* in 1972 and has been associated with both forms of diabetes type 1 and type 2.[28] The main factors contributing to the development of diabetic cardiomyopathy are microangiopathic changes in the small vessel of the heart, autonomic neuropathy, endothelial dysfunction and metabolic alterations in substrate supply.[29,30]

In diabetic cardiomyopathy, myocardial structural and functional abnormalities such as increased left ventricular mass, a reduced endocardial and mid-wall fractional shortening, and, most importantly, a higher prevalence of left ventricular diastolic dysfunction, have been observed.[31] Thus, functionally hallmarks of diabetic cardiomyopathy are left ventricular dilation, myocyte hypertrophy and reduced or preserved systolic function in the presence of ventricular diastolic dysfunction. Furthermore, the electrical and mechanical properties of the heart from diabetic patients with cardiomyopathy are impaired.[32] Importantly, these myo-cardial abnormalities in diabetic patients with cardiomyopathy are present, even in the absence of coronary atherosclerosis or hypertension.[33]

The cellular mechanisms underlying the development of diabetic cardiomyopathy are triggered by non-esterified fatty acids, myocardial insulin resistance, and hyperglycemia.[34] The

molecular mechanisms have been explored in detail in rodent models, and phenotypic changes result in myocardial dysfunction similar to that seen in diabetic human patients suffering from cardiomyopathy such as coronary artery disease and heart attack.[35] Diabetic cardiomyopathy is a multifactorial disease, which is characterized by multiple changes in structure, calcium signaling, lipid metabolism, and function of the myocardium. However, one of the major contributing factors in the pathogenesis of diabetic cardiomyopathy are alterations in myocardial substrate supply and utilization resulting from disrupted insulin signaling.[36] In diabetic hearts, defects in the stimulation of glycolysis and glucose oxidation can be found in particular due to a decreased glucose transport across the sarcolemmal membrane into the myocardium. Moreover, high circulating levels of free fatty acids reduce glucose oxidation by inhibiting fatty acid oxidation at the level of pyruvate dehydrogenase, enhance peripheral insulin resistance, increase endoplasmic reticulum (ER) stress and myocardial dysfunction, and trigger cell death.[37] The accumulation of toxic molecules in the diabetic heart, such as free fatty acids and free radicals, also dysregulate the function of proteins regulating cytosolic calcium metabolism, such as sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) and natrium-calcium exchanger function.[38] These defects include reduced activities and expression of ATPases like SERCA and other exchangers such as natrium-calcium, which causes decreased ability of the sarcoplasmic reticulum to take up calcium.[39] Finally, it has been reported that abnormalities in the protein levels, and activities of myosin isoenzymes and regulatory proteins, and myosin phosphorylation contribute to the development of myofibrillar remodeling in the diabetic heart.[40]

In addition, increased oxidative stress, enhanced inflammatory state and alteration in body mass (obesity) may also play an important role in the development of diabetic cardiomyopathy.[36] In a mouse model of DM2, hyperglycaemia leads to increased glucose oxidation, and mitochondrial and cytosolic generation of reactive oxygen species (ROS), which causes reduced mitochondrial ATP production and induce cardiomyocyte cell-death.[41] Reduced ATP production, due to the increased mitochondrial generation of ROS, affects myocardial contractility. Moreover, ROS can also alter gene expression, for example, cardiac myosin heavy-chain gene expression, by activation of the NF-kB (nuclear factor kappa B) pathway.[42]

Finally, diabetic cardiomyopathy is characterized by changes in cardiac structure including interstitial and perivascular fibrosis. Typical features of interstitial fibrosis are increases in collagen deposition around intramural vessels and between myofibers, which enhance the stiffness of the heart. It has been suggested that myocardial fibrosis is caused by the activation of the RAS (renin-angiotensin) system, which is associated with enhanced oxidative damage and cell death.[38]

1.1.3 Myocardial energy metabolism

One of the major contributing factors in the pathogenesis of diabetic cardiomyopathy are alterations in myocardial substrate supply and utilization.[36] To sustain myocardial contraction, basal metabolic processes and ionic homeostasis, a constant supply of fuel and oxygen is required, in order to maintain intracellular ATP-level in the heart. More than 95 % of the ATP production comes from the mitochondrial oxidation of different substrates such as fatty acids (60-70 %), glucose (20 %) and lactate (10 %).[43] The main ATP-generating pathway in the healthy adult heart is the long chain fatty acid oxidation, but when glucose and insulin plasma concentrations rise in the circulation, glucose becomes the favoured oxidized substrate. In the diabetic heart, almost 80-90 % of the ATP is generated from lipid oxidation, as direct result of increased circulating fatty acids and decreased insulin sensitivity.[44,45] Oxidation of long-chain fatty acids further inhibits glucose oxidation via the Randle cycle.[46] Consequently, when the rate of fatty acid uptake increases the rate of fatty acid oxidation, lipotoxicity may occur, which has been implicated in the development of cardiac dysfunction in DM2.[36]

Insulin, a key regulator of myocardial energy substrate metabolism, was first discovered by Banting and Best in 1921, and it has been shown that this polypeptide hormone is produced and released by the ß-cells of the islets of the Langerhans in the pancreas in response to increased plasma glucose levels.[47] Insulin stimulates the uptake of glucose into striated muscle such as skeletal and cardiac muscle and adipose tissue, and consists of the two dissimilar polypeptide chains A and B, which are linked by two disulfide bonds.[48] In the heart, insulin regulates metabolism by promoting glucose transport, glycolysis, glycogen synthesis, lipid

metabolism, protein synthesis, growth, contractility, and apoptosis in cardiomyocytes.[49,50] Insulin-stimulated glucose uptake involves the translocation of intracellular vesicles containing the glucose transporter (GLUT)4 from intracellular stores to the cell surface.[51-53] In the late 1980s, GLUT4 was found to be expressed uniquely in insulin-sensitive muscle and adipose tissue.[54] In basal cells, like cardiac muscle cells, GLUT4 is slowly exocytosed and rapidly internalized and upon stimulation with insulin, exocytosis is accelerated whereas endocytosis is inhibited. So insulin promotes the abundance of GLUT4 at the sarcolemmal membrane thereby promoting glucose uptake in the heart.[55]

The signaling pathway via which insulin promotes glucose uptake involves binding of insulin to the insulin receptor (IR), a tetrameric protein comprising two extracellular α-subunits and two transmembrane ß-subunits.[56] The IR belongs to the class of tyrosine kinase receptors and activation of this receptor results in an autotransphosphorylation by activation of the intrinsic tyrosine kinase activity of the ß-subunits.[57] Expression studies demonstrated that this receptor is expressed in virtually all vertebrate tissues, in particular in adipocytes and hepatocytes.[58] Subsequently, the cytosolic protein IR substrate proteins (IRS) become phosphorylated at multiple tyrosine residues (Figure 1.1). This leads to the binding and activation of phosphatidylinositol 3-kinase (PI3K), which then translocates to the plasma membrane and phosphorylates phosphoinositol (PI) substrates to generate inositol phospholipid-triphosphate (PIP_3). In cardiac muscle, the main signal that triggers insulin action is the activation of the regulatory subunit p85 of PI3K by IRS1.[59] Phosphorylation of IRS can also contribute to a second pathway, the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is principally involved in cell growth and differentiation.[60,61]

Activation of the PI3K pathway induces the increase in PIP_3 at the plasma membrane, which leads to the recruitment and co-localization of the PI3K-dependent serine/threonine kinase (PDK1) and protein kinase B (PKB)/Akt. During the last 20 years, it could be demonstrated that the PKB/Akt pathway plays a crucial role in insulin signaling. In fact, PIP_3 binds to the PH domain of PKB/Akt and this leads to translocation of Akt from a mainly cytosolic localisation to the plasma membrane, and phosphorylation at Thr308 by PDK1. PIP_3 triggers also the activation

of the mammalian target of rapamycin complex-2 (mTORC2), which has been implicated in the phosphorylation of Akt on Ser473.[62]

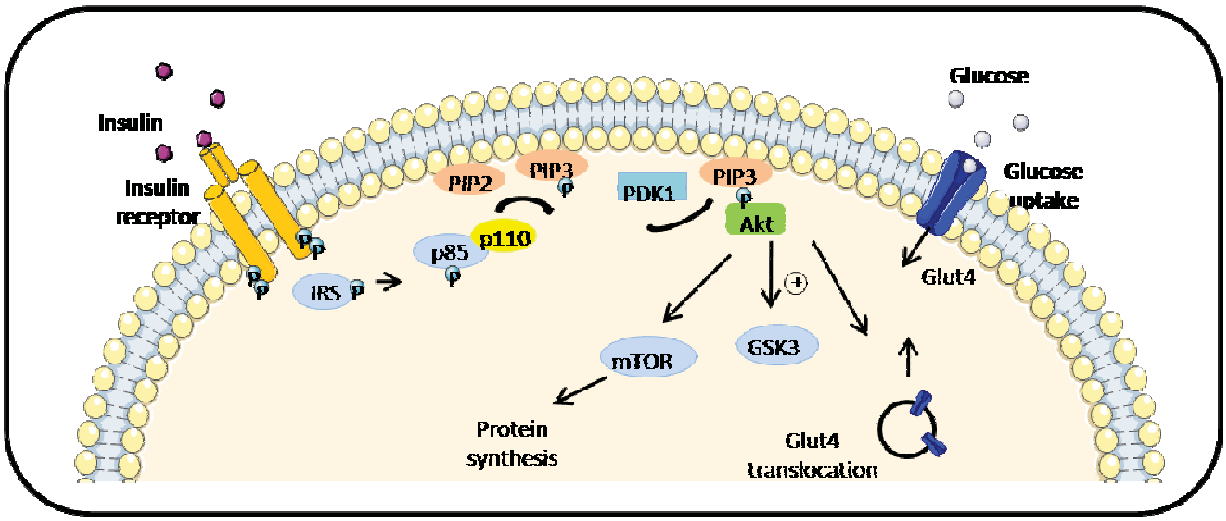

Figure 1.1 Assembly of the insulin signaling pathway (from the "Cell membrane slide kit" from Servier). Glut, glucose transporter; Gsk3; glycogen synthase kinase 3, IRS, insulin receptor substrate; mTOR, mammalian target of rapamycin, P, phosphate; PDK, PI3K-dependent serine/threonine kinase, PIP2, phosphatidylinositol (4,5)-bisphosphate; TGF, tumor necrosis factor.

PKB/Akt, named so because of its high homology with protein kinase A (PKA) and C (PKC), exists in three isoforms, Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ), which have demonstrated to play over-lapping but also differential roles. The effects of Akt on myocardium have been reported in several transgenic mouse models, and cardiac specific overexpression of different mutated forms of Akt results in varying phenotypes.[63] For example, in transgenic mice, it could be shown that cardiomyocyte size is enhanced and either maintained or improved cardiac function.[64] In the family of Akt-proteins, one of the most important proteins is Akt2, which functions primarily as a regulator of glucose metabolism. Akt 1 plays an important role on growth and anti-apoptosis, and until now, the specific role of the third Akt-protein Akt3 is not completely clear. However, previous studies showed that Akt3 is highly expressed in the brain and not required for maintenance of normal carbohydrate metabolism.[65] Activation of the serine-threonine kinase Akt2 regulates the transmission of the insulin signal by phosphorylation of components of the GLUT4 complex and the α-subunit at Ser21 and ß-subunit at Ser9 of

glycogen synthese kinase-3 (GSK-3). The phosphorylation and inactivation of GSK-3 causes activation of glycogen synthase, which catalyses the final step in glycogen synthesis. In addition, PKB/Akt phosphorylates AS160 (also called TBC1D4), which is essential for basal GLUT4 intracellular retention. This substrate has a Rab GAP (GTPase-activating protein) domain and increasing evidence suggested that AS160 plays an important role in GLUT4 trafficking as a positive regulator. In adipocytes, AS160 knockdown leads to a partial redistribution of GLUT4 from intracellular compartments to the plasma membrane, a concomitant increase in basal glucose uptake, and a increase in basal GLUT4 exocytosis.[66] This indicates that the substrate of Akt AS160 is required for membrane trafficking. However, recent studies in adipocytes and myocytes suggest, that the Akt-AS160 axis is probably not the sole mechanism, which regulates the insulin-stimulated GLUT4 translocation.[67,68] The newly identified PKB/Akt substrate TBC1D1, which is also a Rab GAP, or the phosphoinositide 5-kinase PIKfive and the SNARE-associated protein synip, two other substrates of PKB/Akt, could also play a role in GLUT4 translocation.[69,70] However, the specific mechanisms of TBC1D1, PIKfive and synip remain to be elucidated.

Akt 2, as a key downstream substrate of PI3K, also regulates the activity of many other downstream targets. For instance, it promotes cell survival and inhibits apoptosis by phosphorylating and inhibiting Forkhead transcription factors. These Forkhead proteins regulate the transcription of target genes involved in metabolism, but it also mediates the survival factor function of growth factors by controlling expression of apoptosis genes. A major negative regulator of the PI3P/Akt signaling pathway is the PTEN (Phosphatase and Tensin Homolog) phosphatase by dephosphorylation of PIP3.[71]

Interestingly, there is ample evidence indicating that insulin signaling and action are disturbed under multiple pathological states, including insulin resistance, DM2, the metabolic syndrome and cardiac disease.

1.1.4 Pathophysiology of insulin resistance

Insulin resistance, a decreased ability to respond to the effects of insulin, leads to the release of free fatty acids from adipose tissue, increased hepatic production of very-low-density lipoproteins and decreased high-density lipoproteins. Multiple studies demonstrated that insulin resistance is associated with defects in the action of insulin on PI3K activation, Akt-phosphorylation at Thr308 and Ser473, PI3K activity and glucose transport.[72] In the last years, a series of studies demonstrated that increased release of free fatty acids, inflammatory cytokines such as TNF-α, and mitochondrial dysfunction could contribute to decreased striated muscle glucose uptake, increased hepatic gluconeogenesis, and β-cell dysfunction, leading to hyperglycemia.[73-75]

In the setting of insulin resistance and obesity, it has been suggested that increased circulating free fatty acid and very-low-density lipoprotein concentrations cause an elevated lipid supply to the cardiac myocytes that leads to a cytosolic accumulation of lipid metabolites such as ceramides.[43] Furthermore, it has been assumed that increased ceramide levels resulting from lipid accumulation contribute to apoptosis of cardiomyocytes that in turn leads to contractile dysfunction.[43] Finally, enhanced fatty acid oxidation contributes to lipotoxicity in the heart, which causes feedback inhibition of pyruvate dehydrogenase (catalyzes oxidative decarboxylation of pyruvate) and subsequent glucose oxidation, ultimately resulting in the development of myocardial insulin resistance.[76] Thus insulin resistance, but also hyperglycemia and impaired insulin secretion not only form the basis for the development of DM2 and the metabolic syndrome, but also contribute to cardiovascular disease in these pathways.[77,78]

1.1.5 Mass of adipose tissue as potential predictor for cardiovascular and metabolic disease

In the early 1980s, a group of scientists revealed that the waist-hip ratio and the ectopic fat accumulation are one of the best predictors of cardiovascular and metabolic diseases such as insulin resistance.[78-80] Moreover, it has been shown that individuals with increased visceral

adiposity have more obesity-related complications than people with increased subcutaneous obesity.[81] Interestingly, since the 1980s the prevalence of obesity has tripled in Europe and according to World Health Organisation Europe, it is expected that more than 150 million adults and 15 million children become obese by the year 2010. In general, subcutaneous adipose tissue (SAT) represents about 82-97 % of total fat, whereas visceral fat (10-15 %) and cardiac fat (0-3 %) represents a minor amount of total body fat. Importantly, adipose tissue not only acts as a lipid storage organ, but also functions as an endocrine tissue by secreting adipokines. Expansion of adipose tissue is highly associated with a switch towards the secretion of pro-inflammatory adipokines, which amongst others have negative effects on insulin sensitivity and vascular function.[82] A consequence of adipose tissue dysfunction is the enhanced storage of triglycerides in non-adipose tissues, such as the muscle, liver, pancreas and heart.[83] This ectopic fat accumulation or steatosis of non-adipose tissue has been shown to impact cardiovascular, respiratory, gastro-intestinal, metabolic, urinary, oncologic and osteoarticular function.[84,85] Consequently, adipose tissue dysfunction has been linked to endothelial dysfunction, coronary artery disease, hypertension, congestive heart failure, hyperventilation, obesity-associated gastroesophageal reflux disease, insulin resistance, DM2, hyperfiltration, osteoarthritis and increased incidence of cancer.[86-88] Therefore, visceral fat and ectopic fat accumulation can be considered as potential predictor for multiple pathological states, including cardiovascular disease, metabolic syndrome and DM2.[89,90]

1.2.6 Crosstalk between adipose tissue and other organs

In the late 1980s, Spiegelman and co-workers revealed for the first time that adipocytes can release a number of different factors, and that the release of some of these factors is affected by metabolic dysregulation, such as obesity.[91-93] These adipose tissue-derived factors play a role in fat mass regulation, adipocyte differentiation, vascular and blood flow regulation, lipid and cholesterol metabolism, and in immune system function.[94,95] These observations explain why it has been suggested that adipose tissue-derived factors may play a role in disease processes such as insulin resistance, cardiovascular disease, metabolic syndrome, and altered

lipid homeostasis.[96-99] Because adipose tissue-derived peptide hormones have cytokine-like properties, they are called adipocytokines. An adipocytokine is defined as a hormone, cytokine, chemokine, growth factor, matrix protein, enzyme, or complement factor, produced from adipose tissue.[100,101] However, studies on the source of cytokine secretion from adipose tissue have shown that adipocytes are not the only adipokine producer, but that also other cell types like macrophages, pre-adipocytes, mast cells, fibroblasts, endothelial cells and lymphocytes secrete adipokines.[102-107] The non-adipose cells, which are often called stromal vascular fraction, produces factors, like IL-1ß, IL-6, IL-8, IL-10, TNF-α, resistin, plasminogen activator inhibitor (PAI-1), and vascular endothelial growth factor (VEGF). In contrast, adiponectin, leptin and chemokines such as monocyte chemotactic protein (MCP)-1, macrophage migration inhibitory factor (MMIF) and nerve growth factors (NGF) are predominantly released by adipocytes (~50 %).[104,108]

Adipokines have pleiotropic effects on multiple organs, leading to fine tuning of fuel utilization, energy homeostasis, and cardiovascular function through autocrine, endocrine and paracrine processes.[109] These mechanisms involve both a nutrient-sensing mechanism within adipocytes and stromal vascular cells (autocrine effects), and intercellular (paracrine) or interorgan (endocrine) crosstalk, representing a propagation of signals, particularly from adipose tissue, to entrain the metabolic cooperation of other target organs, such as skeletal muscle and heart. A potent example of a paracrine interaction is the communication of adipocytes and macrophages from the stromal vascular fraction. It is noteworthy that MCP-1 and TNF-α are important adipokines, which are involved in this interaction.[110] The interaction between adipose tissue and specific neurones in the hypothalamus, which caused the inhibition of food intake by the release of leptin, could be a powerful illustration of the endocrine role of adipose tissue.[111]

It has also been reported that disturbances in skeletal muscle insulin signaling and insulin resistance, are caused by a negative crosstalk between excess body fat and skeletal muscle. In the recent years, the scientific interest of the crosstalk between adipose tissue and internal organs has been increased and studied by using co-culture models of, for example, human adipocytes and myocytes.[112] Moreover, several other co-culture studies have been performed to analyse the role of adipose tissue-derived factors on different cell types such as

macrophages, neurons, and adrenocortical cells.[113-115] Thus, it has been shown that adipose tissue is involved in a complex network of paracrine and endocrine crosstalk circuits, involving a number of tissues including the liver, muscle, the pancreas, the brain and the vascular wall.[116] Adipokines have also been implicated in the regulation of contractile function of the heart.[117,118] However, these studies were mainly confined to factors derived from SAT. Because, the human heart is surrounded by a visceral fat depot, called epicardial adipose tissue (EAT), which shares its blood supply with the myocardium, the physiological relevance of studies on factors derived from SAT remains to be clairified.

1.2 Epicardial adipose tissue

1.2.1 Fat depots around the heart and the cardiac vasculature

In addition to EAT, also perivascular adipose tissue (PVAT) and pericardial adipose tissue (PAT) surround the human heart. PVAT is a fat depot on the surface of vascular structures, such as blood vessels, arteries and the aorta irrespective of their location. However, infiltration of perivascular adipocytes has also been observed at the adventitial border.[119] PVAT around coronary arteries, called peri-coronary epicardial fat, has been considered as part of the EAT compartment.[120] The term EAT is usually used for the adipose tissue, commonly found around the heart inside the pericardium.[121,122] Anatomically, it is mostly located between the myocardium and the visceral pericardium, which is the double-walled, fibroserous, inelastic outermost layer of the heart. Cardiovascular magnetic resonance and echocardiography have indicated that EAT is present over the base of the heart, the atrioventricular and the interventricular grooves, along coronary arteries, the great vessels and the acute margial branch and at the apex of the heart.[100,123,124] Although, it could be observed that there is three- to fourfold more EAT mass associated with the right than with the left ventricle, the absolute amount of EAT is similar in both ventricles.[125] Microscopic images showed that a small amount of EAT can also be seen within the subepicardial myocardium.[126] In contrast to other fat depots, EAT and PVAT are visceral fat depots that are not separated by fascial boundaries from the underlying tissues, although both may have functional differences. Quantification studies

revealed that both cover nearly 80 % of the total heart and in particular EAT represents approximately 20 % of the total ventricular weight and 14 % of the total heart.[127-130] Between the visceral pericardium and the parietal pericardium, a conical sac of fibrous tissue that surrounds the heart and the roots of the great blood vessels, the intra-thoracic PAT can be found.[124] In the literature, EAT is often incorrectly referred to as PAT.[131,132] However, PAT and EAT are not supplied by the same vascular system and do not have the same embryologic origin. While pericardial adipocytes are derived from primitive thoracic mesenchyme splits, adipocytes from EAT are derived from the splanchnopleuric mesoderm associated with the gut.[125] Compared to EAT, which share the same microcirulation with the underlying myocardium, PAT receives its blood supply through branches of the internal thoracic and phrenic arteries and PVAT from the adjacent coronary artery through the vasa vasorum.[100,123,133]

1.2.2 Physiology of epicardial adipose tissue

William Harvey was the first physician describing EAT in the 17th century. Later, in the 18th and 19th century, several physicians wrote about the fatty heart in obese patients and that fatty heart was frequently held to be responsible for a sudden death.[134,135] Interestingly, EAT has been poorly studied since then, presumably because the amount of this tissue is relatively scarce in laboratory rats and mice.[125,136] Consequently, most of putative physiological functions of EAT are based on observational data and speculation from a limited amount of studies on models which do have EAT, such as larger mammals, guinea pigs and rabbits.[125] Therefore, the normal physiological role of EAT is not completely clear.

Since the myocardium, including the coronary arteries, is surrounded by EAT, it has been assumed that EAT may function as cushion to buffer the coronary artery against the torsion induced by the arterial pulse wave and cardiac contraction.[123] In 1990, an animal study with guinea pigs showed that fatty acid synthesis, rate of fatty acid incorporation and rate of fatty acid breakdown is significantly higher in EAT than in other fat depots.[137] Furthermore, in EAT the activity of the both key enzymes lipoprotein lipase and acetyl-CoA carboxylase, which are involved in coordinate regulation of fatty acid synthesis, is lower. In the normal healthy adult

heart, the long chain fatty acid oxidation is the main ATP-generating pathway to maintain contractile function. Thus, on the one hand, EAT may act as local energy source for the heart by providing fatty acids to meet increased myocardial demands, especially under ischemic conditions, and on the other hand, it may function as a buffering system against high toxic levels of fatty acids by its ability to rapidly take up and incorporate fatty acids.[136] Interestingly, EAT is the anatomic site where the intrinsic cardiac nervous system, including numerous ganglia and interconnecting plexuses, is located.[138] Stimuli such as transient coronary artery occlusion can modify the activity generated by the intrinsic cardiac nervous system and thereby mediate the pain of angina pectoris. Thus, it has been assumed that EAT protects the ganglia of intrinsic cardiac nervous system.[139] Furthermore, it is assumed that EAT may play a role in coronary artery vasomotion and remodeling.[140] In general, positive coronary artery remodeling is characterized by an asymmetrical expansion of the coronary vessel wall. However, if these vessels were constrained by myocardium, the ability to expand or accommodate an atherosclerotic plaque is limited. Thus, coronary artery remodeling is more likely to occur when coronary lesions are surrounded by or adjacent to EAT which permits expansion rather than in those lesions embedded by the myocardium.[141]

Finally, compared to other fat depots such as thoracic SAT, higher mRNA levels of uncoupling protein (UCP)-1 and brown adipocytes differentiation transcription factor PR-domain-missing 16 (PRDM16), both specific markers of the thermogenesis in brown adipocytes, has been observed in EAT and it may be hypothesized that EAT may function as brown adipose tissue to protect the myocardium against hypothermia.[142]

1.2.3 Role of epicardial fat thickness as a predictor for cardiovascular and metabolic diseases

In the last few years, several autopsy and imaging studies could show that EAT is more than just a silent storage organ. In fact, EAT thickness can be measured and used as diagnostic clinical marker for some pathological states, including risk factors for visceral adiposity, insulin resistance, DM2, coronary artery disease, and the metabolic syndrome. In normal and healthy

individuals, EAT thickness is slightly higher in men (3-5.5 mm) than in woman (2-4.5 mm).[143] Several autopsy and computed-tomography studies revealed that the amount of EAT varies widely between 4 % to 52 %, and that EAT mass and volume is also significant higher in healthy males than in healthy females.[144,145] Although, some animal and human studies demonstrated that the amount of EAT increases with age, there has been some controversy whether EAT mass correlates with age in human subjects.[130] Human autopsy studies on obese and lean patients revealed that there was no relationship between the amount of EAT and age, while some other studies found that there was an relationship in age.[121,122,128,146] Schejbal *et al.* concluded in a study with histological sections that EAT mass over the right ventricle, in patients older than 40 years of age, was not affected by age, whereas a study with Caucasian and Asian individuals revealed that there are correlations between markers of visceral obesity such as BMI, visceral adipose tissue mass, and the amount of EAT.[147,148] To summarize, it can be said that there is likely an increase in EAT mass until age 20-40 years. Thereafter, the amount of EAT is not depending on age.

Moreover, it could be demonstrated that EAT volume in Caucasians is higher than in Asians, Hispanics and Blacks and increased by impaired glucose tolerance and DM2 compared to normal glucose tolerance and non-diabetic.[149,150] Furthermore, it has been suggested that EAT thickness, as measured by standard cardiac ultrasound, may serve as predictor of myocardial fat content better than waist circumference and other more traditional biochemical risk factors.[151] The estimation of insulin sensitivity by euglycaemic hyperinsulinemic clamps and surrogate markers such as HOMA (homeostatic model assessment) revealed that the amount of EAT was related to fasting plasma insulin, fasting plasma glucose, and insulin sensitivity [152]. Furthermore, it has been shown that EAT mass correlates with the grade of hepatic steatosis, a common feature of insulin resistance, and alanine and aspartate aminotransferases, both indicators of hepatic steatosis.[153] However, there is just one report in the literature describing EAT thickness in DM1. In a study with non-obese young adult DM1 women, which exhibit central adiposity and/or clinical parameters of the metabolic syndrome, it has been reported that EAT thickness is increased.[154]

EAT thickness, determined by echocardiography, correlates with visceral adipose tissue mass, and it has been suggested that visceral obesity is a cardiovascular disease risk factor per

se. Due to the close relationship of EAT to the heart and the coronary arteries and its anatomic proximity to the adventitia, it has been hypothesized that alteration in EAT may play a role in the pathogenesis of atherosclerotic coronary artery disease.[155,156] Moreover, it has been reported that EAT thickness was significantly correlated with the severity of coronary artery disease in patients with known coronary artery disease.[157,158] The amount of EAT was found to be higher in patients with coronary artery disease and unstable angina in relation to patients without coronary artery disease and stable angina or atypical chest pain.[159-161] Moreover, it has been shown that EAT mass correlates with increased subclinical markers of atherosclerosis, such as carotid intima media thickness and carotid artery stiffness and also with waist circumference and diastolic blood pressure.[162-164] Because hypertrophy is an independent risk factor for the development of coronary artery disease, it has been demonstrated that the ventricular EAT mass is related to the myocardial mass in normal and hypertrophied hearts.[127] Studies performed on obese and morbidly obese patients reported an association of EAT thickness with left ventricular mass, right ventricular cavity size, atrial dilation and myocardial steatosis, which is an independent predictor of diastolic dysfunction.[165,166]

Interestingly, pathological states, such as visceral obesity, insulin resistance and coronary artery disease, are associated with a state of low-grade chronic inflammation.[167] EAT thickness in obese patients or patients with angina have been found to correlate with plasma level of some pro-inflammatory markers such as IL-6, PAI-1, TNF-α and visfatin, whereas adiponectin levels correlates negatively with EAT thickness.[163,168]

It has been reported that the amount of EAT, imaged by magnetic resonance imaging, is also an important risk marker of the metabolic syndrome.[154] Some clinical markers for the metabolic syndrome are visceral adiposity, increased triglyceride levels, low high-density lipoprotein levels, hypertension, and also enhanced fasting glucose level. In several clinical settings, the amount of EAT was associated with the metabolic syndrome and also some clinical markers for the metabolic syndrome.[143,150,163] For instance, Iacobellis *et al.* reported that the median values of 9.5 and 7.5 mm should be considered the threshold values for high-risk echocardiographic EAT thickness in white European men and women.[143] In patients with an EAT thickness $\geq$5.2 mm, hypertension, increased triglyceride levels and decreased high-density lipoprotein-cholesterol levels have been seen.[160] Although, some studies in obese and Chinese

patients demonstrated that the amount of EAT correlates with triglyceride levels, there has been some controversy whether EAT mass correlates with triglyceride levels.[152,160] Natale *et al.* revealed that there was no relationship between EAT thickness and triglyceride levels, whereas Eroglu *et al.* showed that EAT thickness correlates with triglyceride level.[160,162,163]

To conclude, it has been demonstrated that echocardiographic EAT thickness may play a role in predicting visceral obesity, insulin resistance, fasting glucose, heart morphology, subclinical atherosclerosis, metabolic syndrome and in serving as accurate therapeutic target.[169]

1.2.4 Epicardial adipose tissue as an endocrine organ

Adipokine expression studies in EAT have identified this depot as a source of both pro-inflammatory adipokines, including IL-1β, IL-6, IL-8, PAI-1, MCP-1, and TNF-α, as well as potential protective factors, like adiponectin and omentin.[103,170-173] The expression of pro-inflammatory factors was paralleled by the presence of inflammatory cell infiltrates in EAT stores.[103] Analysis of cDNA microarrays further demonstrated that 1751 genes are overexpressed in EAT compared to SAT from patients with coronary artery disease.[174] From these 1751 genes, more than 270 were identified that were encoding secretory proteins, which can thus potentially affect the function of underlying tissues.

In patients with coronary artery disease, the protein and mRNA expression of adiponectin and adrenomedullin in EAT was significantly decreased compared to EAT from patients without coronary artery disease.[170,175-177] Adrenomedullin has anti-inflammatory properties and it has been suggested that adrenomedullin released by EAT may play a protective role on the vasculature, including the coronary arteries.[178] The decreases in adiponectin expression in EAT was paralleled by decreases in intracoronary adiponectin levels in patients with coronary artery disease.[179] There are also studies indicating that EAT from coronary artery disease patients also expresses and releases higher levels of adipose tissue-derived factors like the pro-inflammatory markers IL-1ß, IL-6, TNF-α, and MCP-1, than for example SAT from the same patients or EAT from patients without coronary artery disease.[103,176,180,181] Finally, the secretion of the pro-inflammatory cytokine RANTES, also known

as CCL5 (Chemokine (C-C motif) ligand 5) from EAT, which plays an active role in recruiting leukocytes into inflammatory sites, was increased in coronary artery disease patients compared to control patients.[182]

In patients with the metabolic syndrome, also alterations in EAT have been found. Compared to patients without the metabolic syndrome, the expression of fatty acid binding protein (FABP) 4 was significantly increased in EAT from patients with metabolic syndrome.[172] Furthermore, proteomic analysis observed that oxidative stress is higher in EAT than in SAT from patients with cardiovascular disease. In particular, production of inflammatory ROS, which are known to be associated with cardiovascular disease, was higher in EAT than in SAT.[183,184] Interestingly, overload of lipids in adipose tissue may initiate a state of cellular stress (adipose tissue ER stress), and enhance production and activation of pro-inflammatory cytokines, both common features of obesity, insulin resistance and diabetes.[185] Moreover, it has been observed that adipose tissue-ER stress additionally enhances ROS production and thus, may induce inflammation through activation of NF-κB and JNK (c-Jun N-terminal kinase) pathways.[186,187] However, adipokine expression and secretion studies in EAT, in relation to DM2, are limited. Only a recent study indicates that EAT from diabetic and non-diabetic subjects expresses similar adiponectin and leptin levels, and that adiponectin and leptin mRNA levels did not differ between EAT and SAT.[188]

As in other fat depots, EAT is also a source of a large number of fatty acids, primarily palmitate and oleate. It has been shown that inappropriately high levels of free fatty acids are correlated with lipotoxicity in many organs including the heart.[189] Interestingly, in EAT from coronary artery disease patients, both secretion and expression of the secretory type II phospholipase A2 (sPLA-IIA) was increased in comparison with patients without coronary artery disease. More recently, sPLA-IIA, which hydrolyzes phospholipids at the sn-2 position, has been recognized as an independent predictor of cardiovascular events.[190] Hydrolysis of the sn-2-ester bond is necessary for generating lysophospholipids and fatty acids. However, it is still unclear whether higher production of sPLA-IIA are paralleled by a higher secretion of fatty acids from EAT.

1.2.5 Epicardial adipose tissue dysfunction in disease

Cultures of adipose tissue have facilitated the analysis of the pathophysiology of adipose tissue depots.[191] Using the explant culture technology, conditioned media from small amounts of adipose tissue, like EAT biopsies, can be generated. Several studies reported that conditioned media from adipose tissue is a complex mixture of different adipokines and seems to be the best method to analyse the secretory profile of fat depots.[182,192,193] To study the effect of adipose tissue-derived factors, cells from target tissues can be exposed to conditioned media generated from fat depots. Furthermore, the ability to culture adipose tissue in a defined medium provides a powerful tool to study a wide array of factors, including adipokines that are released by adipose tissue. EAT is of particular interest in this respect because factors released from the depot communicate with the underlying tissue in the absence of the fascial boundaries. Thus, EAT-derived factors can directly affect the heart and coronary arteries. However, data on the crosstalk between EAT and the underlying tissues are limited. Interestingly, a small number of studies revealed that conditioned media, generated from adipose tissue such as EAT, induce atherogenic changes in relevant target cells such as human monocytes and coronary artery endothelial cells.[194] However, the cross talk between EAT and the heart has not been studied yet.

1.3 Objectives

Although expansion of EAT has been identified as a risk marker for multiple pathological states such as DM2, coronary artery disease and the metabolic syndrome, studies toward qualitative alterations in the secretory profile of EAT are scarce. Yet, adipose tissue-derived factors have been implicated in the regulation of insulin sensitivity and contractile function in cardiomyocytes. Therefore, alterations in the secretory profile of EAT may contribute to the pathogenesis of cardiac dysfunction in DM2 and the metabolic syndrome. Studying the crosstalk between EAT and myocardium may be a key issue in understanding the pathogenesis of cardiomyopathy. Furthermore, the identification of EAT-released factors that are crucial to this crosstalk, could lead to the discovery of new pharmacological treatments of diabetes and the metabolic syndrome, or of new biomarkers for the early detection of person at risk of becoming diabetic later in life.

Thus, the first objective of this thesis was to profile the soluble factors released from adipose tissue, including EAT, PAT and SAT, and whether the release of these factors is affected by a pathological state. Because adipose tissue consists of different cell types, we applied the fat explant culture technology to study the whole-tissue secretion. Since human EAT is limited and mostly from multimorbid patients, we initially used an animal model to study disease-related alterations in secretory profile of adipose tissue-derived factors from EAT. Therefore, adipose tissue biopsies were collected from female guinea pigs fed with either a standard diet or a high-fat diet to induce insulin resistance and contractile dysfunction. In a second study, we extended our studies to human adipose tissue biopsies from patients with and without DM2.

The second aim of this thesis was to study the role of specific EAT-derived factors, showing dysregulated secretion in DM2, in the induction of insulin resistance in primary adult rat cardiomyocytes.

Because it has been shown that SAT releases a variety of factors which induce cardiodepressant effects in cardiomyocytes, the third aim of this study was to analyse the effect of EAT-derived factors on contractile function and SERCA2a-expression in cardiomyocytes.

Finally, several studies have reported that omentin is a new adipokine that is preferentially expressed in EAT compared to SAT and may regulate insulin action. Thus, the

fourth objective of this thesis was to determine whether omentin expression and secretion is reduced in pathological states, and whether omentin protects against the detrimental effects induced by conditioned media from adipose tissue in primary adult rat cardiomyocytes.

2

Secretory products of guinea pig epicardial fat induce insulin resistance and impair primary adult rat cardiomyocyte function

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Chapter 2

Study 1

Secretory products of guinea pig epicardial fat induce insulin resistance and impair primary adult rat cardiomyocyte function

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Abstract

Epicardial adipose tissue (EAT) has been implicated in the development of heart disease. Nonetheless, the crosstalk between factors secreted from EAT and cardiomyocytes has not been studied. Here, we examined the effect of factors secreted from EAT on contractile function and insulin signaling in primary rat cardiomyocytes. EAT and subcutaneous adipose tissue (SAT) were isolated from guinea pigs fed a high-fat (HFD) or standard diet. HFD feeding for 6 months induced glucose intolerance, and decreased fractional shortening and ejection fraction (all $P < 0.05$). Conditioned media (CM) generated from EAT and SAT explants were subjected to cytokine profiling using antibody arrays, or incubated with cardiomyocytes to assess the effects on insulin action and contractile function. Eleven factors were differentially secreted by EAT when compared to SAT. Furthermore, secretion of 30 factors by EAT was affected by HFD feeding. Most prominently, activin A-immunoreactivity was 6.4-fold higher in CM from HFD versus standard diet-fed animals and, 2-fold higher in EAT versus SAT. In cardiomyocytes, CM from EAT of HFD-fed animals increased SMAD2-phosphorylation, a marker for activin A-signaling, decreased sarcoplasmic-endoplasmic reticulum calcium ATPase 2a expression, and reduced insulin-mediated phosphorylation of Akt-Ser473 versus CM from SAT and standard diet-fed animals. Finally, CM from EAT of HFD-fed animals as compared to CM from the other groups markedly reduced sarcomere shortening and cytosolic Ca^{2+}-fluxes in cardiomyocytes. These data provide evidence for an interaction between factors secreted from EAT and cardiomyocyte function.

Introduction

Cardiovascular disease (CVD) is a common characteristic of type 2 diabetes (DM2) and the metabolic syndrome.[1] Risk factors for the metabolic syndrome, including hypertension, dyslipidemia, increased visceral adipose tissue mass, obesity, increased plasma glucose, and insulin resistance, also associate with expansion of the epicardial adipose tissue (EAT).[2-5] EAT is a visceral thoracic fat depot, located at the aortic arch, along the large coronary arteries and on the surface of the ventricles and the apex of the human heart.[6] Since EAT is not separated by a fascia from the myocardium, factors secreted from EAT can directly affect the myocardium and coronary vessels.[2,6,7]

EAT is a source of various adipokines, including adiponectin, fatty acid binding protein 4 (FABP4), interleukin (IL) 1, IL6, monocyte chemoattractive protein-1 (MCP1), leptin, resistin, tumor necrosis factor α (TNFα).[5,8-14] Patients with obesity, DM2, and coronary artery disease (CAD) show elevated plasma levels and altered expression and secretion of pro-inflammatory adipokines in EAT.[7,15] Conversely, expression and circulating levels of protective factors, like adiponectin, is lower in patients with CAD.[9,16] Furthermore, FABP4 suppresses contractile function *in vitro* in isolated rat cardiomyocytes.[17] Although these data indicate that secretory products from EAT may contribute to the pathogenesis of CVD, studies toward diabetes-related alterations in adipokine secretion by EAT are limited.

Here, we studied the interaction between secretory products from EAT and cardiomyocyte function and insulin signaling. Therefore, EAT and subcutaneous adipose tissue (SAT) were isolated from guinea pigs, which were fed a high-fat diet (HFD) to induce glucose intolerance and contractile dysfunction.[18,19] In contrast to laboratory rats and mice, guinea pigs contain abundant amounts of EAT, which increases with age.[6,20] Conditioned media (CM) generated from adipose tissue explants were profiled for adipokine secretion using antibody arrays. Primary adult rat cardiomyocytes were used to assess the effects of CM *in vitro* on insulin signaling, contractile function and cytosolic Ca^{2+}-fluxes. Our data provide evidence for a detrimental effect of factors secreted from the EAT on myocardial function, and suggest a role for EAT in the pathogenesis of heart disease.

Materials and Methods

Animal experiments

Animal experiments were performed in accordance with the "Principle of laboratory animal care" (NIH publication No. 85-23, revised 1996) and the current version of the German Law on the protection of animals. Seven week old female guinea pigs (Crl:HA, Dunkin Hartley) were purchased from Charles River (Sulzfeld, Germany), and housed under standard conditions at a temperature of 18-20^{o}C and a day-night rhythm of 12 h, and fed either a HFD or standard diet (SD). The SD was obtained from Ssniff (Soest, Germany), while the HFD diet was obtained from Altromin (Lage, Germany). The composition of the diets is listed in Supplementary Table 1.

Echocardiography

In vivo cardiac function was measured using non-invasive transthoracic echocardiography with a 6- to 15-MHz transducer (SONOS 5500, Hewlett Packard) in guinea pigs anaesthetized with 1.5 % isoflurane in oxygen-enriched air (95 % O_2) at 28 weeks after initiation of the diet. All echocardiographic images were recorded by the same investigator who was blinded for the animal group. Two-dimensional (2D) guided M-mode images in the parasternal short axis of the left ventricle (LV) were obtained just below the level of the midpapillary muscles. At end systole and end diastole, LV lumen diameter, LV ventricular diameter, and posterior wall and interventricular septum wall thicknesses were determined for five cardiac cycles and averaged. The LV dimensions were used to calculate the left ventricular mass and the cardiac systolic parameters fractional shortening and ejection fraction as described.[19]

Glucose tolerance test

A glucose tolerance test (GTT) was performed after 29 weeks on the diet. Guinea pigs were food-deprived for 20 h, and blood glucose levels were determined before (0 min) and after (15, 30, 60, 120, 180 and 240 min) intraperitoneal injection of 2g glucose/kg body weight in blood taken from the ear vein.

Preparation and characterization of conditioned media

Thirty weeks after initiation of the diets, guinea pigs were sacrificed by CO_2 inhalation, whereafter EAT and SAT were collected and used to generate conditioned media (CM) as described.[21,22] Briefly, on day 1 adipose tissue were washed 3 times with PBS, supplemented with antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA) at 37°C, and cut with scissors into 10 mg small fat explant pieces. Subsequently, adipose tissue explants were washed 3 times with PBS and centrifuged 1 min at 1200 rpm at room temperature. Then, fat explants were cultured in adipocyte media (AM) (DMEM F12 containing 10 % fetal calf serum, 33 µmol/l biotin, 17 µmol/l panthothenate (all from Invitrogen, Carlsbad, CA, USA) and antibiotic-antimycotic) overnight in a humidified atmosphere (95 % air and 5 % CO_2) at 37°C. To generate CM, 100 mg of fat tissue explants were cultured in 1 ml AM without serum for 24 h. Then, CM was collected and stored as aliquots at -80^{o}C until further use.
Antibody arrays (RayBio human cytokine antibody G series 2000; Ray Biotech, Inc., Norcross GA, USA) were used to determine the secretory profile of the CM exactly according to the supplier's instructions.

Effects of conditioned media on primary adult rat cardiomyocytes

Cardiomyocytes were isolated from male Lewis rats (Lew/Crl) as described,[23] and cultured for 24 h on laminin-coated dishes before exposure to CM. The effects of CM on protein expression and phosphorylation in cardiomyocytes were determined by Western blotting. The effects of CM on sarcomere shortening and cytosolic Ca^{2+}-fluxes were determined following electric stimulation of the cells with 1 Hz on a contractility and fluorescence system from IonOptix (Dublin, Ireland) using Indo-1 (Invitrogen, Carlsbad, CA, USA) as Ca^{2+}-indicator.

Isolation and culture of adult primary rat cardiomyocytes

Cardiomyocytes were prepared from male Lewis rats (LEW/Crl, Charles River, Sulzfeld, Germany) weighing 250-350 gram using a Langendorff perfusion system as described.[23] Briefly, rats were sacrificed following anaesthesia with ketamine (100 mg/kg) (Ratiopharm, Ulm,

Germany) and xylazine (Rompun, 5 mg/kg) (Bayer Healthcare, Leverkusen, Germany). The anaesthetic further contained heparin (666 µl/kg) (Biochrom AG, Berlin, Germany). Isolated hearts were retrograde perfused through the aorta for 5 min with Ca^{2+}-free Krebs-Ringer bicarbonate buffer (KRBB) (composition: NaCl, 35 mM; KCl, 4.75 mM; KH_2PO_4, 1.19 mM; Na_2HPO_4, 16 mM; $NaHCO_3$; 25 mM; sucrose, 134 mM; Hepes, 10 mM; glucose, 10 mM) gassed with 95 % O_2 and 5 % CO_2. After the addition of 0.1 mmol/L $CaCl_2$, the isolated heart was perfused for 5 min with Krebs-Ringer bicarbonate buffer (KRBB),[23] followed by KRBB, containing 1.25 g/l bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 0.7 g/l collagenase (Worthington, Lakewood, NJ, USA) and 0.1 g/l hyaluronidase (Applichem, Darmstadt, Germany). Perfusion medium was gassed with O_2. After 20 min, the softened heart was minced and incubated for 5 min at 37°. Then, the dispersion was filtered through a nylon mesh, and centrifuged for 5 min at 500 rpm. After centrifugation, cell pellet was washed with HEPES-buffer (composition: NaCl, 130 mM; KCl, 4.7 mM; KH_2PO_4, 1.2 mM; Hepes, 25 mM; glucose, 5 mM, equilibrated with O_2), containing 3 g/l bovine serum albumin (Carl Roth GmbH, Karlsruhe, Germany). Subsequently, cells were incubated for 7 min at 37°C in HEPES-buffer containing 0.059 units/ml trypsin and treated as described.[23] Isolated cells were seeded on laminin-coated dishes ($1x10^5$ cells per 35 mm plate) in Medium 199 with Hank's salts, supplemented with ITS (insulin, transferrin, selenium), 100 U/ml penicillin, 100 mg/ml streptomycin and 10 % fetal calf serum (all from PAA laboratories, Pasching, Austria) on laminin-coated 35 mm culture dishes (for signaling experiments: Greiner Bio-One GmbH, Solingen, Germany; for fluorescence analysis: ibidi GmbH, Martinsried, Germany). The medium was renewed after 4 h, and culture was continued overnight.

Western blot analysis

Ventricular biopsies collected from SD- or HFD-fed guinea pigs were homogenized in Triton X-100 lysis buffer, containing 50 mM Tris.HCl [pH 7.5]; 150 mM NaCl; 0.5 % Triton X-100; 1 mM NaF; 1 mM Na_3VO_4; 2 mM $MgCl_2$, 1 mM DTT; and protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany). Following a 2 h incubation at 4°C under gentle rotation, homogenates were cleared by centrifugation for 15 min at 12.000 rpm and 4^oC. Cultured

cardiomyocytes were incubated for 24 h with CM (diluted 1:4 with AM) or AM. Then, cells were stimulated for 10 min with insulin (100 nM), washed twice with ice-cold PBS and lysed for 2 h at 4°C in Triton X-100 lysis buffer under gentle rotation. Lysates were cleared by centrifugation (15 min; 12.000 rpm; 4^{o}C), and protein content was determined using Bradford reagent (Biorad Laboratories, München, Germany). Ten microgram of protein was loaded onto 10 % SDS-Page gels, and transferred to polyvinylidene difluoride (PVDF) membranes. After blotting, membranes were blocked with Tris-buffered saline (TBS), containing 0.1 % Tween 20 and 5 % non fat dry milk for 2 h at room temperature and then incubated overnight at 4^{o}C with primary antibody. After washing, membranes were incubated with appropriate secondary HRP-conjugated antibody for 2 h at room temperature and washed again. All antibodies and dilutions are listed in Supplementary Table 2. Bound antibodies were visualized using enhanced chemiluminescence and quantified by using a LUMI Imager system (Roche Diagnostics, Mannheim, Germany).

Measurement of sarcomere shortening and Ca^{2+} transients

For analysis of the effects of CM, cells were preloaded with Indo-1 (Invitrogen, Carlsbad, CA, USA) for 15 min at 37°C, washed once with AM and then incubated for 30 min with CM or AM. For reversibility experiments, CM was removed and cells were incubated with AM for 2 h. Subsequently, contractile function and Ca^{2+}-transients were analyzed in cells showing an intact rod-shaped morphology and sarcomere length >1.6 µm. Before measurement was started, cells were electrically pre-stimulated for 5-10 min with 1 Hz to reach a steady-state level for sarcomere shortening and Indo-1 fluorescence. Then, cells were paced with bipolar pulses of 5 ms duration at 1 Hz. The cytosolic Ca^{2+} concentration was monitored as a ratio of the fluorescence emission peaks at 475 and 400 nm. In each experimental condition, data files were recorded of 10 consecutive beats for at least 8 different cells. Sarcomere shortening and Ca^{2}-transients were calculated using IonWizard (IonOptix).

Data analysis

Data are presented as mean ± S.E.M. Significant differences between experimental conditions were evaluated by one-way ANOVA or unpaired Student´s *t* test using Graphpad Prism software (version 5). A value of $P < 0.05$ was considered as statistically significant.

Results

HFD-feeding induces cardiac contractile dysfunction in guinea pigs

In comparison to SD-fed animals, feeding guinea pigs a HFD for 29 weeks induced mild glucose intolerance as determined by a GTT (Fig. 1A,B). Table 1 shows the physiological and cardiac parameters after 28 weeks on the diets. There were no significant differences in body weight, epicardial adipose tissue mass, left ventricular mass and the diastolic parameters. In the end systolic phase, the lumen diameter of the left ventricle (LV) was increased in HFD-fed guinea pigs ($P < 0.05$). Accordingly, end systolic volume was increased ($P < 0.05$), and fractional shortening and the ejection fraction were decreased in HFD-fed animals (both $P < 0.02$) (Table 1). At the molecular level, protein expression of calreticulin (Fig. 1C) was higher, and phosphorylation of phospholamban (Fig. 1D) was lower in hearts from HFD-fed versus SD-fed animals. Furthermore, expression of SERCA2a tended to be decreased in hearts from HFD- versus SD-fed animals (Fig. 1E). Finally, protein expression of the insulin-regulated glucose transporter GLUT4 was substantially lower in HFD-hearts (Fig. 1F). Collectively, these data indicate contractile dysfunction in hearts from HFD-fed animals.

Characterization of conditioned medium from epicardial and subcutaneous adipose tissue

To study the interaction between EAT and the myocardium, conditioned media (CM) were generated from EAT, and characterized using antibody arrays. The factors secreted by EAT were compared with CM generated from SAT of the same SD-fed animals. Supplementary Table 3 shows the relative immunoreactivity obtained for the 174 factors present on the arrays for CM-

EAT and CM-SAT. Eleven factors were differentially secreted by EAT when compared to SAT. Specifically, adiponectin (ADIPOQ) immunoreactivity was decreased in EAT versus SAT, while immunoreactivity for cardiotrophin (CTF1), activin A, endoglin (ENG), E-selectin (SELE), IL2Rγ, IL5Rα, PDGFA, PDGFRA, PECAM1, VEGFR2 (KDR) was increased in EAT versus SAT (all $P< 0.05$). The immunoreactivity of thirty factors in EAT was affected by HFD-feeding (Fig. 2). Specifically, twelve factors, i.e. activin A, ALCAM, SIGLEC5, CTF1, PDGFA, IL10, AREG, VEGF, FGF6, IL12B, THPO, and CCL5, were selectively increased by HFD-feeding in EAT versus SAT (Fig. 2A). Immunoreactivity of CXCL5, and CSF2 was selectively decreased by HFD-feeding in EAT versus SAT (Fig. 2A). Sixteen factors were affected by HFD-feeding in CM from both EAT and SAT. Figure 2B shows an increased immunoreactivity for CCL18, EGF, PDGFB, CXCL12, CSF1R, IL16, FGF7, and PECAM1 in CM-EAT from HFD- versus SD-fed animals (all $P< 0.05$). Figure 2C shows a decreased immunoreactivity for TNFRSF1B, IL9, IGFBP3, FASLG, IL1R1, SELE, PDGFRB, and ICAM3 in CM-EAT from HFD- versus SD-fed animals (all $P< 0.05$). These data show that HFD-feeding differentially affects the secretion of multiple factors by EAT and SAT, with most notable changes observed for activin A. Activin A, a homodimer of two activin β_A –subunits encoded by the INHBA-gene, is a member of the TGFβ superfamily, which utilizes the SMAD2/3-signaling pathway [24]. Therefore, we examined whether CM affected SMAD2-phosphorylation levels in rat cardiomyocytes. Incubation of cardiomyocytes with CM-EAT from HFD-fed animals increased SMAD2-phosphorylation by 4-fold ($P< 0.05$) when compared to control adipocyte medium (AM) and 1.7-fold when compared to CM-EAT from SD-fed animals (Fig. 3). Exposure of cardiomyocytes to CM-SAT had no significant effect on SMAD2-phosphorylation (Fig. 3). CM had no effect on the expression of the activin receptor type 1B (also known as Alk4) and SMAD2/3 (Fig. 3A). Thus, the enhanced activin A-immunoreactivity in CM-EAT from HFD-fed animals is paralleled by the ability to induce SMAD2-phosphorylation in rat cardiomyocytes.

Effect of conditioned media from epicardial and subcutaneous adipose tissue on insulin action

Short-term exposure (3 h) of cardiomyocytes to CM had no effect on insulin-stimulated Akt-phosphorylation (data are not shown). However, when cardiomyocytes were exposed for 24 h to CM-EAT from HFD-fed animals, the induction of insulin-stimulated Akt-phosphorylation was

reduced by ~60 % as compared to CM-EAT from SD-fed animals or AM (Fig. 4A/B) (both $P< 0.05$). CM-SAT from either SD- or HFD-fed had no inhibitory effect on insulin-stimulated Akt-phosphorylation (Fig. 4A/B). Protein expression levels of Akt (Fig. 4A) were not affected by exposure to CM.

Effect of conditioned media from epicardial and subcutaneous adipose tissue on cardiomyocyte contractile function

Time-dependent alterations in sarcomere length were recorded in primary rat cardiomyocytes incubated with AM or CM from the various groups (Fig. 5A). Exposure to CM did not affect cell morphology and resting sarcomere length. Following electric stimulation of the cells with 1 Hz, peak sarcomere shortening and return velocity (Fig. 5C/D) were reduced following a 30 min exposure to CM-EAT from HFD-fed animals. Departure velocity was not affected by CM-EAT from HFD-fed animals (Fig. 5B). CM from the other experimental groups had no significant effect on sarcomere length when compared to AM (Fig. 5B-D). When the concentration of CM was increased by 2-fold, a further reduction of peak sarcomere shortening and return velocity was found following exposure of cardiomyocytes to CM-EAT from HFD-fed animals (Supplementary Fig. 1; both $P< 0.05$). Also departure velocity was significantly lower in cells incubated with a higher concentration of CM-EAT from HFD-fed animals as compared to AM and CM-SAT from HFD-fed animals (both $P< 0.05$; (Supplementary Fig. 1). Finally, the effects elicited by more concentrated CM-EAT from HFD-fed animals on determinants of sarcomere length were significantly lower when compared to CM-EAT from SD-fed animals (Supplementary Fig. 1).

Time-dependent alterations in cytosolic [Ca^{2+}] are presented in Figure 6A. A 30 min exposure to CM-EAT from HFD-fed animals lowered peak [Ca^{2+}] as compared to cells exposed to AM and CM-SAT from HFD-fed animals (both $P< 0.05$), without affecting departure- and return velocities (Fig. 6). However, when the concentration of CM-EAT was doubled, departure- and return velocity for alterations in cytosolic [Ca^{2+}] were reduced in cells exposed to CM-EAT from HFD-fed animals versus AM and CM-EAT from SD-fed animals (Supplementary Fig. 2).

Long term incubation for 24 h of the cells with CM showed no further impairment of sarcomere shortening and cytosolic Ca^{2+}-fluxes when compared to short term incubation for 30

minutes (data not shown). Analysis of the contractile parameters two hours after replacement of the CM by AM showed that peak sarcomere shortening and peak [Ca^{2+}] were still reduced in cardiomyocytes that were exposed to CM from EAT of HFD-fed animals in the lowest dilution tested (Supplementary Fig. 3). In all other experimental conditions, contractile function was restored.

Effect of conditioned media from epicardial and subcutaneous adipose tissue on sarcoplasmic-endoplasmic reticulum calcium ATPase 2a-expression

Expression of sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA)2a, a key regulator of cardiac Ca^{2+}-metabolism, was reduced in cardiomyocytes incubated with CM-EAT from HFD-fed animals when compared to AM and CM-EAT from SD-fed animals. CM-SAT from HFD-fed animals also slightly lowered SERCA2a-expression whereas CM from the other groups had no effect (Fig. 7). Thus, the detrimental effects of CM-EAT from HFD-fed animals on contractile function are paralleled by a reduction in SERCA2a-expression.

Discussion

The present study shows that the secretory profile of EAT is distinct from that of SAT. Furthermore, HFD-induced alterations in the factors secreted by EAT cause dysfunction of primary rat cardiomyocytes as illustrated by impairments in insulin signaling, sarcomere shortening, cytosolic Ca^{2+}-metabolism and SERCA2a-expression. Collectively, these findings show that diet-induced changes in EAT could contribute to the development of cardiomyocyte dysfunction.

Accumulating evidence from epidemiological studies has identified associations between EAT and clinical markers of the metabolic syndrome, DM2, CAD, and cardiac function.[3] A recent report shows that obesity and CAD also affect the secretory profile of EAT in humans.[15] Specifically, CM from EAT of these patients was found to induce atherogenic changes in monocyte migration and endothelial cell adhesion.[15] Yet, studies on qualitative alterations of

EAT in relation to disease are often hampered by the lack of appropriate human controls, and the scarce amounts of EAT in frequently used laboratory rodents, like rat and mice.[4,6] Therefore, in the current study, guinea pigs were used, in which EAT expands with age.[20,25] HFD-fed guinea pigs showed glucose intolerance, indicative of reduced insulin sensitivity, in the absence of obesity, as previously also reported in HFD-fed rats.[18,19] Although the HFD contained more calories than the SD, the HFD-group consumed less of the diet as compared to the SD group, resulting in similar caloric intake, but with different food ingredients. For example, the concentration of saturated fatty acids was much higher in HFD than in SD. In addition, HFD-fed guinea pigs showed abnormalities in left ventricular function, like reductions in ejection fraction and fractional shortening similar to HFD-fed rats.[19] The observed alterations in GLUT4-, SERCA2a-, and calreticulin expression, and phospholamban phosphorylation provide further support for a reduced cardiac function on HFD-fed guinea pigs.

A key finding in the present study is that secretory products from EAT from HFD-fed animals exert a cardiodepressant and negative inotropic effect on primary adult rat cardiomyocytes. This detrimental effect was dose-dependent, and occurred within minutes after the addition of CM to the cardiomyocytes. Furthermore, the effect could be reversed by replacing CM by control medium. In support of a previous study, which demonstrated that primary human subcutaneous adipocytes secrete a cardiodepressant factor,[26] we observed that cardiomyocyte dysfunction, could also be induced by a higher dose of CM-SAT from HFD-fed animals although to a significantly lower extent when compared to the same dose of CM-EAT from HFD-fed animals. In contrast to SAT, EAT is not separated by facial boundaries from the myocardium, and factors secreted from the EAT could directly affect the function of the underlying myocardium. The functional significance of the cardiodepressant activity secreted by SAT remains to be elucidated since it may only affect cardiomyocyte function via the systemic circulation. The negative effects of CM-EAT from HFD-fed animals on cytosolic Ca^{2+} transients in rat cardiomyocytes were accompanied by a reduction of protein expression of SERCA2a, a key regulator of cytosolic [Ca^{2+}] in the heart.[27] Decreases in myocardial SERCA2a expression are a common characteristic of cardiopathological states in humans,[27] but have also been reported in animal models for diabetes-related heart disease.[28] Collectively, these findings indicate that secretory products from EAT regulate myocardial function, and that HFD-induced alterations in

the EAT secretory profile could contribute to the diet-induced alterations in *in vivo* cardiac function in HFD-fed guinea pigs.

In patients with DM2, alterations in cardiac structure and function which are found even in the absence of hypertension and CAD are ascribed to diabetic cardiomyopathy (DCM). DCM often co-exists with myocardial insulin resistance.[29,30] Here, we show for the first time that exposure of cardiomyocytes to secretory products from EAT of HFD-fed animals abrogates insulin-mediated phosphorylation of Akt, a key regulator of myocardial glucose uptake.[30,31] In contrast to the effects on contractile function, the detrimental effects on insulin action were only observed upon prolonged incubation of the cardiomyocytes with CM, indicating that this detrimental effect is caused by a different mechanism. Abrogation of insulin-mediated Akt-phosphorylation in cardiomyocytes was also described when cells were exposed to CM from epididymal adipose tissue from diabetic rats as compared to control rats.[32] In this study, the inhibition of Akt-phosphorylation was paralleled by a reduced ability of insulin to stimulate glucose uptake. In obese Zucker diabetic rats, a decrease in insulin-mediated Akt-phosphorylation could be linked to a decrease in insulin-mediated myocardial glucose utilisation *in vivo* as determined by positron emission tomography under hyperinsulinaemic euglycaemic clamp conditions.[33] Collectively, these findings provide strong evidence that secretory products of EAT from HFD-fed animals contribute to the induction of insulin resistance at the level of the Akt-pathway regulating myocardial glucose uptake.

In order to identify potential factors responsible for the detrimental effects caused by CM-EAT from HFD-fed animals, CM from the various experimental groups was further characterized. Glycerol and free fatty acids levels was similar in the CM of the experimental groups (data not shown). Also lactate content, before and after incubation with cardiomyocytes, did not differ between CM-EAT and CM-SAT, and was not affected by the dietary intervention (data are not shown). Therefore, it seems unlikely that an increased utilization of lactate as myocardial energy substrate, which negatively affects myocardial performance,[34] contributes to the observed effects caused by CM-EAT from HFD-fed animals. In support of previous studies, which have ascribed cardiosuppressive effects to adipokines,[17,35-38] like FABP4, IL1β, IL6, and TNFα, boiling was found to destroy the detrimental effect of CM on cardiomyocyte function. Therefore, the CM was evaluated for diet-induced alterations in adipokine secretion by EAT and

SAT. With the use of antibody arrays, we found that the secretory profile of EAT differs from that of SAT, and that the secretory profiles in both depots were affected by HFD-feeding. Although we could identify these differences, this approach has limitations. First, not all factors secreted by adipose tissue are present on the array, like FABP4. In addition, there are no specific arrays available for guinea pigs. Instead, we used human antibody arrays, and cannot exclude problems related to cross-reactivity between factors from guinea pigs and humans. Similarly, some adipokines identified in the antibody array may not act on rat cardiomyocytes because of species differences. In this respect, we have attempted to examine the effects of CM on primary guinea pig cardiomyocytes. However, in our hands, the viability of the isolated cells was too low to allow the experiments described in this study.

Despite the limitations of our approach, in CM-EAT from HFD-fed animals, a selective increase in immunoreactivity for 12 factors, and loss of immunoreactivity for 2 factors was found. The most notable change in CM-EAT was observed for activin A. Immunoreactivity for activin A was 6.4-fold higher in CM from HFD-fed versus SD-fed animals and 2-fold higher in CM-EAT versus CM-SAT. Although the sequence for guinea pig activin A is still unknown, the activin A protein sequence is highly conserved (95.8 % identical) among humans, rats and mice. Furthermore, the alterations in activin A immunoreactivity were accompanied by biological activity of the CM, as illustrated by the induction of SMAD2-phosphorylation by CM-EAT from HFD-fed animals only. Although other factors present on the arrays like TGFβ1, TGFβ2, TGFβ3, HGF, and IGFBP3, are also capable to regulate SMAD2-phosphorylation, immunoreactivity of these factors did not differ among the experimental groups. Multiple studies have linked activin A to cardiac dysfunction. Serum levels of activin A are elevated in patients with heart failure.[39] Furthermore, activin A increases the expression of genes involved in myocardial remodeling, like atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), TGFβ1, and MCP1,[39] and inhibits the organization of sarcomeric proteins induced by leukemia inhibitory factor in neonatal rat cardiomyocytes.[40] In line with this, recombinant activin A exerts a cardiodepressant activity on rat adult cardiomyocytes (SG, DMO, and JE, submitted for publication). Finally, in neonatal cardiomyocytes activin A has been found to induce the expression of SOCS3,[40] a key repressor of

insulin action.[41] Importantly, in the present study SOCS3 expression was elevated in hearts from HFD-fed guinea pigs (data not shown).

Beside activin A, also the immunoreactivity of other factors was selectively affected in CM-EAT from HFD-fed animals. Although alterations in serum levels have been reported in patients with cardiac dysfunction for some factors, like IL10 and CXCL5,[42-44] the role of most factors in relation to myocardial function and insulin sensitivity remains to be elucidated.

Another question that remains to be addressed is what underlies the diet-induced changes in the secretory profile from EAT. In the present study, CM was generated from adipose tissue explants. In addition to adipocytes, adipose tissue also contains other cell types, like pre-adipocytes, macrophages, fibroblasts, endothelial cells and lymphocytes, which can release a variety of chemo- and cytokines.[45,46] Hypertrophy of adipose tissue in obesity and DM2 is closely linked to low-grade inflammation.[47] This can be ascribed to accumulation of immune cells into the adipose tissue, and secretion of pro-inflammatory cytokines.[45,46] We could not obtain histological evidence for immune cell infiltration in EAT from HFD-fed guinea pigs, because of the unavailability of antibodies cross-reacting with guinea pig macrophage markers. However, a study performed on humans with CAD reports the infiltration of immune cells in EAT.[11] Furthermore, a recent study shows that activin A expression is elevated in adipose tissue from obese subjects, and dramatically increased by factors secreted by macrophages isolated from obese adipose tissue.[48] Based on these findings, it seems likely that the observed alterations in the secretory profile of EAT from HFD-fed guinea pigs can be ascribed to infiltration of immune cells.

In conclusion, the present study shows an interaction between factors secreted from EAT and the cardiomyocytes. Our data demonstrate that HFD-feeding of guinea pigs induces specific alterations in the secretory profile of EAT that are responsible for the induction of contractile dysfunction and insulin resistance in primary adult rat cardiomyocytes.

Achnowledgement

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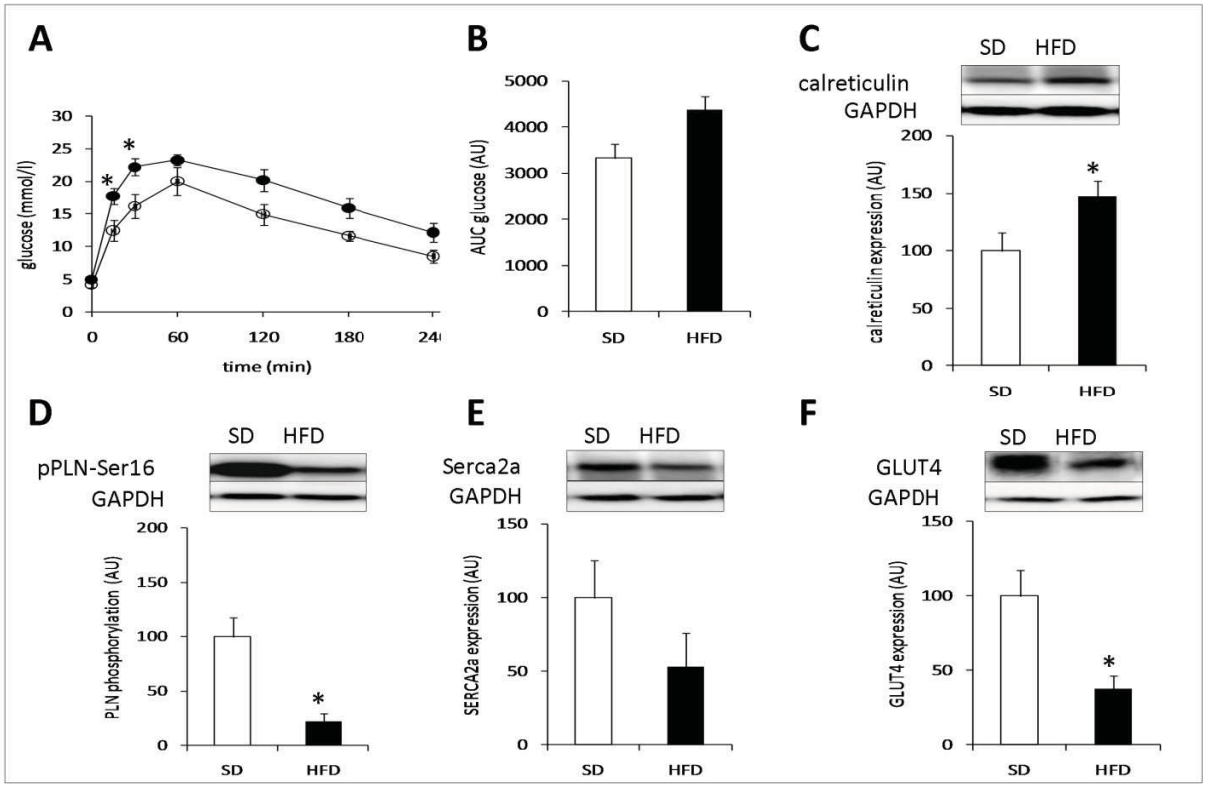
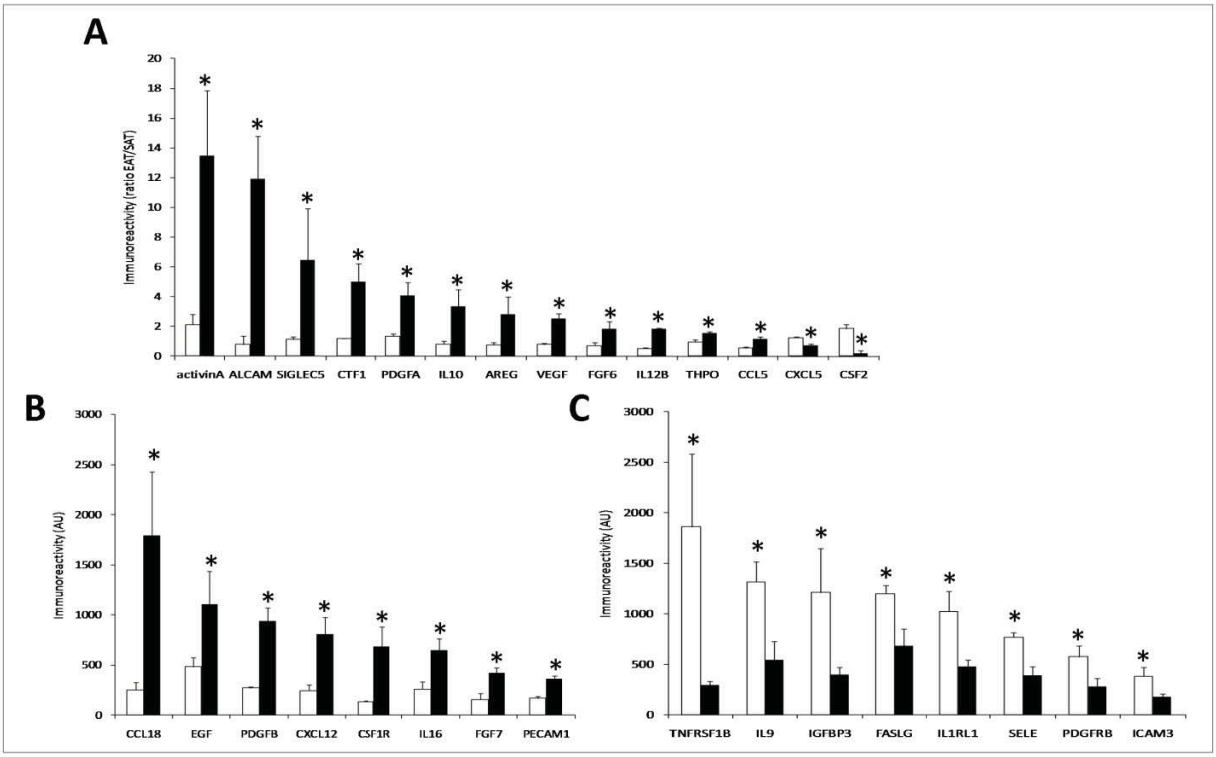
Figure 1. Animal characteristics



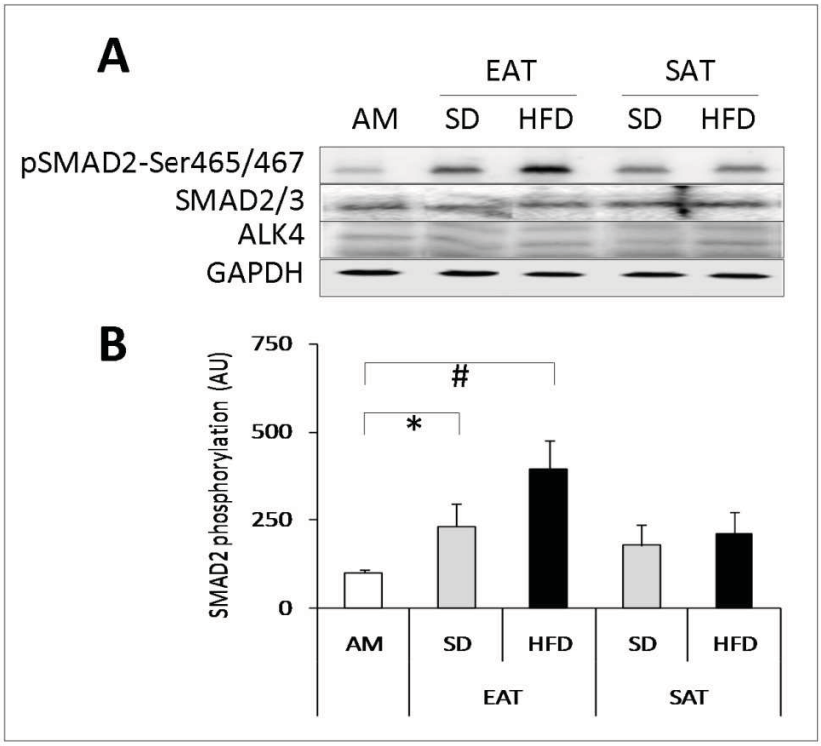
(A) Blood glucose levels and (B) area under the curve of blood glucose levels after a glucose tolerance test in guinea pigs fed a SD (open circles; open bars; n=5) or HFD (closed circles; filled bars; n=10) for 29 weeks. (C-F) Representative immunoblots and quantification of calreticulin expression (C), phosphorylation of PLN (D), expression of Serca2a (E) and Glut4 (F) in ventricular tissue from guinea pigs fed a SD (open bars) or HFD (black bars) for 30 weeks. Equal loading was verified by probing the immunoblots with GAPDH-antibody. All data are expressed as mean ± S.E.M.

Figure 2. Characterization of secretory products of epicardial and subcutaneous adipose tissue



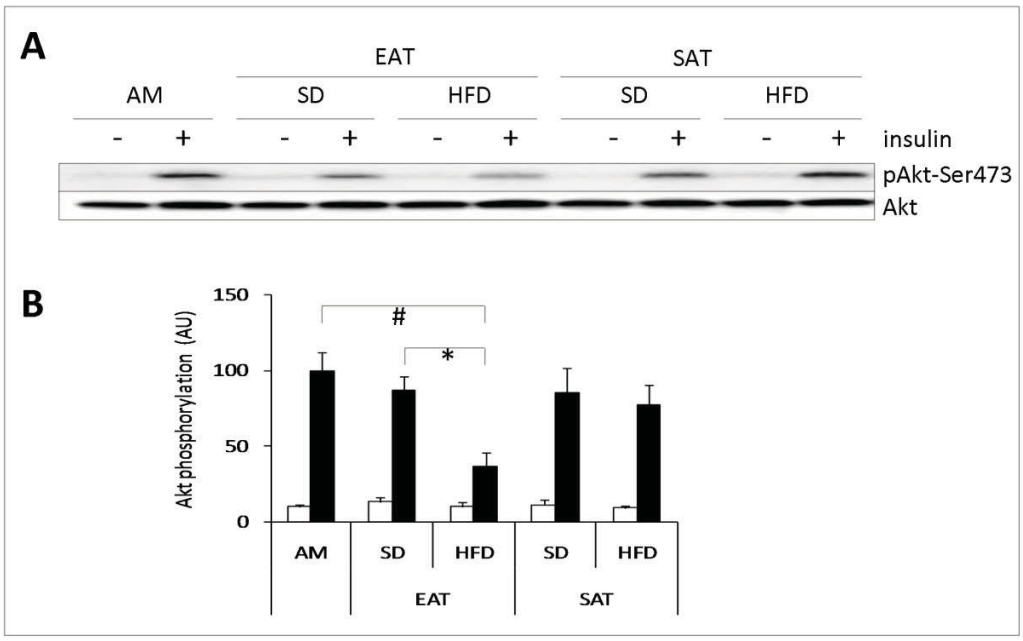
Conditioned media (CM) generated from EAT and SAT from SD- and HFD-fed guinea pigs were profiled using antibody arrays. (A) Ratio of immunoreactivity for secretory products from EAT over SAT that are selectively altered in EAT by the HFD compared to SD. White bars; SD-CM; black bars, HFD-CM. (B/C) Immunoreactivity of secretory products in EAT that are upregulated (B) or downregulated (C) by the HFD in CM from EAT. All data are expressed as mean ± S.E.M. (n=4 per group). Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. *, $P< 0.05$ HFD versus SD.

Figure 3. Effect of conditioned medium from epicardial and subcutaneous adipose tissue on SMAD2-phosphorylation



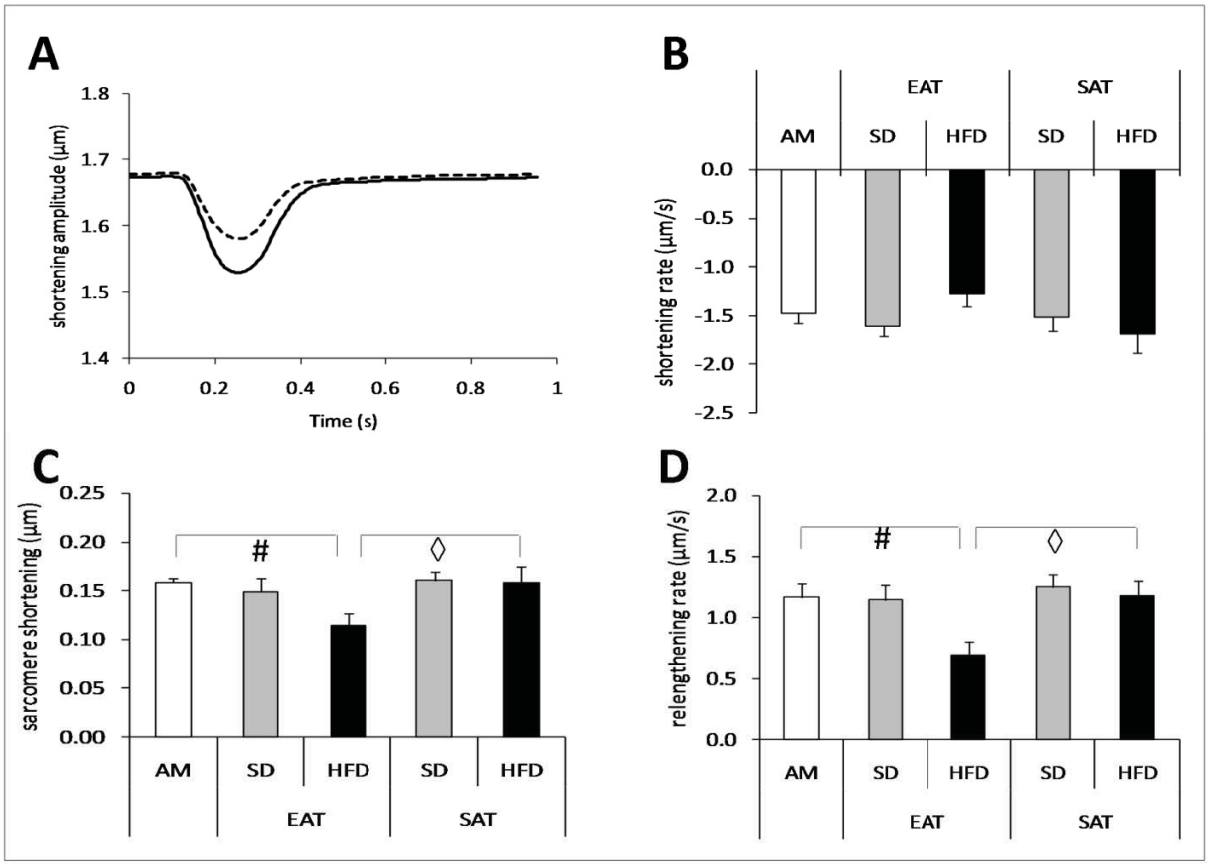
Lysates prepared from rat cardiomyocytes incubated with control adipocyte media (AM) or conditioned media (CM, diluted 1:4) from EAT and SAT from SD- and HFD-fed guinea pigs for 24 h were analyzed by Western blotting with antibodies recognizing phosphorylated SMAD2-Ser465/467, total SMAD2/3 and activin A receptor type 1B (Alk4). Equal loading was verified by probing the immunoblots with GAPDH-antibody. Representative immunoblots (A) together with quantification (B) are shown. Data are presented as mean ± S.E.M. (n=5 per group). Open bars, AM; gray bars, CM from SD-fed animals; black bars, CM from HFD-fed animals. Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P< 0.05$ versus AM; *$P< 0.05$ HFD versus SD.

Figure 4. Effect of conditioned medium from epicardial and subcutaneous adipose tissue on insulin action



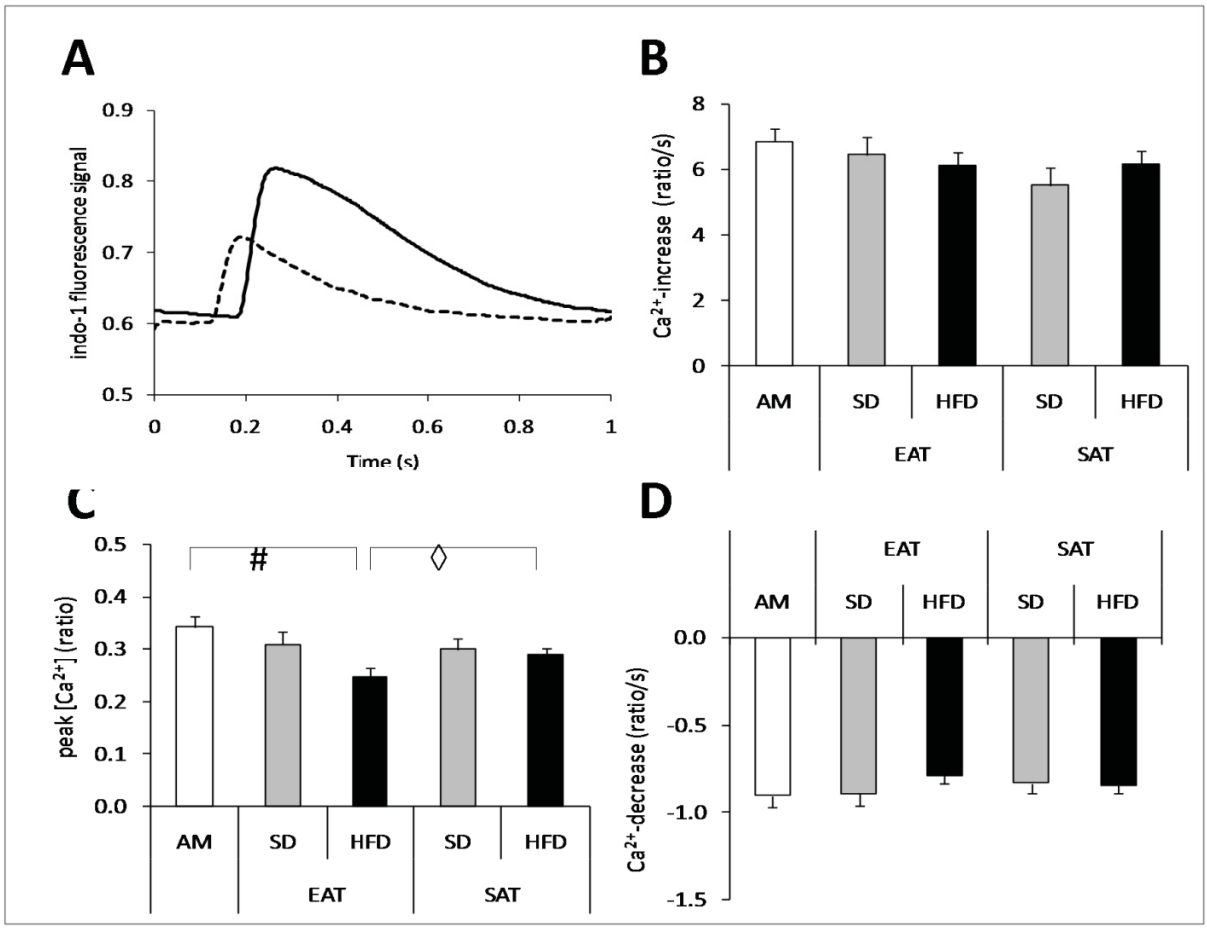
Lysates prepared from rat cardiomyocytes incubated with control adipocyte media (AM) or conditioned media (CM, diluted 1:4) from EAT and SAT from SD- and HFD-fed guinea pigs for 24 h were analyzed by Western blotting with antibodies recognizing total and Ser473-phosphorylated Akt. Representative immunoblots (A) and quantification (B) of insulin-induced Akt-Ser473 phosphorylation. Data are presented as mean ± S.E.M. (n=4 per group). Open bars, basal; filled bars, insulin stimulated cells (10 min; 100 nM). Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P< 0.05$ versus AM; * $P< 0.05$ HFD versus SD.

Figure 5. Effect of conditioned medium from epicardial and subcutaneous adipose tissue on sarcomere shortening in cardiomyocytes



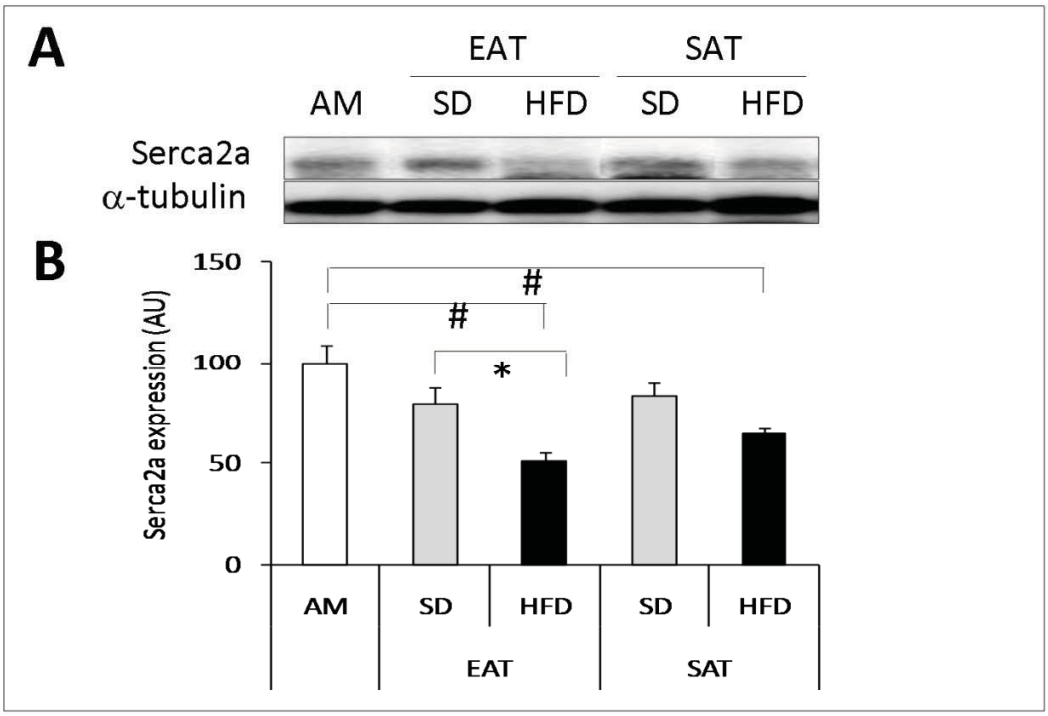
Rat cardiomyocytes were incubated with control adipocyte media (AM) or conditioned media (CM, diluted 1:4) from EAT and SAT from SD- and HFD-fed guinea pigs for 30 min before analysis of contractile function. (A) Representative chart recording of alterations in sarcomere length in time. Black line, AM; dashed line, CM-EAT from HFD-fed animals. (B-D) Effect of exposure of cardiomyocytes to CM on departure velocity of contraction (B), peak sarcomere shortening (C), and return velocity of contraction (D). Open bars, AM; gray bars, CM from SD-fed animals; black bars, CM from HFD-fed animals. Data are expressed as mean ± S.E.M. of at least 8 independent experiments. Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P< 0.05$ versus AM; ◊, $P< 0.05$ EAT versus SAT.

Figure 6. Effect of conditioned medium from epicardial and subcutaneous adipose tissue on cytosolic [Ca^{2+}] in cardiomyocytes



Rat cardiomyocytes were incubated with control adipocyte media (AM) or conditioned media (CM, diluted 1:4) from EAT and SAT from SD- and HFD-fed guinea pigs for 30 min before analysis of cytosolic [Ca^{2+}]. (A) Representative chart recording of alterations in indo-1 fluorescence in time. Black line, AM; dashed line, CM-EAT from HFD-fed animals. (B-D) Effect of exposure of cardiomyocytes to CM on [Ca^{2+}] increase, (B), peak [Ca^{2+}] (defined as the ratio between Ca^{2+}-bound Indo 1 and unbound free Indo 1) (C), and [Ca^{2+}] decrease (D). Open bars, AM; gray bars, CM from SD-fed animals; black bars, CM from HFD-fed animals. Data are expressed as mean ± S.E.M. of at least 8 independent experiments. Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P< 0.05$ versus AM; ◊, $P< 0.05$ EAT versus SAT.

Figure 7. Effect of conditioned media from epicardial and subcutaneous adipose tissue on SERCA2a expression



Lysates prepared from rat cardiomyocytes incubated with control adipocyte media (AM) or conditioned media (CM, diluted 1:4) from EAT and SAT from SD- and HFD-fed guinea pigs for 24 h were analyzed by Western blotting for SERCA2a- and α-tubulin-expression. Representative western blots (A) and quantitative analysis (B) are also shown. Open bars, AM; gray bars, CM from SD-fed animals; black bars, CM from HFD-fed animals. Data are expressed as mean ± S.E.M. (n=3 per group). Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P< 0.05$ versus AM; *, $P< 0.05$ HFD versus SD.

Supplementary Table 1. Composition of the diets

	Standard diet	***High Fat diet***
Energy content (kcal/kg)	2508	3701
Protein (g/kg)	200	170
Carbohydrate (g/kg)	720	670
Total fat (g/kg)	80	160
	Saturated fatty acids	
Capric acid (g/kg)	0.0	4.7
Lauric acid (g/kg)	0.0	33.4
Myristic acid (g/kg)	0.1	15.7
Palmitic acid (g/kg)	5.2	28.3
Stearic acid (g/kg)	0.9	19.2
Arachidic acid (g/kg)	0.1	0.5
	Unsaturated fatty acids	
Palmitoleic acid (g/kg)	0.2	2.63
Oleic acid (g/kg)	5.1	29.0
Linoleic acid (g/kg)	15.1	11.2
Linolenic acid (g/kg)	3.2	2.5
Eicosaenoic acid (g/kg)	0.1	0.9
Arachidonic acid (g/kg)	0	0.4

Supplementary Table 2. List of Antibodies

Primary Antibody	***Species***	***Dilution***	***Manufacturer***	***Corresponding Secondary Antibody & Dilution***
Akt	Rabbit	1:1000	Cell Signaling Technology	HRP anti-rabbit, 1:2500
p-Akt-Ser473	Rabbit	1:1000	Cell Signaling Technology	HRP anti-rabbit, 1:2500
α-Tubulin	Mouse	1:1000	Calbiochem	HRP anti-mouse, 1:2500
Glut 4 (C-20)	Goat	1:1000	Santa Cruz Technology	HRP anti-goat, 1:2500
GAPDH	Rabbit	1:10000	Abcam	HRP anti-rabbit, 1:2500
Serca2a	Mouse	1:1000	Abcam	HRP anti-mouse, 1:2500
p-Smad-Ser465/467	Rabbit	1:500	Cell Signaling Technology	HRP anti-rabbit, 1:2500
p-Phospholamban	Mouse	1:200	Santa Cruz Technology	HRP anti-mouse, 1:2500
Calreticulin	Rabbit	1:1000	Stressgen Bioreagents	HRP anti-rabbit, 1:2500

Supplementary Table 3. Immunoreactivity in conditioned medium collected from epicardial and subcutaneous adipose tissue explants from guinea pigs

Swiss Prot	*Name on array*	*Symbol*	*EAT (n=7)*	*SAT (n=7)*
Q15848	Acrp30	ADIPOQ	182 ± 42	519 ± 166
P08476	Activin A	INHBA	1718 ± 468	536 ± 212
O00253	AgRP	AGRP	1434 ± 521	1076 ± 141
Q13740	ALCAM	ALCAM	8149 ± 6594	1286 ± 621
P15514	Amphiregulin	AREG	1998 ± 568	1629 ± 557
P03950	Angiogenin	ANG	208 ± 68	193 ± 64
O15123	Angiopoietin-2	ANGPT2	2576 ± 754	1805 ± 342
P30530	Axl	AXL	712 ± 220	258 ± 65
P33681	B7-1 (CD80)	CD80	496 ± 119	438 ± 83
P23560	BDNF	BDNF	820 ± 222	829 ± 144
P09038	bFGF	FGF2	1328 ± 617	1359 ± 498
O43927	BLC	CXCL13	388 ± 85	268 ± 74
P12644	BMP-4	BMP4	719 ± 253	919 ± 227
P22003	BMP-5	BMP5	1090 ± 342	958 ± 300
P22004	BMP-6	BMP6	1087 ± 305	1310 ± 297
P18075	BMP-7	BMP7	358 ± 73	245 ± 80
P01138	b-NGF	NGF	4372 ± 1794	2995 ± 723
P35070	BTC	BTC	1037 ± 330	649 ± 127
Q16619	Cardiotrophin-1	CTF1	941 ± 289	400 ± 88
Q9NRJ3	CCL-28	CCL28	307 ± 128	290 ± 72
P08571	CD14	CD14	371 ± 80	270 ± 58
P55773	CK b 8-1	CCL23	1059 ± 401	1031 ± 246
P26441	CNTF	CNTF	459 ± 79	513 ± 61
Q9Y4X3	CTACK	CCL27	626 ± 110	767 ± 314
Q9H2A7	CXCL-16	CXCL16	1252 ± 268	943 ± 222
O75509	DR6 (TNFRSF21)	TNFRSF21	559 ± 224	440 ± 138
Q06418	Dtk	TYRO3	637 ± 157	574 ± 179
P01133	EGF	EGF	730 ± 162	876 ± 235
P00533	EGF-R	EGFR	347 ± 113	445 ± 176
P42830	ENA-78	CXCL5	966 ± 445	969 ± 313
P17813	Endoglin	ENG	515 ± 101	326 ± 77
P51671	Eotaxin	CCL11	570 ± 144	532 ± 138
O00175	Eotaxin-2	CCL24	875 ± 264	1046 ± 295
Q9Y258	Eotaxin-3	CCL26	670 ± 211	551 ± 146
P21860	ErbB3	ERBB3	834 ± 170	500 ± 88
P16581	E-Selectin	SELE	440 ± 100	308 ± 85
P48023	Fas Ligand	FASLG	843 ± 146	621 ± 143
Q549F0	Fas/TNFRSF6	FAF1	2641 ± 1082	2142 ± 856
P08620	FGF-4	FGF4	3175 ± 1002	3931 ± 1427
P10767	FGF-6	FGF6	283 ± 46	299 ± 95
P21781	FGF-7	FGF7	273 ± 48	282 ± 65
P31371	FGF-9	FGF9	846 ± 296	736 ± 151

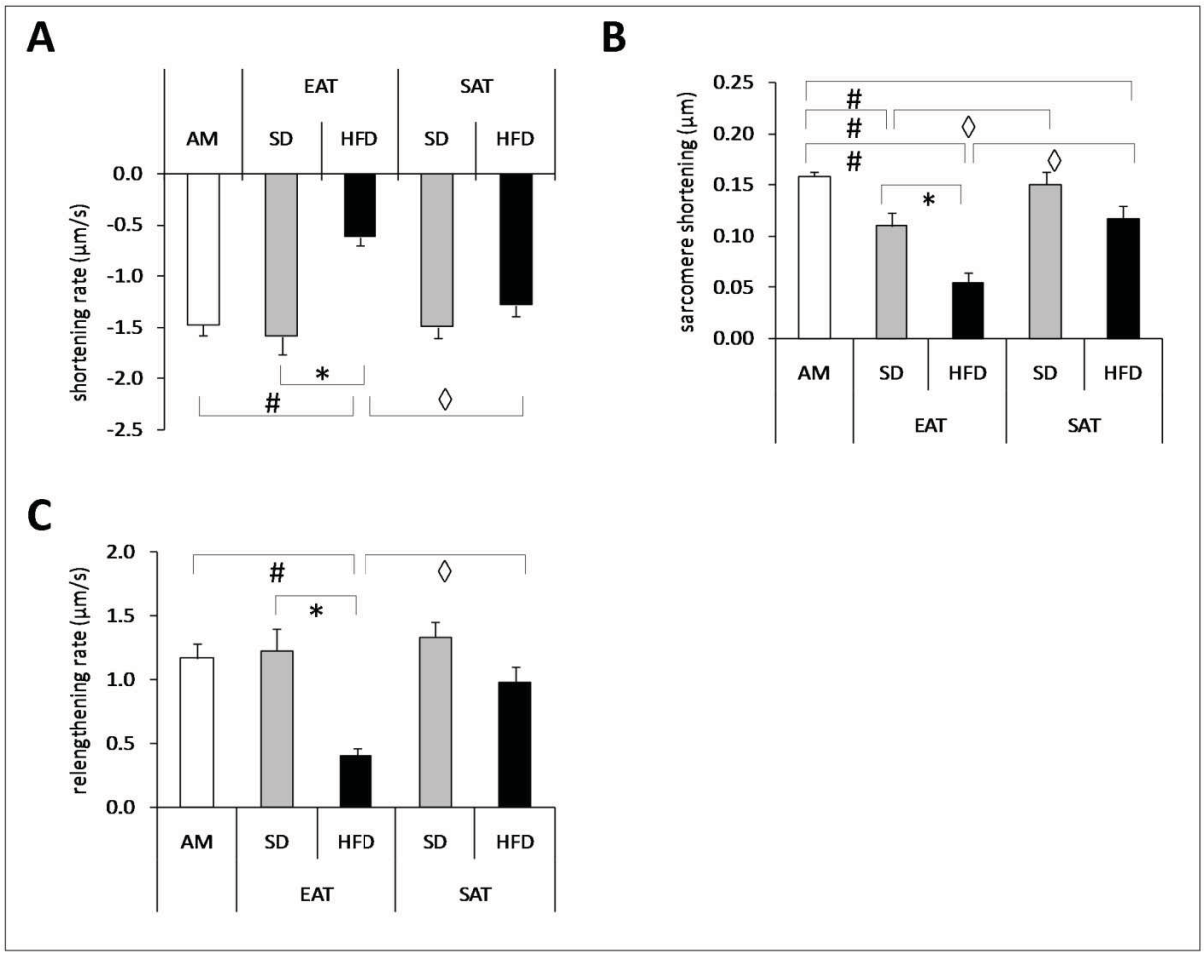
P49771	Fit-3 Ligand	FLT3LG	601 ± 313	555 ± 242
P78423	Fractalkine	CX3CL1	302 ± 68	295 ± 63
P80162	GCP-2	CXCL6	339 ± 60	415 ± 79
P09919	GCSF	CSF3	513 ± 208	474 ± 246
P39905	GDNF	GDNF	3799 ± 2962	2904 ± 1646
Q9Y5U5	GITR	TNFRSF18	672 ± 185	2198 ± 1605
Q9UNG2	GITR-Ligand	TNFSF18	596 ± 241	692 ± 466
P04141	GM-CSF	CSF2	196 ± 72	206 ± 44
P09341	GRO	CXCL1	699 ± 122	549 ± 94
P09341	GROα	CXCL1	217 ± 51	286 ± 107
O15467	HCC-4	CCL16	693 ± 206	765 ± 423
P14210	HGF	HGF	254 ± 64	215 ± 63
P22362	I-309	CCL1	292 ± 97	456 ± 110
P05362	ICAM-1	ICAM1	521 ± 134	493 ± 108
P13598	ICAM-2	ICAM2	15372 ± 5139	13015 ± 3916
P32942	ICAM-3	ICAM3	703 ± 474	281 ± 136
P01343	IGF-1	IGF1	443 ± 145	282 ± 49
P08833	IGFBP-1	IGFBP1	531 ± 147	590 ± 136
P18065	IGFBP-2	IGFBP2	348 ± 78	403 ± 85
P17936	IGFBP-3	IGFBP3	794 ± 240	952 ± 579
P22692	IGFBP-4	IGFBP4	582 ± 287	342 ± 145
P24592	IGFBP-6	IGFBP6	1070 ± 314	951 ± 246
P08069	IGF-I SR	IGF1R	290 ± 117	326 ± 93
P01344	IGF-II	IGF2	5358 ± 3475	537 ± 156
P27930	IL-1 R II	IL1R2	4512 ± 3582	2587 ± 1867
Q01638	IL-1 RA/ST2	IL1RL1	683 ± 154	711 ± 270
P14778	IL-1 RI	IL1R1	362 ± 114	337 ± 64
P22301	IL-10	IL10	489 ± 57	404 ± 138
Q08334	IL-10 Rβ	IL10RB	352 ± 56	234 ± 115
P20809	IL-11	IL11	280 ± 151	443 ± 243
P29459	IL-12 p40	IL12A	893 ± 344	798 ± 211
P29460	IL-12 p70	IL12B	695 ± 213	708 ± 431
P35225	IL-13	IL13	326 ± 105	204 ± 78
Q14627	IL-13 Rα2	IL13RA2	847 ± 340	402 ± 132
P40933	IL-15	IL15	234 ± 61	223 ± 57
Q14005	IL-16	IL16	426 ± 78	382 ± 97
Q16552	IL-17	IL17A	422 ± 234	551 ± 354
Q13478	IL-18 BPα	IL18R1	224 ± 47	157 ± 37
O95256	IL-18 Rβ	IL18RAP	802 ± 203	486 ± 90
P01583	IL-1a	IL1A	670 ± 183	970 ± 215
P01584	IL-1b	IL1B	192 ± 64	280 ± 60
P18510	IL-1ra	IL1RN	191 ± 41	325 ± 75
P60568	IL-2	IL2	114 ± 34	133 ± 20
P31785	IL-2 Rγ	IL2RG	606 ± 102	429 ± 123
P01589	IL-2 Rα	IL2RA	493 ± 109	263 ± 38
P14784	IL-2 Rβ	IL2RB	715 ± 300	838 ± 482

Q9HBE5	IL-21R	IL21R	614 ± 161	311 ± 54
P08700	IL-3	IL3	178 ± 82	281 ± 58
P05112	IL-4	IL4	264 ± 132	380 ± 186
P05113	IL-5	IL5	201 ± 43	128 ± 38
Q01344	IL-5 Rα	IL5RA	514 ± 133	137 ± 52
P05231	IL-6	IL6	519 ± 192	264 ± 96
P08887	IL-6R	IL6R	1185 ± 311	1324 ± 214
P13232	IL-7	IL7	150 ± 19	214 ± 81
P10145	IL-8	IL8	420 ± 131	406 ± 209
P15248	IL-9	IL9	831 ± 202	742 ± 188
P01579	INFγ	IFNG	168 ± 28	270 ± 47
P02778	IP-10	CXCL10	990 ± 396	451 ± 123
O14625	I-TAC	CXCL11	507 ± 138	593 ± 133
P17676	LAP	CEBPB	693 ± 229	297 ± 98
P41159	Leptin	LEP	491 ± 135	501 ± 89
P48357	Leptin R	LEPR	838 ± 196	713 ± 167
P15018	LIF	LIF	646 ± 117	574 ± 159
O43557	LIGHT	TNFSF14	682 ± 194	710 ± 183
P14151	L-Selectin	SELL	511 ± 115	382 ± 139
P47992	Lymphotactin	XCL1	1019 ± 287	1406 ± 476
P13500	MCP-1	CCL2	304 ± 109	319 ± 64
P80075	MCP-2	CCL8	454 ± 170	354 ± 73
P80098	MCP-3	CCL7	218 ± 109	290 ± 74
Q99616	MCP-4	CCL13	562 ± 364	185 ± 44
P09603	M-CSF	CSF1	498 ± 161	807 ± 191
P07333	M-CSF R	CSF1R	376 ± 113	165 ± 52
O00626	MDC	CCL22	629 ± 105	890 ± 224
P14174	MIF	MIF	2203 ± 491	2891 ± 1565
Q07325	MIG	CXCL9	379 ± 148	347 ± 112
P10147	MIP-1a	CCL3	1017 ± 359	921 ± 115
P13236	MIP-1b	CCL4	1172 ± 283	1320 ± 539
Q16663	MIP-1d	CCL15	852 ± 468	738 ± 199
P78556	MIP-3a	CCL20	237 ± 85	281 ± 90
Q99731	MIP-3b	CCL19	229 ± 41	330 ± 62
P03956	MMP-1	MMP1	1355 ± 171	1194 ± 276
P45452	MMP-13	MMP13	405 ± 96	207 ± 76
P08254	MMP-3	MMP3	478 ± 116	314 ± 73
P14780	MMP-9	MMP9	406 ± 179	228 ± 63
P55773	MPIF-1	CCL23	262 ± 70	170 ± 35
P26927	MSPa	MST1	559 ± 140	431 ± 131
P02775	NAP-2	PPBP	3162 ± 1314	4162 ± 1257
P08138	NGF R	NGFR	272 ± 72	240 ± 20
P20783	NT-3	NTF3	594 ± 239	721 ± 182
P34130	NT-4	NTF4	617 ± 122	485 ± 116
P13725	Oncostatin	OSM	575 ± 120	537 ± 91
O00300	Osteoprotegerin	TNFRSF11B	552 ± 192	496 ± 119

P55774	PARC	CCL18	858 ± 362	1571 ± 322
P04085	PDGF AA	PDGFA	897 ± 142	453 ± 149
P16234	PDGF Rα	PDGFRA	395 ± 120	168 ± 44
P09619	PDGF Rβ	PDGFRB	386 ± 86	563 ± 182
na	PDGF-AB		661 ± 98	489 ± 77
P01127	PDGF-BB	PDGFB	479 ± 119	505 ± 118
P16284	PECAM-1	PECAM1	278 ± 36	168 ± 48
Q07326	PIGF	PIGF	982 ± 290	1058 ± 286
P01236	Prolactin	PRL	554 ± 151	302 ± 85
P13501	RANTES	CCL5	2158 ± 472	2727 ± 885
P21583	SCF	KITLG	563 ± 117	391 ± 93
P10721	SCF R	KIT	679 ± 137	554 ± 87
P48061	SDF-1	CXCL12	470 ± 316	474 ± 207
P48061	SDF-1β	CXCL12	389 ± 105	158 ± 45
P40189	sgp 130	IL6ST	1782 ± 526	1324 ± 370
O15389	Siglec-5	SIGLEC5	406 ± 106	196 ± 54
P19438	sTNFR I	TNFRSF1A	2271 ± 1064	2308 ± 1287
P20333	sTNFR II	TNFRSF1B	1194 ± 430	792 ± 288
Q92583	TARC	CCL17	359 ± 78	323 ± 36
O15444	TECK	CCL25	746 ± 203	498 ± 149
P01135	TGFα	TGFA	728 ± 224	615 ± 222
P01137	TGFβ1	TGFB1	916 ± 731	594 ± 397
P61812	TGFβ2	TGFB2	1264 ± 328	1461 ± 364
P10600	TGFβ3	TGFB3	398 ± 98	274 ± 31
P40225	Thrombopoeitin	THPO	729 ± 151	498 ± 85
P35590	Tie-1	TIE1	516 ± 122	488 ± 119
Q02763	Tie-2	TEK	734 ± 187	381 ± 90
P01033	TIMP-1	TIMP1	882 ± 408	958 ± 553
P16035	TIMP-2	TIMP2	47654 ± 16491	35681 ± 92975
Q99727	TIMP-4	TIMP4	3368 ± 1237	5444 ± 3623
P01375	TNFα	TNF	248 ± 69	251 ± 66
P01374	TNFβ	LTA	814 ± 285	770 ± 179
O14798	TRAIL R3	TNFRSF10C	361 ± 77	299 ± 87
Q9UBN6	TRAIL R4	TNFRSF10D	628 ± 121	526 ± 86
Q03405	uPAR	PLAUR	450 ± 148	416 ± 102
P33151	VE-Cadherin	CDH5	437 ± 104	348 ± 111
P15692	VEGF	VEGFA	727 ± 208	1092 ± 723
P35968	VEGF R2	KDR	536 ± 96	230 ± 54
P35916	VEGF R3	FLT4	4452 ± 1326	3822 ± 1781
O43915	VEGF-D	FIGF	2536 ± 1190	2702 ± 1816

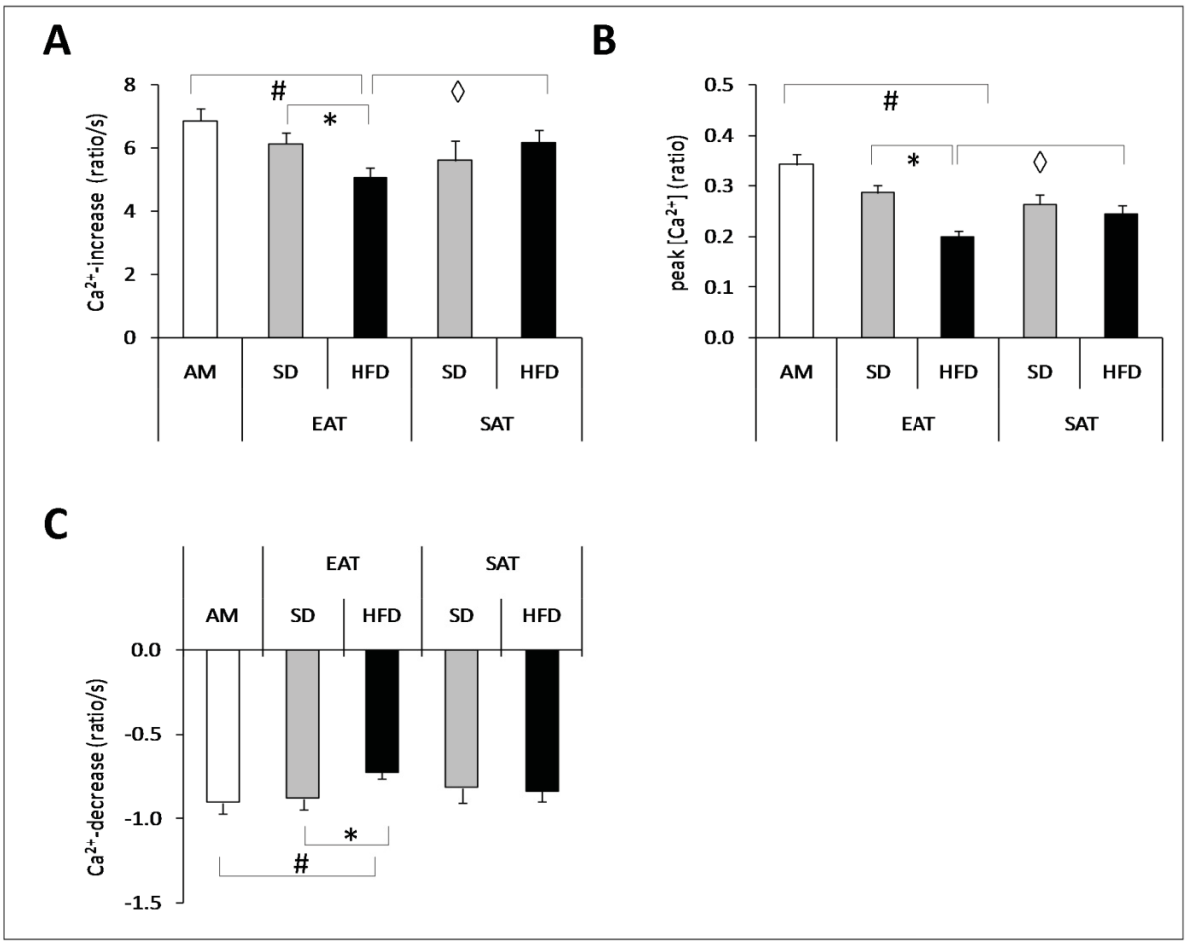
*, $P<0.05$ epicardial adipose tissue (EAT) versus subcutaneous adipose tissue (SAT)

Supplementary Figure 1. Effect of conditioned medium from epicardial and subcutaneous adipose tissue on sarcomere shortening in cardiomyocytes



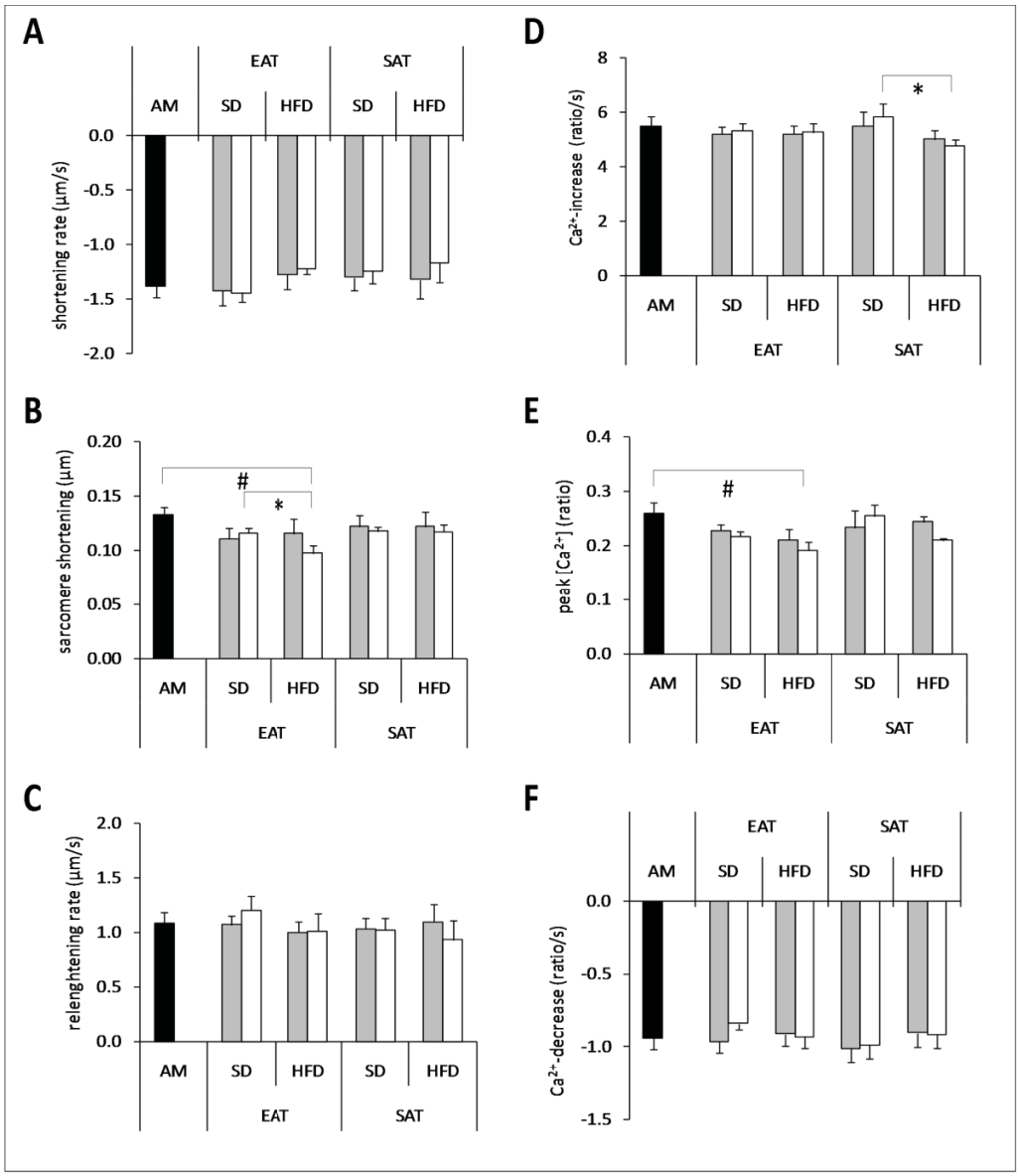
Primary adult rat cardiomyocytes were incubated with control AM or CM (diluted 1:2) from EAT and SAT from SD- and HFD-fed guinea pigs for 30 min before analysis of contractile function. A-C. Effect of exposure of cardiomyocytes to CM on departure velocity of contraction (A), peak sarcomere shortening (B), and return velocity of contraction (C). Open bars, AM; gray bars, CM from SD-fed animals; black bars, CM from HFD-fed animals. Data are expressed as mean ± S.E.M. of at least 8 independent experiments. Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P<0.05$ versus AM; *, $P<0.05$, HFD versus SD; ◊, $P<0.05$ EAT versus SAT.

Supplementary Figure 2. Effect of conditioned medium from epicardial and subcutaneous adipose tissue on cytosolic [Ca^{2+}] in cardiomyocytes



Primary adult rat cardiomyocytes were incubated with control AM or CM (diluted 1:2) from EAT and SAT from SD- and HFD-fed guinea pigs for 30 min before analysis of cytosolic [Ca^{2+}]. A-C. Effect of exposure of cardiomyocytes to CM on [Ca^{2+}] increase (A), peak [Ca^{2+}] (B), and [Ca^{2+}] decrease (C). Open bars, AM; gray bars, CM from SD-fed animals; black bars, CM from HFD-fed animals. Data are expressed as mean ± S.E.M. of at least 8 independent experiments. Differences between the experimental groups were calculated by one-way ANOVA and unpaired students t-tests. #, $P<0.05$ versus AM; *, $P<0.05$, HFD versus SD; ◊, $P<0.05$ EAT versus SAT.

Supplementary Figure 3. Contractile function 2 hours after removal of conditioned media



Primary adult rat cardiomyocytes were incubated with control AM or CM from EAT and SAT from SD- and HFD-fed guinea pigs for 30 min. Then, the CM were replaced by control AM. Following a 2 h incubation, contractile function and cytosolic [Ca^{2+}] was analyzed. A-C. Reversibility effect of exposure of cardiomyocytes to CM on departure velocity of contraction (A), peak sarcomere shortening (B), and return velocity of contraction (C). D-F. Reversibility effect of exposure of cardiomyocytes to CM on [Ca^{2+}] increase (D), peak [Ca^{2+}] (E), and [Ca^{2+}] decrease (F). Black bars, AM; gray bars, cells exposed to CM diluted 1:4; open bars, cells exposed to CM diluted 1:4. Data are mean ± S.E.M. from at least 8 independent experiments. Groups were compared by one-way ANOVA and unpaired students t-test. #, $P<0.05$ versus AM; *, $P<0.05$, HFD versus SD.

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3

Enhanced secretion of activin A from epicardial adipose tissue of patients with type 2 diabetes induces cardiomyocyte dysfunction

Manuscript submitted for publication

Chapter 3

Study 2

Enhanced secretion of activin A from epicardial adipose tissue of patients with type 2 diabetes induces cardiomyocyte dysfunction

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Abstract

Secreted factors from epicardial adipose tissue (EAT) have been implicated in the development of cardiomyocyte dysfunction. However, the effect of type 2 diabetes mellitus (DM2) on the cross-talk between EAT and the myocardium has not been studied. To assess whether DM2-related alterations in the secretory profile of EAT affect contractile function and insulin action in cardiomyocytes.

Contractile function and insulin action was analyzed in primary adult rat cardiomyocytes incubated with conditioned media (CM) generated from explants of EAT biopsies obtained from patients without (ND) and with DM2. CM from subcutaneous (SAT) and pericardial adipose tissue (PAT) biopsies from the same patients served as control. Cardiomyocytes treated with CM (EAT) from DM2-patients showed reductions in sarcomere shortening and cytosolic Ca^{2+}-fluxes, and decreased insulin-mediated Akt-Ser473-phosphorylation as compared to CM from the other groups. Profiling of the CM showed that secretion of six factors was selectively enhanced in CM-EAT-DM2 versus CM-EAT-ND and CM from the other fat depots. Most notable changes were observed for activin A. Accordingly, SMAD2-phosphorylation, a downstream target of activin A signaling was elevated in cardiomyocytes treated with CM (EAT) from DM2-patients. Furthermore, the detrimental effects of CM (EAT) from DM2-patients were partially abolished in cardiomyocytes pretreated with an inhibitor of the activin A receptor. Finally, recombinant activin A impaired cardiomyocyte function and insulin-mediated Akt-Ser473-phosphorylation.

Collectively, our data implicate DM2-related alterations in the secretory profile of EAT, and in particular activin A, in the pathogenesis of diabetes-related heart disease.

Introduction

Cardiac contractile dysfunction and myocardial insulin resistance frequently occur in patients with type 2 diabetes (DM2) and the metabolic syndrome.[1] Risk factors for diabetes-related heart disease, like hypertension, dyslipidemia, increased visceral adipose tissue mass, and insulin resistance, also associate with expansion of the epicardial adipose tissue (EAT).[2-5] EAT is a visceral thoracic fat depot, surrounding the aortic arch, the large coronary arteries, the ventricles and the apex of the human heart.[6] Because EAT shares the coronary blood supply with the myocardium, and no structures resembling a fascia separate the adipose and myocardial layers, factors released from EAT may directly affect the underlying tissues.[2,6,7]

Like other adipose tissue depots, EAT is a source of pro-inflammatory adipokines, like interleukin (IL) 1, IL6, IL8, monocyte chemoattractive protein-1 (MCP1), leptin, plasminogen activator inhibitor 1 (PAI-1), resistin, RANTES, and tumor necrosis factor α (TNFα), as well as potential protective factors, like adiponectin and omentin.[5,8-16] Patients with coronary artery disease (CAD) show a dysregulated expression and release of these factors by EAT.[7,11,15] For example, expression and intracoronary levels of adiponectin are lower in patients with CAD.[17] Furthermore, conditioned media (CM) from EAT from patients with CAD show an enhanced potential to induce atherogenic changes in monocytes and endothelial cells.[15] While these data implicate secretory products from EAT in the pathogenesis of CAD, studies toward the interaction between EAT and myocardial function in DM2 are limited.

Since adipose tissue of patients with DM2 is characterized by a state of low-grade inflammation, we propose that DM2-related alterations in the secretory profile of EAT may affect myocardial function. In the present study, we analyzed contractile function and insulin action in primary rat cardiomyocytes following exposure to CM generated from explants of EAT biopsies obtained from patients without (ND) and with DM2. CM from subcutaneous (SAT) and pericardial adipose tissue (PAT) biopsies from the same patients served as control. In addition, we used antibody arrays to profile the CM from the various experimental groups for changes in adipokine secretion. Our data provide evidence for a cardiodepressant activity that can at least partially be ascribed to activin A in CM derived from EAT from patients with DM2, and implicate

DM2-related alterations in the secretory profile of EAT in the pathogenesis of diabetes-related heart disease.

Materials and Methods

Adipose tissue biopsies

Biopsies from EAT (obtained from above the right ventricle), PAT and intrathoracal SAT were collected from males of Caucasian origin undergoing open heart surgery (coronary artery bypass or valve replacement) after written informed consent. The procedure to obtain adipose tissue samples was approved by the ethical committee of the Heinrich-Heine-University (Duesseldorf, Germany). Patients of other ethnic origins, diagnosed as having HIV infection, lipodystrophy or chronic coexistent inflammatory disease were excluded from participation. Participants were distributed into two groups, ND or DM2, on the basis of the diagnosis DM2 in the status of the patient. Anthropomorphic characteristics and medication use are listed in supplementary Table 1.

Preparation and characterization of conditioned media (CM)

Adipose tissue biopsies were used to generate conditioned media (CM) as previously described.[16,18,19] Antibody arrays (RayBio human cytokine antibody G series 2000; Ray Biotech, Inc., Norcross GA, USA) were used to determine the secretory profile of the CM.[16]

Effects of conditioned media on primary adult rat cardiomyocytes

Cardiomyocytes were isolated from Lewis rats (Lew/Crl) as described.[20] and cultured for 24 h on laminin-coated dishes (ibidi GmbH, Martinsried, Germany) before exposure to CM.[16] The effects of CM on protein expression and phosphorylation in cardiomyocytes were determined by Western blotting.[16] The effects of CM on sarcomere shortening and cytosolic Ca^{2+}-fluxes were

determined following electric stimulation of the cells with 1 Hz on a contractility and fluorescence system from IonOptix (Dublin, Ireland) using Fura-2 (Merck chemicals, Darmstadt, Germany) as Ca^{2+}-indicator.[16]

Data analysis

Data are presented as mean ± standard error of the mean. Significant differences between experimental conditions were evaluated by one-way ANOVA or unpaired Student´s *t* test using Graphpad Prism software (version 5). A value of $P< 0.05$ was considered as statistically significant.

Results

Effect of conditioned media on sarcomere shortening, cytosolic Ca^{2+}-fluxes and sarcoplasmic-endoplasmic reticulum calcium ATPase 2a expression in cardiomyocytes

Compared to control adipocyte medium (AM), exposure of primary rat cardiomyocytes to CM-EAT from patients with DM2 (CM-EAT-DM2) markedly impaired contractile function, as illustrated by reductions in departure velocity of contraction, peak sarcomere shortening, and return velocity of contraction (Figure 1 A-C). CM-SAT and CM-PAT from DM2-patients induced minor reductions in cardiomyocyte function (Figure 1 A-C). Contractile parameters were not affected by CM generated from fat depots from ND-patients (Figure 1 A-C). Abrogation of cytosolic Ca^{2+}-transients, as illustrated by reductions in departure and return velocities and a lower peak Fura-2 fluorescence signal was only induced by CM-EAT-DM2 (Figure 1 D-F). In line with this, only CM-EAT-DM2 significantly lowered protein levels of sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) 2a in primary rat cardiomyocytes (Figure 2A).

Effect of conditioned media on insulin signaling in cardiomyocytes

CM-EAT-DM2 and to a minor extend CM-SAT-DM2 markedly blunted insulin-stimulated Akt-phosphorylation in adult rat cardiomyocytes (Figure 2B). In contrast, CM-PAT-DM2 or CM generated from fat depots from ND-patients had no inhibitory effect on insulin-stimulated Akt-phosphorylation (Figure 2B).

Characterization of conditioned media

In order to identify the factor(s) responsible for the selective induction of cardiomyocyte dysfunction by CM-EAT-DM2, alterations in adipokine secretion by CM from the various groups were profiled using antibody arrays. The immunoreactivity of the 174 factors present on the array for CM-EAT, CM-SAT, and CM-PAT from patients with and without DM2 is presented in supplementary tables 2-4. In CM-EAT-DM2, immunoreactivity of activin A, agouti-related protein (AgRP), angiopoietin-2, cluster of differentiation 14 (CD14), eotaxin-2 (also known as CCL24 (chemokine C-C- motif ligand 24)), and TRAIL-receptor 4 (TRAILR4) was increased versus CM-EAT-ND (all $P<0.05$) (Supplementary Table 2, Figure 3). Notably, the increases in activin A, angiopoietin-2, AgRP and CD14 immunoreactivity were selective for CM-EAT-DM2, indicating that DM2 differentially affects the secretion of these factors in the various fat depots (Figure 3).

Effect of conditioned media on SMAD2-phosphorylation

Since we recently identified activin A as the predominant factor to be affected by high-fat feeding in EAT from guinea pigs,[16] we decided to evaluate whether this adipokine could be responsible for the detrimental effects induced by CM-EAT-DM2 in cardiomyocytes. Using ELISA, the amount of activin A in CM-EAT-DM2 was determined at 2.4 ± 0.8 ng/ml. To verify whether activin A in CM-EAT-DM2 is biologically active, we analyzed cardiomyocytes incubated with CM for phosphorylation of SMAD2, a downstream target of activin A signaling [21]. As shown in Figure 4A, only primary rat cardiomyocytes exposed to CM-EAT-DM2 showed a significantly increased SMAD2-phoshorylation. Pretreating cardiomyocytes with the activin-receptor-like kinase

inhibitor SB4311542 prevented the increase in SMAD2-phosphorylation induced by CM-EAT-DM2 and activin A (Figure 4B).

Effect of inhibition of activin A signaling on the detrimental effects induced by CM-EAT in cardiomyocytes

We used SB4311542 to further detail the importance of activin A signaling for the induction of cardiomyocyte dysfunction by CM-EAT-DM2. Pretreatment of cardiomyocytes with SB4311542 partially improved the reductions in sarcomere shortening and cytosolic Ca^{2+}-fluxes induced by CM-EAT-DM2 (Figure 5A-B, supplementary Figure 1). Also the inhibitory effect of CM-EAT-DM2 on insulin-mediated Akt-phosphorylation was partially reversed in cardiomyocytes incubated with SB4311542 (Figure 5C). SB4311542 had no effect on these parameters in cardiomyocytes incubated with control adipocytes medium (AM) or CM-EAT-ND (Figure 5 A-C, supplementary Figure 1).

Effect of human recombinant activin A on contractile function in cardiomyocytes.

Finally, we determined whether activin A could mimic the detrimental effects of CM-EAT-DM2 in cardiomyocytes. Incubation of cardiomyocytes with recombinant activin A caused a dose-dependent reduction in the parameters of sarcomere shortening and cytosolic Ca^{2+}-fluxes, which became significant at a concentration of 1 ng/ml activin A (Figure 6). The inhibitory effects of activin A were fully reversed in cells pretreated with SB4311542 (supplementary figure 2). Insulin-mediated Akt-phosphorylation was also blunted in cardiomyocytes exposed to activin A (Figure 7). However, significance for the inhibitory effect on insulin action was reached at 10 ng/ml of activin A (Figure 7). Collectively, these findings suggest that enhanced secretion of activin A by CM-EAT-DM2 may contribute to the induction of cardiomyocyte dysfunction.

Discussion

This study demonstrates that EAT from DM2-patients secretes factors that induce contractile dysfunction and insulin resistance in primary rat cardiomyocytes. Moreover, we show that the secretory profile of EAT from patients with DM2 differs from that of SAT and PAT, and from ND. Finally, we identified activin A as a major factor responsible for the detrimental effects on cardiomyocyte function induced by CM-EAT-DM2. Collectively, these findings provide evidence for a cardiodepressant activity that can be ascribed to activin A in CM derived from EAT from patients with DM2, and implicate DM2-related alterations in the secretory profile of EAT in the pathogenesis of diabetes-related heart disease.

Multiple epidemiological studies have demonstrated associations between expansion of EAT and clinical markers of the metabolic syndrome, DM2, CAD, and cardiac function.[3,5] Recently, we demonstrated that high-fat feeding of guinea pigs induces qualitative alterations in the secretory profile of EAT which contribute to the induction of cardiomyocyte dysfunction.[16] Also obesity and CAD have been associated with qualitative alterations in the secretory profile of EAT that underlie the induction of atherogenic changes in monocytes and endothelial cells.[15] Here, we extend these observations by showing that CM generated from EAT of age- and BMI-matched DM2-patients exerts a strong cardiodepressant and negative inotropic effect on primary rat cardiomyocytes. The reductions in sarcomere shortening and cytosolic Ca^{2+}-fluxes were paralleled by a decreased protein expression of SERCA2a in cardiomyocytes incubated with CM-EAT from DM2-patients. SERCA2a is a key regulator of myocardial Ca^{2+} metabolism, and decreases in SERCA2a expression are a common characteristic of cardiopathological states in humans and rodents.[22,23] Therefore, it seems likely that the reduction in SERCA2a expression may account for the reduction of Ca^{2+}-transients in cardiomyocytes treated with CM-EAT from patients with DM2.

We also found that CM from SAT and PAT of DM2-patients exhibited minor inhibitory effects on parameters of sarcomere shortening in the absence of significant effects on cytosolic Ca^{2+}-transients and SERCA2a expression. Previous study also reported cardiosuppressive effects induced by CM from human SAT in isolated cardiomyocytes and hearts under Langendorff perfusion.[24-26] These cardiosuppressive effects of adipose tissue have been ascribed to

adipokines which are elevated in DM2, including fatty acid binding protein 4 (FABP4), IL1β and 6, and TNFα.[27-31] However, the physiological relevance of the cardiodepressant factor(s) secreted by other adipose tissue depots remains to be elucidated since it may only affect cardiomyocyte function via the systemic circulation. In contrast, factors secreted from the EAT can directly affect the function of the underlying myocardium because to the absence of facial boundaries.[2,4,5]

In addition to induction of cardiomyocyte dysfunction, we observed that CM-EAT-DM2 abrogates insulin-mediated phosphorylation of Akt, a key regulator of myocardial glucose uptake. In patients with DM2, alterations in cardiac structure and function which are found even in the absence of hypertension and CAD are ascribed to diabetic cardiomyopathy. The latter syndrome often co-exists with alterations in myocardial substrate metabolism, such as decreased insulin stimulation of myocardial glucose uptake.[32,33] Although our study provides strong evidence that secretory products of EAT from DM2-patients contribute to the induction of myocardial insulin resistance, it should be noted that the detrimental effects on insulin action were only demonstrated upon prolonged incubation of the cardiomyocytes with CM. Since the effects on contractile function could already be observed within minutes after the addition of CM, it is likely that the induction of insulin resistance by CM-EAT-DM2 is established through distinct mechanisms.

To identify factors responsible for the detrimental effect of CM-EAT-DM2, we characterized the secretory profile of CM-EAT as compared to CM from the other experimental groups. Using antibody arrays, we observed that the secretory profile of CM-EAT differs from CM-SAT and CM-PAT. Furthermore, we observed the EAT-specific accumulation of four factors, i.e. activin A, AgRP, angiopoietin-2, and CD14 in CM-EAT-DM2 versus CM-EAT-ND. Accumulation of activin A in CM-EAT was also observed in guinea pigs upon high-fat diet feeding versus animals fed a standard diet.[16] The alterations in activin A immunoreactivity were accompanied by biological activity of the CM, as illustrated by the induction of SMAD2-phosphorylation by CM-EAT-DM2 only. Although other factors present on the arrays like tumor growth factor (TGF) β1, TGFβ2, TGFβ3, hepatocyte growth factor (HGF), and insulin-like growth factor binding protein (IGFBP) 3, are also capable to regulate SMAD2-phosphorylation, immunoreactivity of these factors did not differ among the experimental groups. In line with previous reports

demonstrating that activin A increases the expression of genes involved in myocardial remodeling, like atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1), and TGFβ1,[34] and inhibits the organization of sarcomeric proteins induced by leukemia inhibitory factor in neonatal rat cardiomyocytes,[35] we observed that incubation of primary adult rat cardiomyocytes with activin A at a concentration comparable to that found in CM-EAT-DM2 reduced cardiomyocyte contractile parameters up to 30-50 % as compared to the inhibition caused by CM-EAT-DM2. Accordingly, the cardiosuppressive effects of CM-EAT-DM2 could be partially reversed by pharmacological inhibition of the activin A receptor. Thus, while these findings highlight the involvement of activin A in the induction of cardiomyocyte dysfunction by CM-EAT-DM2, they also indicate an involvement of as yet unknown secreted factors.

Recombinant activin A was also able to abrogate insulin-mediated Akt-Ser473-phosphorylation in primary adult rat cardiomyocytes. In line with this activin A has been found to induce the expression of SOCS3,[35] a key repressor of insulin action in neonatal cardiomyocytes.[36] However, both the abrogation of insulin-mediated Akt-phosphorylation and induction of SOCS3 expression occurred at a higher concentration of activin A than was found in CM-EAT-DM2. Furthermore, like CM-EAT-DM2, the inhibitory effects of activin A were only observed after prolonged exposure of the cardiomyocytes. These findings therefore suggest that the detrimental effect of CM-EAT-DM2 is mainly established through other mechanisms.

With the use of the antibody arrays, we could indeed identify other factors that are selectively increased in CM-EAT-DM2, including angiopoietin-2, AgRP, and CD14. Although serum levels of CD14 and angiopoietin-2 are raised in patients with DM2 and cardiovascular dysfunction,[37,38] the role of these factors in relation to myocardial function and insulin sensitivity remains to be elucidated. Furthermore, not all factors potentially secreted by EAT are present on the array used in this study, such as FABP4 and omentin. Expression of FABP4 is elevated in EAT of patients with the metabolic syndrome,[13] and recombinant FABP4 has been found to acutely suppress contractile function in cardiomyocytes.[27] In a separate study, we observed that omentin expression and secretion in EAT is decreased in DM2, and that addition of omentin could reverse the detrimental effects induced by CM on cardiomyocyte function (Greulich et al., manuscript in preparation).

Another question that remains to be addressed is what underlies the alterations in the secretory profile of EAT from patients with DM2. In the present study, CM was generated from adipose tissue explants. In addition to adipocytes, adipose tissue also contains other cell types, like pre-adipocytes, macrophages, monocytes, endothelial cells and lymphocytes, which can release a variety of chemo-, cytokines and other factors.[39,40] Hypertrophy of adipose tissue in obesity and DM2 is closely linked to low-grade inflammation,[41] which can be ascribed to accumulation of immune cells in the adipose tissue, and secretion of pro-inflammatory cytokines.[39,40] A study performed on humans with CAD reports the infiltration of immune cells in EAT.[11] Although we did not assess the cellular composition of the EAT biopsies in the present study, two lines of evidence indicate that also the accumulation of activin A in CM-EAT-DM2 can be ascribed to infiltration of immune cells in EAT. First, CD14 is mainly expressed in macrophages and secreted in its soluble form by monocytes. In addition, it has been demonstrated that activin A expression is elevated in adipose tissue from obese subjects, and dramatically increased by factors secreted by macrophages isolated from obese adipose tissue.[42]

In conclusion, the present study indicates that CM-EAT from patients with DM2 causes myocardial dysfunction and induces insulin resistance in primary rat cardiomyocytes. Our data also demonstrate that the secretory profile of EAT is considerably different from SAT and PAT and that this secretion pattern differs between ND- and DM2-patients. Furthermore, in this study we report for the first time that the release of activin A is elevated by EAT from DM2-subjects and that this cytokine causes cardiomyocyte dysfunction. Therefore, the present study suggests that DM2-related alterations in the secretory profile of EAT, and in particular activin A, could contribute to the pathogenesis of cardiac dysfunction in DM2.

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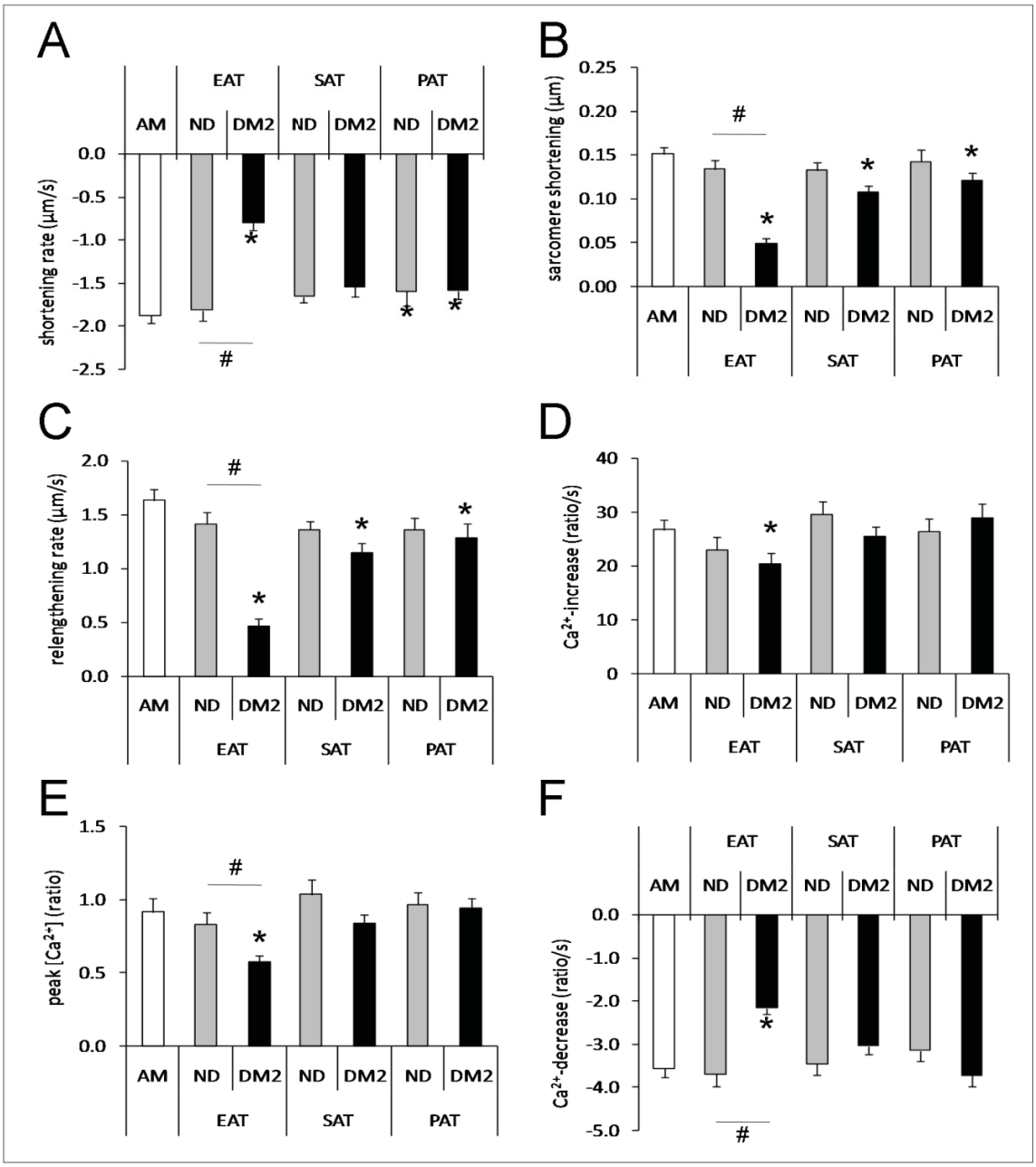
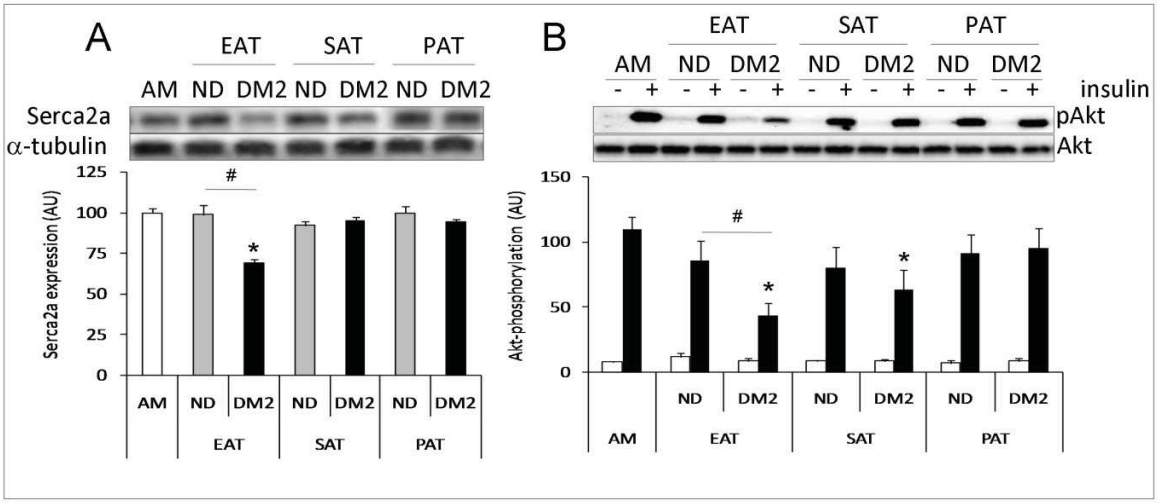
Figure 1. Effect of conditioned media from human epicardial, subcutaneous and pericardial adipose tissue on cardiomyocyte contractile function



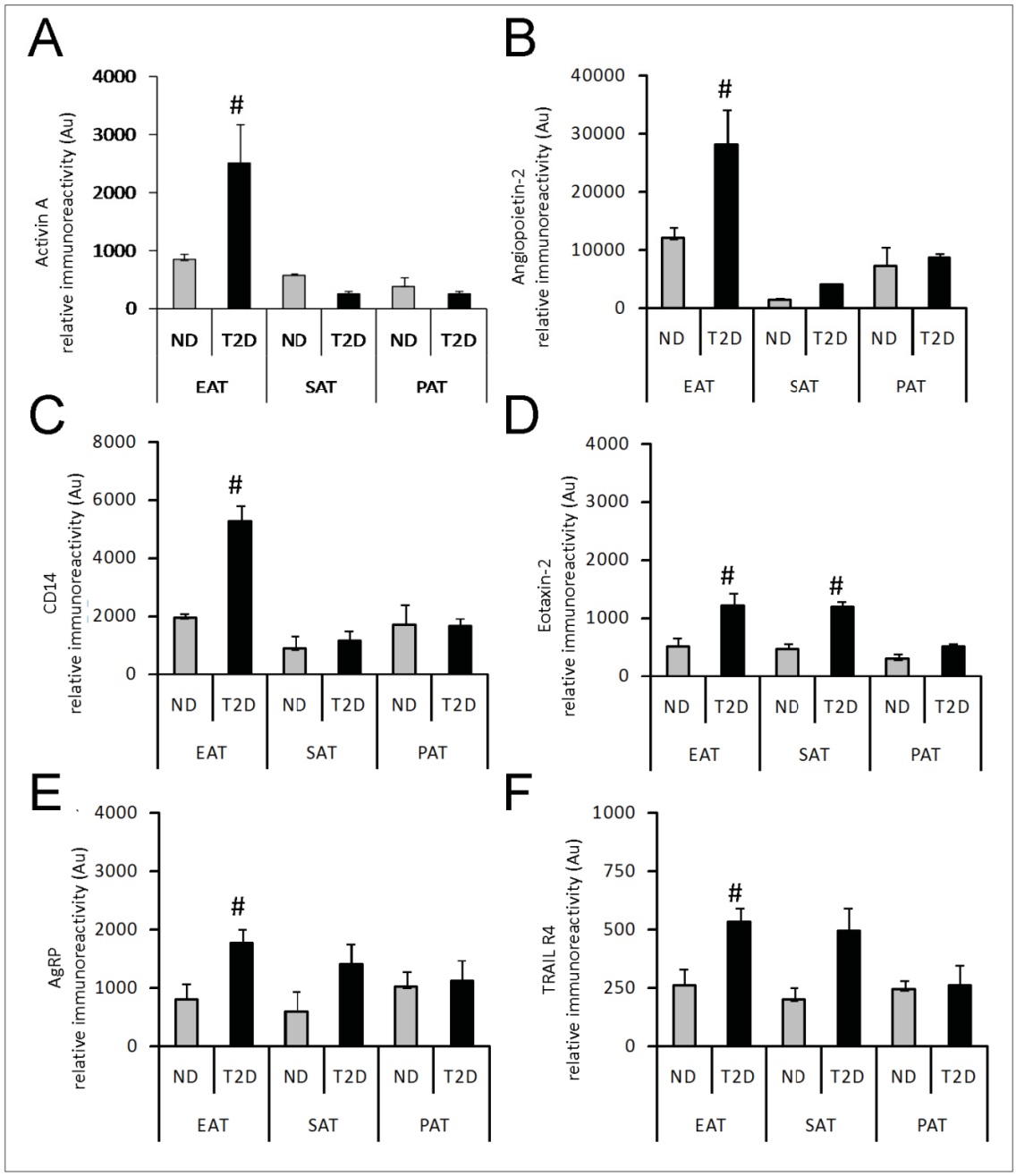
Primary adult rat cardiomyocytes were exposed to control adipocyte medium (AM) or conditioned media (CM, diluted 1:4) from EAT, SAT and PAT from DM2- or ND-patients for 30 min prior to analysis of sacromere shortening and Ca^{2+}-fluxes. Effect of AM or CM on departure velocity of contraction (A), peak sarcomere shortening (B), and return velocity of contraction (C). Effect of cardiomyocytes to AM or CM on departure velocity of cytosolic [Ca^{2+}] increases (D), peak Fura-2 fluorescence signal (E), and cytosolic [Ca^{2+}] decreases (F). Open bars, AM; gray bars, CM from ND patients; black bars, CM from DM2 patients. Values are expressed as mean ± S.E.M. of at least 10 independent experiments. *, $P< 0.05$ versus AM; #, $P< 0.05$ ND versus DM2.

Figure 2. Effect of conditioned medium on Serca2a expression and insulin action in primary adult rat cardiomyocytes



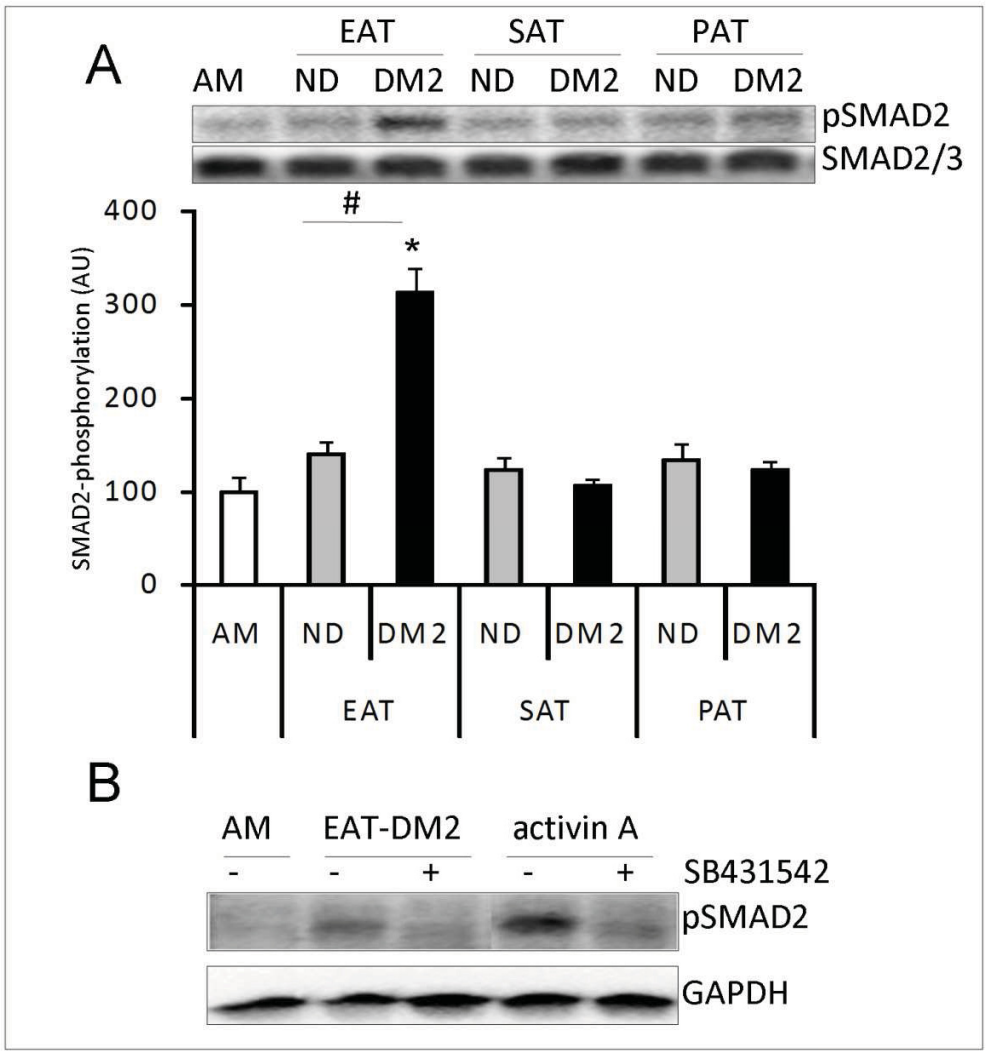
Representative Western blots and quantification of Serca2a expression (A) and insulin induced Akt-Ser473-phosphorylation (B) in cardiomyocytes treated with control adipocyte medium (AM) or conditioned media (CM, diluted 1:6) from EAT, SAT and PAT from DM2- or ND-patients for 24 h. Data are presented as mean ± S.E.M. (n=6 per group). Open bars, basal; filled bars, insulin stimulated cells (10 min; 100 nM). *, $P< 0.05$ versus AM; # $P< 0.05$ ND versus DM2.

Figure 3. Adipokine immunoreactivity in conditioned media from EAT, SAT and PAT in ND- and DM2-patients



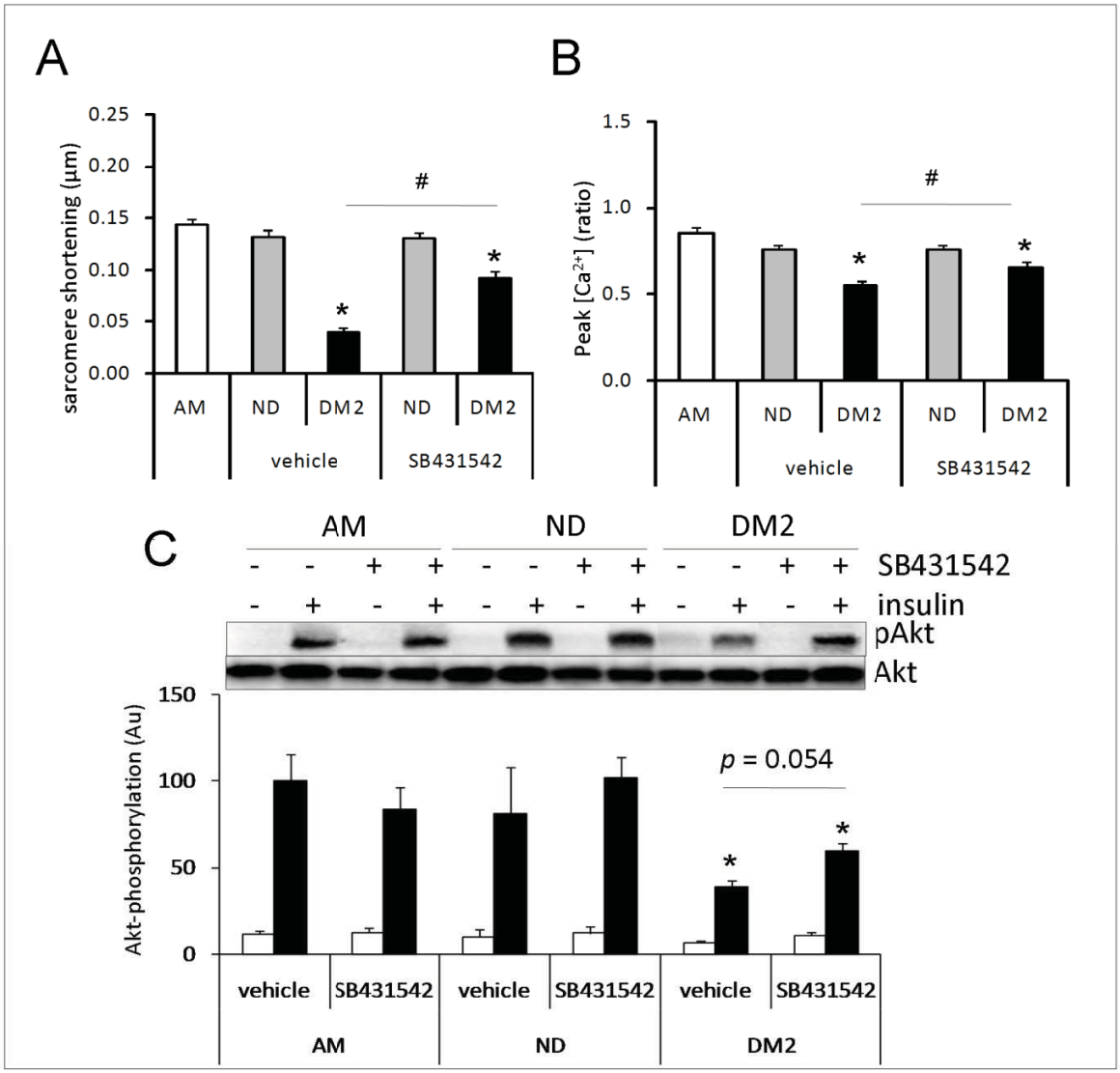
CM from EAT, SAT and PAT from ND- and DM2-patients was profiled using antibody arrays. Shown is the immunoreactivity for activin A (A), angiopoietin-2 (B), CD14 (C), Eotaxin-2 (D), AgRP (E), and TRAIL R4 (F). Gray bars, CM from ND-patients; black bars, CM from DM2-patients. Values are expressed as mean±S.E.M. (n=4 per group). #, $P< 0.05$ ND versus DM2.

Figure 4. Effect of conditioned medium on SMAD2 phosphorylation



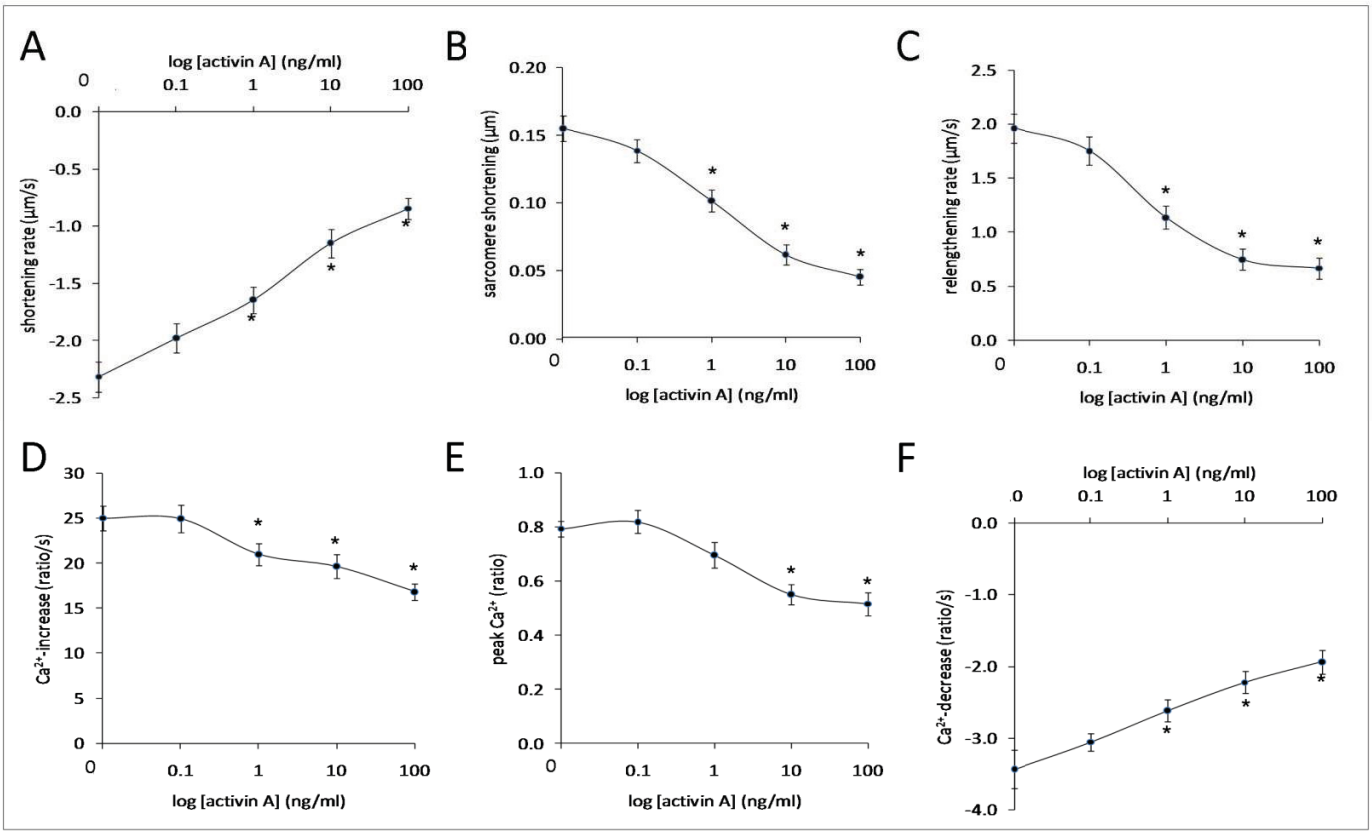
(A) Representative Western blot and quantification of SMAD2-Ser465/Ser467-phosphorylation in primary adult rat cardiomyocytes treated with control adipocyte medium (AM) or conditioned medium (CM) (diluted 1:6; 24 h). Data are presented as mean ± S.E.M. (n=5 per group). Open bars, AM; gray bars, CM from ND-patients; black bars, CM from DM2-patients. *, *P*< 0.05 versus AM; #*P*< 0.05 ND versus DM2. (B) Representative Western blot for the effect of pretreating primary adult rat cardiomyocytes with 10 µM SB431542 (1 h) on the induction of SMAD2-Ser465/Ser467-phosphorylation in response to to CM-EAT-DM2 or activin A (100 ng/ml) for 24 h.

Figure 5. Effect of inhibition of activin A signaling on the detrimental effects induced by CM-EAT in cardiomyocytes



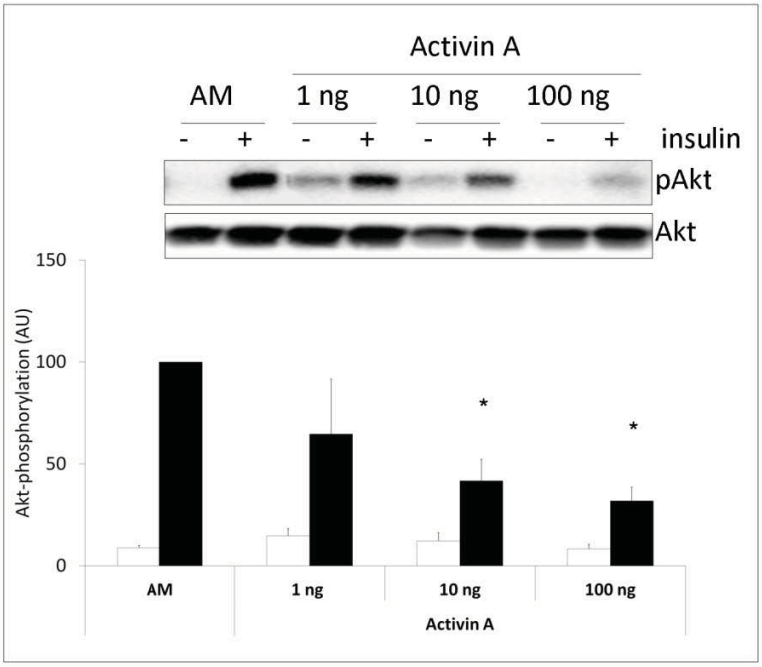
Primary adult rat cardiomyocytes were pretreated with the activin A receptor-kinase inhibitor SB431542 (10 µM, 1 h) and then incubated with control adipocyte medium (AM), or conditioned media (CM) from EAT of DM2- or ND-patients. Effect of SB431542 on peak sarcomere shortening (A), peak Fura-2 fluorescence signal (B), and insulin-mediated pAkt-Ser473-phosphorylation (C). For Figure A and B, open bars, AM; gray bars, CM-EAT from ND-patients; black bars, CM-EAT from DM2-patients. For Figure C, open bars, basal; filled bars, insulin-stimulated cells (10 min ; 100 nM). Values are expressed as mean ± S.E.M. (n=5-10 per group). *, $P< 0.05$ versus AM; #, $P< 0.05$ vehicle versus SB431542.

Figure 6. Effect of recombinant activin A on contractile function and cytosolic Ca^{2+} transients



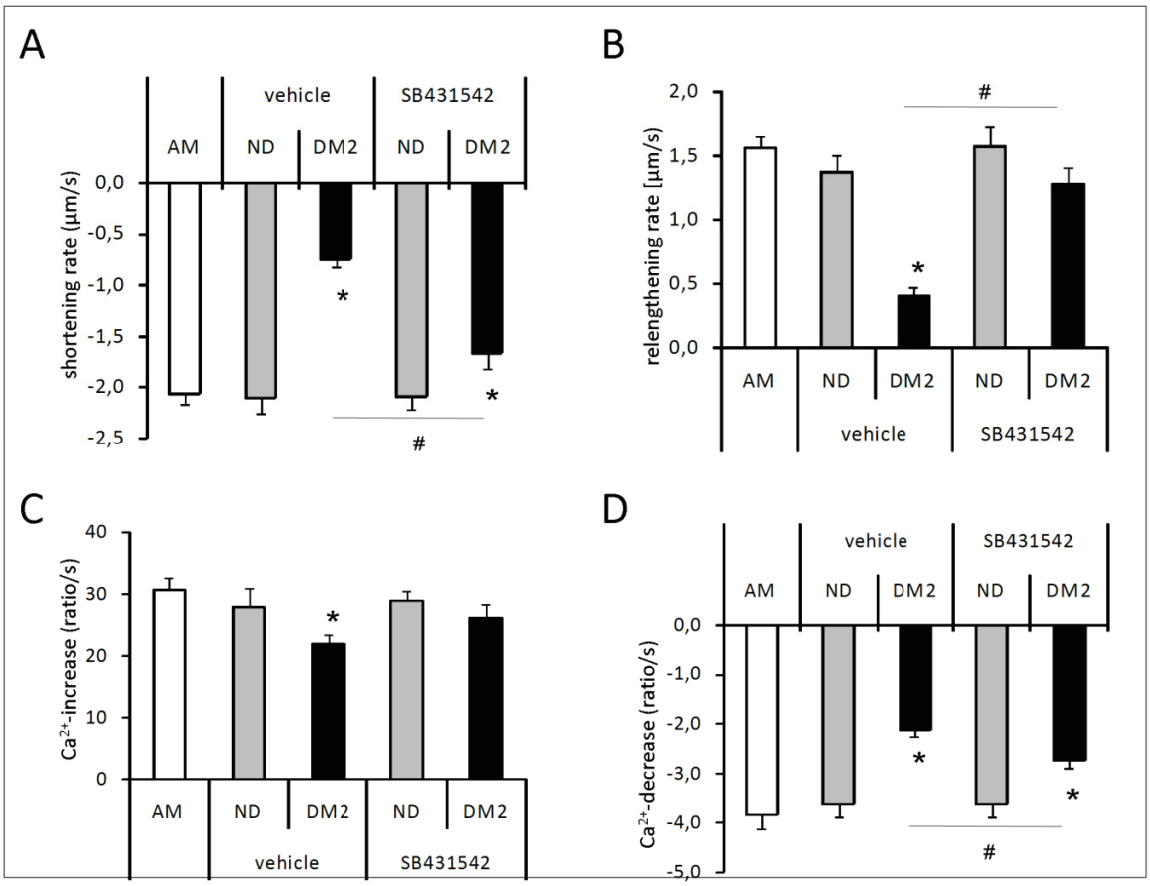
Dose-dependent effect of a 30 min exposure of primary adult rat cardiomyocytes to activin A on departure velocity of contraction (A), peak sarcomere shortening (B), return velocity of contraction (C), [Ca^{2+}] increase (D), peak Fura-2 fluorescence signal (E), and [Ca^{2+}] decrease (F) as compared to control adipocyte medium (AM). Open bars, AM; gray bars, CM from ND-patients; black bars, CM from DM2-patients. Values are expressed as mean ± S.E.M. of at least 10 independent experiments. *, P< 0.05 versus AM (0).

Figure 7. Effect of recombinant activin A on insulin-mediated Akt-Ser473-phosphorylation



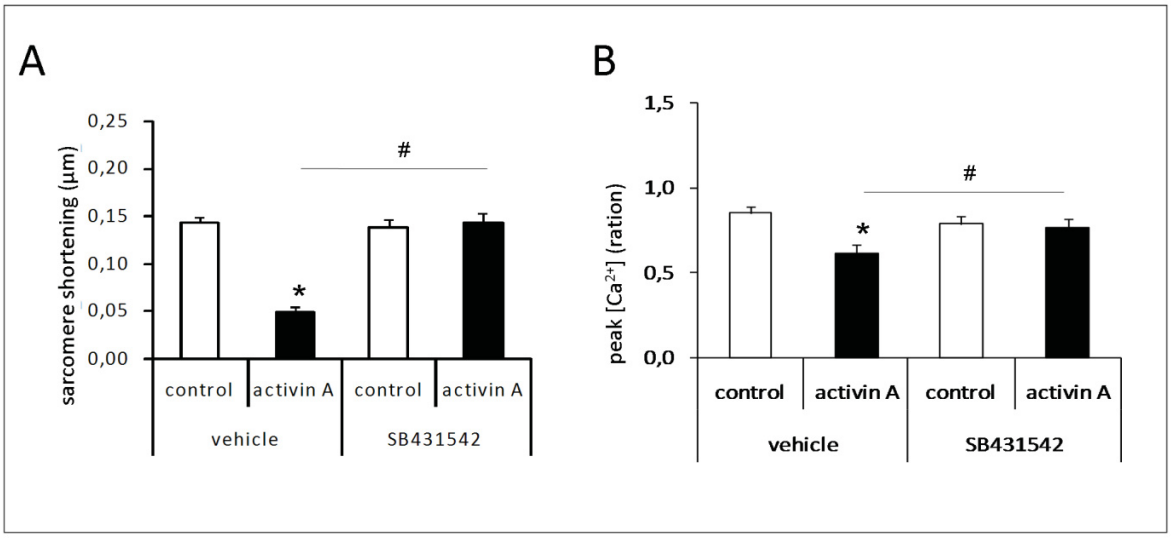
Lysates from primary adult rat cardiomyocytes exposed for 24 h to adipocyte medium (AM) or increasing concentrations of recombinant activin A (ng/ml) were analyzed for insulin-induced Akt-Ser473-phosphorylation. Data are presented as mean ± S.E.M. (n=6 per group). Open bars, basal; filled bars, insulin-stimulated cells (10 min; 100 nM). *, $P< 0.05$ versus AM.

Supplementary Figure 1. Effect of inhibition of activin A signaling on the detrimental effects induced by CM-EAT in cardiomyocytes.



Primary adult rat cardiomyocytes were pretreated with the activin A receptor-kinase inhibitor SB431542 (10 µM, 1 h) and then incubated with control adipocyte medium (AM), or conditioned media (CM) from EAT of DM2- or ND-patients. Effect of SB431542 on departure (A) and return velocities (B) of sarcomere shortening, and increases (C) and decreases (D) in cytosolic [Ca^{2+}]. Open bars, AM; gray bars, CM-EAT from ND-patients; black bars, CM-EAT from DM2-patients. Values are expressed as mean ± S.E.M. (n=5-10 per group). *, $P< 0.05$ versus AM; #, $P< 0.05$ vehicle versus SB431542.

Supplementary Figure 2. Effect of inhibition of activin A signaling on the detrimental effects induced by activin A in cardiomyocytes.



Primary adult rat cardiomyocytes were pretreated with the activin A receptor-kinase inhibitor SB431542 (10 µM, 1 h) and then incubated with control adipocyte medium (AM), or activin A. Effect of SB431542 on peak sarcomere shortening (A) and peak Fura-2 fluorescence signal (B). Open bars, AM; filled bars, activin A-treated cells (30 min; 10 ng/ml).

Supplementary Table 1. Patient characteristics

	ND (n=8)	**DM2 (n=8)**	***P***
Age (years)	72.7 (64-80)	73 (59-86)	0.73
Blood glucose level (mg/dL)	94.4	177	0.01
BMI (kg/m^2)	28.5	29.3	0.95
Medication use			
Statins	6/8	4/8	
ACE	5/8	5/8	
β-blockers	5/8	4/8	
diuretics	4/8	5/8	
glucose lowering medication	0/8	4/8	

Data are expressed as median (1st-3rd quartile) or mean ± standard error of the mean. The *p*-value indicates differences between anthropomorphic parameters. ND, non-diabetic; DM2, type 2 diabetes; ACE, angiotensin-converting enzyme inhibitor; BMI, body mass index.

Supplementary Table 2. Immunoreactivity in conditioned media generated from epicardial adipose tissue explants from patients without (ND) and with type 2 diabetes (DM2).

Swiss Prot	*Name on array*	*Symbol*	*ND (n=4)*	*DM2 (n=4)*	*P*
Q15848	Acrp30	ADIPOQ	103593 ± 36671	56030 ± 24285	0.321
P08476	Activin A	INHBA	842 ± 115	3023 ± 623	**0.010**
O00253	AgRP	AGRP	817 ± 248	1757 ± 245	**0.036**
Q13740	ALCAM	ALCAM	540 ± 116	536 ± 314	0.991
P15514	Amphiregulin	AREG	540 ± 202	677 ± 206	0.650
P03950	Angiogenin	ANG	3477 ± 1153	11415 ± 7505	0.336
O15123	Angiopoietin-2	ANGPT2	12111 ± 1819	28157 ± 6077	**0.045**
P30530	Axl	AXL	466 ± 254	610 ± 305	0.729
P33681	B7-1 (CD80)	CD80	795 ± 122	744 ± 321	0.888
P23560	BDNF	BDNF	246 ± 62	384 ± 153	0.435
P09038	bFGF	FGF2	1296 ± 220	854 ± 288	0.268
O43927	BLC	CXCL13	78 ± 29	76 ± 17	0.951
P12644	BMP-4	BMP4	291 ± 64	439 ± 211	0.526
P22003	BMP-5	BMP5	590 ± 77	408 ± 235	0.490
P22004	BMP-6	BMP6	482 ± 63	929 ± 427	0.341
P18075	BMP-7	BMP7	276 ± 56	316 ± 163	0.803
P01138	b-NGF	NGF	641 ± 444	257 ± 43	0.423
P35070	BTC	BTC	838 ± 496	799 ± 267	0.947
Q16619	Cardiotrophin-1	CTF1	530 ± 40	611 ±378	0.811
Q9NRJ3	CCL-28	CCL28	630 ± 541	251 ± 145	0.586
P08571	CD14	CD14	1952 ± 154	5260 ± 555	**0.001**
P55773	CK b 8-1	CCL23	363 ± 172	435 ± 152	0.765
P26441	CNTF	CNTF	164 ± 51	127 ± 44	0.608
Q9Y4X3	CTACK	CCL27	366 ± 103	122 ± 31	0.064
Q9H2A7	CXCL-16	CXCL16	631 ± 121	787 ± 184	0.507
O75509	DR6 (TNFRSF21)	TNFRSF21	283 ± 41	408 ± 222	0.546
Q06418	Dtk	TYRO3	240 ± 50	214 ± 41	0.717
P01133	EGF	EGF	772 ± 142	817 ± 205	0.863
P00533	EGF-R	EGFR	214 ± 71	379 ± 131	0.309
P42830	ENA-78	CXCL5	44663 ± 23528	9349 ± 6047	0.196
P17813	Endoglin	ENG	571 ± 108	338 ± 232	0.398
P51671	Eotaxin	CCL11	171 ± 36	80 ± 21	0.072
O00175	Eotaxin-2	CCL24	521 ± 158	1213 ± 221	**0.044**
Q9Y258	Eotaxin-3	CCL26	129 ± 39	207 ± 93	0.467
P21860	ErbB3	ERBB3	1001 ± 117	705 ± 428	0.530
P16581	E-Selectin	SELE	392 ± 84	252 ± 172	0.492
P48023	Fas Ligand	FASLG	393 ± 57	254 ± 122	0.341
Q549F0	Fas/TNFRSF6	FAF1	3542 ± 2478	3283 ± 2028	0.938
P08620	FGF-4	FGF4	7238 ± 2279	5411 ± 1856	0.557
P10767	FGF-6	FGF6	240 ± 101	288 ± 75	0.718

P21781	FGF-7	FGF7	188 ± 58	203 ± 73	0.875
P31371	FGF-9	FGF9	584 ± 126	579 ± 23	0.967
P49771	Fit-3 Ligand	FLT3LG	165 ± 34	97 ± 35	0.212
P78423	Fractalkine	CX3CL1	129 ± 18	72 ± 36	0.178
P80162	GCP-2	CXCL6	2709 ± 1182	2535 ± 1976	0.942
P09919	GCSF	CSF3	326 ± 122	434 ± 189	0.648
P39905	GDNF	GDNF	590 ± 79	823 ± 292	0.470
Q9Y5U5	GITR	TNFRSF18	3981 ± 1242	2204 ± 663	0.254
Q9UNG2	GITR-Ligand	TNFSF18	281 ± 105	343 ± 131	0.726
P04141	GM-CSF	CSF2	675 ± 107	2005 ± 1882	0.507
P09341	GRO	CXCL1	303092 ± 103372	199124 ± 49973	0.400
P09341	GROα	CXCL1	78485 ± 25344	23553 ± 14967	0.111
O15467	HCC-4	CCL16	455 ± 121	548 ± 195	0.699
P14210	HGF	HGF	36768 ± 11706	53397 ± 12016	0.360
P22362	I-309	CCL1	208 ± 77	174 ± 25	0.685
P05362	ICAM-1	ICAM1	4064 ± 1173	11417 ± 2852	0.054
P13598	ICAM-2	ICAM2	6275 ± 2610	4168 ± 1031	0.481
P32942	ICAM-3	ICAM3	335 ± 120	398 ± 153	0.758
P01343	IGF-1	IGF1	121 ± 30	322 ± 149	0.235
P08833	IGFBP-1	IGFBP1	235 ± 47	467 ± 97	0.076
P18065	IGFBP-2	IGFBP2	385 ± 69	485 ± 35	0.239
P17936	IGFBP-3	IGFBP3	602 ± 69	523 ± 153	0.653
P22692	IGFBP-4	IGFBP4	928 ± 48	1545 ± 665	0.391
P24592	IGFBP-6	IGFBP6	1318 ± 462	774 ± 333	0.376
P08069	IGF-I SR	IGF1R	153 ± 30	224 ± 104	0.535
P01344	IGF-II	IGF2	3045 ± 324	3978 ± 1184	0.476
P27930	IL-1 R II	IL1R2	7058 ± 1691	4856 ± 1553	0.375
Q01638	IL-1 RA/ST2	IL1RL1	1006 ± 272	545 ± 217	0.234
P14778	IL-1 RI	IL1R1	105 ± 24	115 ± 41	0.842
P22301	IL-10	IL10	1140 ± 491	3709 ± 3023	0.434
Q08334	IL-10 Rβ	IL10RB	409 ± 78	374 ± 254	0.900
P20809	IL-11	IL11	142 ± 61	151 ± 105	0.940
P29459	IL-12 p40	IL12A	1780 ± 869	1046 ± 210	0.443
P29460	IL-12 p70	IL12B	253 ± 44	156 ± 22	0.097
P35225	IL-13	IL13	150 ± 105	194 ± 82	0.748
Q14627	IL-13 Rα2	IL13RA2	1205 ± 444	1312 ± 1051	0.928
P40933	IL-15	IL15	99 ± 40	149 ± 133	0.695
Q14005	IL-16	IL16	236 ± 48	410 ± 127	0.208
Q16552	IL-17	IL17A	157 ± 51	648 ± 3121	0.171
Q13478	IL-18 BPα	IL18R1	377 ± 15	398 ± 297	0.934
O95256	IL-18 Rβ	IL18RAP	1168 ± 302	1024 ± 704	0.858
P01583	IL-1a	IL1A	479 ± 118	622 ± 189	0.546
P01584	IL-1b	IL1B	143 ± 29	277 ± 142	0.391
P18510	IL-1ra	IL1RN	310 ± 94	181 ± 22	0.230

P60568	IL-2	IL2	87 ± 24	39 ± 20	0.200
P31785	IL-2 Rg	IL2RG	545 ± 51	573 ± 354	0.928
P01589	IL-2 Rα	IL2RA	344 ± 53	425 ± 26	0.738
P14784	IL-2 Rβ	IL2RB	294 ± 108	436 ± 72	0.315
Q9HBE5	IL-21R	IL21R	522 ± 44	299 ± 166	0.241
P08700	IL-3	IL3	138 ± 36	55 ± 14	0.078
P05112	IL-4	IL4	259 ± 101	116 ± 5	0.286
P05113	IL-5	IL5	3714 ± 1001	2140 ± 481	0.206
Q01344	IL-5 Rα	IL5RA	624 ± 123	396 ± 264	0.463
P05231	IL-6	IL6	888441 ± 30874	605767 ± 177670	0.458
P08887	IL-6R	IL6R	9270 ± 3607	1949 ± 685	0.093
P13232	IL-7	IL7	345 ± 127	584 ± 335	0.529
P10145	IL-8	IL8	209915 ± 79553	74004 ± 33505	0.166
P15248	IL-9	IL9	434 ± 29	288 ± 158	0.400
P01579	INFg	IFNG	128 ± 24	143 ± 26	0.702
P02778	IP-10	CXCL10	2767 ± 517	1620 ± 733	0.248
O14625	I-TAC	CXCL11	201 ± 45	406 ± 177	0.306
P17676	LAP	CEBPB	979 ± 187	908 ± 410	0.880
P41159	Leptin	LEP	5657 ± 4143	39186 ± 38128	0.416
P48357	Leptin R	LEPR	461 ± 116	552 ± 336	0.784
P15018	LIF	LIF	1138 ± 356	777 ± 250	0.438
O43557	LIGHT	TNFSF14	936 ± 227	1295 ± 449	0.503
P14151	L-Selectin	SELL	331 ± 75	339 ± 180	0.967
P47992	Lymphotactin	XCL1	232 ± 83	424 ± 201	0.411
P13500	MCP-1	CCL2	291590 ± 94218	171828 ± 41996	0.290
P80075	MCP-2	CCL8	71914 ± 33617	54150 ± 32994	0.719
P80098	MCP-3	CCL7	4262 ± 2614	29015 ± 28278	0.417
Q99616	MCP-4	CCL13	210 ± 67	555 ± 364	0.387
P09603	M-CSF	CSF1	627 ± 58	726 ± 222	0.679
P07333	M-CSF R	CSF1R	558 ± 78	618 ± 336	0.869
O00626	MDC	CCL22	247 ± 115	301 ± 97	0.736
P14174	MIF	MIF	1475 ± 235	1648 ± 397	0.722
Q07325	MIG	CXCL9	256 ± 144	230 ± 105	0.888
P10147	MIP-1a	CCL3	40864 ± 9020	19817 ± 18123	0.339
P13236	MIP-1b	CCL4	64582 ± 17447	22821 ± 11863	0.095
Q16663	MIP-1d	CCL15	144 ± 62	319 ± 148	0.316
P78556	MIP-3a	CCL20	1067 ± 803	1207 ± 885	0.910
Q99731	MIP-3b	CCL19	2104 ± 925	765 ± 331	0.222
P03956	MMP-1	MMP1	4604 ± 750	5940 ± 3034	0.684
P45452	MMP-13	MMP13	627 ± 111	641 ± 365	0.971
P08254	MMP-3	MMP3	6138 ± 2079	2882 ± 497	0.178
P14780	MMP-9	MMP9	2292 ± 270	2672 ± 1774	0.839
P55773	MPIF-1	CCL23	394 ± 42	363 ± 236	0.903
P26927	MSPa	MST1	869 ± 135	662 ± 193	0.404

P02775	NAP-2	PPBP	5118 ± 1566	14235 ± 5364	0.154
P08138	NGF R	NGFR	277 ± 76	702 ± 518	0.381
P20783	NT-3	NTF3	476 ± 112	844 ± 224	0.192
P34130	NT-4	NTF4	290 ± 146	410 ± 160	0.605
P13725	Oncostatin	OSM	357 ± 82	272 ± 49	0.411
O00300	Osteoprotegerin	TNFRSF11B	829 ± 278	1502 ± 577	0.301
P55774	PARC	CCL18	129 ± 31	440 ± 208	0.265
P04085	PDGF AA	PDGFA	1500 ± 196	1443 ± 422	0.906
P16234	PDGF Rα	PDGFRA	302 ± 31	272 ± 155	0.829
P09619	PDGF Rβ	PDGFRB	178 ± 24	177 ± 81	0.991
na	PDGF-AB		605 ± 89	496 ± 250	0.663
P01127	PDGF-BB	PDGFB	353 ± 165	1278 ± 795	0.298
P16284	PECAM-1	PECAM1	218 ± 45	360 ± 187	0.486
Q07326	PIGF	PIGF	1789 ± 89	1166 ± 661	0.319
P01236	Prolactin	PRL	666 ± 102	607 ± 367	0.881
P13501	RANTES	CCL5	10758 ± 6352	38902 ± 32987	0.434
P21583	SCF	KITLG	396 ± 74	344 ± 81	0.651
P10721	SCF R	KIT	586 ± 64	590 ± 332	0.992
P48061	SDF-1	CXCL12	136 ± 36	290 ± 142	0.276
P48061	SDF-1β	CXCL12	428 ± 39	368 ± 261	0.826
P40189	sgp 130	IL6ST	928 ± 150	834 ± 193	0.714
O15389	Siglec-5	SIGLEC5	1205 ± 423	1608 ± 526	0.572
P19438	sTNFR I	TNFRSF1A	1239 ± 377	1153 ± 435	0.885
P20333	sTNFR II	TNFRSF1B	6406 ± 1612	3633 ± 1875	0.305
Q92583	TARC	CCL17	115 ± 45	182 ± 32	0.315
O15444	TECK	CCL25	235 ± 56	472 ± 279	0.438
P01135	TGFα	TGFA	521 ± 35	634 ± 348	0.758
P01137	TGFβ1	TGFB1	218 ± 21	306 ± 116	0.421
P61812	TGFβ2	TGFB2	95 ± 19	142 ± 29	0.220
P10600	TGFβ3	TGFB3	109 ± 24	119 ± 54	0.857
P40225	Thrombopoeitin	THPO	361 ± 83	397 ± 132	0.825
P35590	Tie-1	TIE1	508 ± 21	590 ± 343	0.819
Q02763	Tie-2	TEK	812 ± 64	1054 ± 720	0.706
P01033	TIMP-1	TIMP1	37154 ± 7877	21700 ± 6103	0.172
P16035	TIMP-2	TIMP2	13257 ± 2762	10736 ± 4371	0.643
Q99727	TIMP-4	TIMP4	1476 ± 635	1850 ± 750	0.669
P01375	TNFα	TNF	129 ± 14	189 ± 60	0.368
P01374	TNFβ	LTA	330 ± 103	593 ± 119	0.146
O14798	TRAIL R3	TNFRSF10C	251 ± 45	229 ± 47	0.751
Q9UBN6	TRAIL R4	TNFRSF10D	262 ± 69	533 ± 60	**0.024**
Q03405	uPAR	PLAUR	2996 ± 494	3330 ± 1237	0.811
P33151	VE-Cadherin	CDH5	379 ± 58	213 ± 140	0.276
P15692	VEGF	VEGFA	588 ± 184	951 ± 393	0.435
P35968	VEGF R2	KDR	677 ± 94	542 ± 274	0.618

P35916	VEGF R3	FLT4	1333 ± 11	1192 ± 506	0.789
O43915	VEGF-D	FIGF	392 ± 186	547 ± 24	0.441

Supplementary Table 3. Immunoreactivity in conditioned media generated from subcutaneous adipose tissue explants from patients without (ND) and with type 2 diabetes (DM2).

Swiss Prot	*Name on array*	*Symbol*	*ND (n=4)*	*DM2 (n=4)*	*P*
Q15848	Acrp30	ADIPOQ	95333 ± 41502	69382 ± 14531	0.577
P08476	Activin A	INHBA	595 ± 159	259 ± 47	0.088
O00253	AgRP	AGRP	955 ± 338	1405 ± 345	0.387
Q13740	ALCAM	ALCAM	573 ± 89	507 ± 338	0.836
P15514	Amphiregulin	AREG	276 ± 50	231 ± 78	0.645
P03950	Angiogenin	ANG	2840 ± 617	18859 ± 11868	0.226
O15123	Angiopoietin-2	ANGPT2	5448 ± 2559	6209 ± 3434	0.865
P30530	Axl	AXL	208 ± 46	376 ± 141	0.252
P33681	B7-1 (CD80)	CD80	944 ± 114	665 ± 274	0.383
P23560	BDNF	BDNF	240 ± 72	339 ± 127	0.521
P09038	bFGF	FGF2	2310 ± 988	604 ± 80	0.136
O43927	BLC	CXCL13	100 ± 25	115 ± 50	0.785
P12644	BMP-4	BMP4	239 ± 53	510 ± 177	0.194
P22003	BMP-5	BMP5	604 ± 134	475 ± 232	0.647
P22004	BMP-6	BMP6	406 ± 42	1296 ± 528	0.144
P18075	BMP-7	BMP7	320 ± 90	259 ± 124	0.702
P01138	b-NGF	NGF	254 ± 61	366 ± 69	0.270
P35070	BTC	BTC	336 ± 62	910 ± 133	**0.008**
Q16619	Cardiotrophin-1	CTF1	569 ± 41	708 ± 301	0.514
Q9NRJ3	CCL-28	CCL28	101 ± 25	345 ± 124	0.103
P08571	CD14	CD14	1591 ± 434	1163 ± 368	0.481
P55773	CK b 8-1	CCL23	351 ± 101	1068 ± 279	0.052
P26441	CNTF	CNTF	118 ± 29	196 ± 65	0.278
Q9Y4X3	CTACK	CCL27	369 ± 94	259 ± 98	0.445
Q9H2A7	CXCL-16	CXCL16	606 ± 119	650 ± 205	0.859
O75509	DR6 (TNFRSF21)	TNFRSF21	282 ± 74	405 ± 147	0.451
Q06418	Dtk	TYRO3	262 ± 52	200 ± 54	0.437
P01133	EGF	EGF	789 ± 151	729 ± 194	0.815
P00533	EGF-R	EGFR	251 ± 52	283 ± 99	0.782
P42830	ENA-78	CXCL5	57678 ± 19965	10323 ± 5692	0.060
P17813	Endoglin	ENG	569 ± 147	344 ± 230	0.442
P51671	Eotaxin	CCL11	160 ± 35	139 ± 10	0.710
O00175	Eotaxin-2	CCL24	580 ± 93	1186 ± 99	**0.004**
Q9Y258	Eotaxin-3	CCL26	161 ± 91	301 ± 91	0.318
P21860	ErbB3	ERBB3	1118 ± 225	626 ± 340	0.273
P16581	E-Selectin	SELE	449 ± 121	300 ± 118	0.409
P48023	Fas Ligand	FASLG	408 ± 71	328 ± 87	0.497
Q549F0	Fas/TNFRSF6	FAF1	3851 ± 2657	4641 ± 3041	0.851
P08620	FGF-4	FGF4	9438 ± 3044	9921 ± 4074	0.928

P10767	FGF-6	FGF6	272 ± 65	278 ± 99	0.957
P21781	FGF-7	FGF7	167 ± 27	434 ± 115	0.065
P31371	FGF-9	FGF9	745 ± 126	827 ± 121	0.660
P49771	Fit-3 Ligand	FLT3LG	121 ± 31	284 ± 152	0.331
P78423	Fractalkine	CX3CL1	85 ± 39	129 ± 38	0.452
P80162	GCP-2	CXCL6	817 ± 234	153 ± 65	**0.034**
P09919	GCSF	CSF3	407 ± 150	285 ± 126	0.556
P39905	GDNF	GDNF	801 ± 214	1011 ± 322	0.608
Q9Y5U5	GITR	TNFRSF18	2889 ± 1406	3954 ± 1619	0.637
Q9UNG2	GITR-Ligand	TNFSF18	321 ± 143	396 ± 112	0.695
P04141	GM-CSF	CSF2	13881 ± 12945	266 ± 136	0.333
P09341	GRO	CXCL1	332616 ± 98420	292569 ± 122638	0.807
P09341	GROα	CXCL1	91290 ± 35618	26283 ± 18289	0.156
O15467	HCC-4	CCL16	434 ± 94	637 ± 329	0.576
P14210	HGF	HGF	6064 ± 1808	7160 ± 3728	0.800
P22362	I-309	CCL1	370 ± 48	263 ± 49	0.168
P05362	ICAM-1	ICAM1	1689 ± 423	2435 ± 1189	0.576
P13598	ICAM-2	ICAM2	7481 ± 3073	9164 ± 4335	0.762
P32942	ICAM-3	ICAM3	325 ± 135	276 ± 85	0.768
P01343	IGF-1	IGF1	116 ± 33	159 ± 94	0.641
P08833	IGFBP-1	IGFBP1	252 ± 45	458 ± 119	0.157
P18065	IGFBP-2	IGFBP2	719 ± 399	851 ± 344	0.821
P17936	IGFBP-3	IGFBP3	492 ± 88	658 ± 126	0.320
P22692	IGFBP-4	IGFBP4	652 ± 237	554 ± 218	0.772
P24592	IGFBP-6	IGFBP6	936 ± 384	599 ± 119	0.434
P08069	IGF-I SR	IGF1R	235 ± 58	95 ± 12	0.100
P01344	IGF-II	IGF2	3388 ± 195	3482 ± 540	0.874
P27930	IL-1 R II	IL1R2	9732 ± 3008	9021 ± 2975	0.872
Q01638	IL-1 RA/ST2	IL1RL1	613 ± 118	544 ± 96	0.664
P14778	IL-1 RI	IL1R1	174 ± 43	206 ± 96	0.752
P22301	IL-10	IL10	1199 ± 378	593 ± 172	0.195
Q08334	IL-10 Rβ	IL10RB	463 ± 104	484 ± 198	0.929
P20809	IL-11	IL11	97 ± 40	47 ± 33	0.472
P29459	IL-12 p40	IL12A	936 ± 129	1575 ± 167	**0.023**
P29460	IL-12 p70	IL12B	284 ± 60	192 ± 67	0.344
P35225	IL-13	IL13	37 ± 10	201 ± 84	0.125
Q14627	IL-13 Rα2	IL13RA2	1353 ± 545	1280 ± 834	0.944
P40933	IL-15	IL15	49 ± 34	213 ± 127	0.280
Q14005	IL-16	IL16	215 ± 38	384 ± 35	**0.016**
Q16552	IL-17	IL17A	175 ± 34	530 ± 317	0.307
Q13478	IL-18 BPα	IL18R1	336 ± 45	322 ± 191	0.945
O95256	IL-18 Rβ	IL18RAP	1381 ± 439	1216 ± 388	0.788

P01583	IL-1a	IL1A	473 ± 116	771 ± 248	0.318
P01584	IL-1b	IL1B	218 ± 87	263 ± 86	0.725
P18510	IL-1ra	IL1RN	302 ± 89	380 ± 117	0.618
P60568	IL-2	IL2	221 ± 119	106 ± 25	0.398
P31785	IL-2 Rg	IL2RG	739 ± 98	596 ± 233	0.590
P01589	IL-2 Rα	IL2RA	351 ± 75	502 ± 198	0.500
P14784	IL-2 Rβ	IL2RB	301 ± 74	693 ± 142	**0.050**
Q9HBE5	IL-21R	IL21R	562 ± 118	431 ± 205	0.599
P08700	IL-3	IL3	171 ± 48	193 ± 28	0.782
P05112	IL-4	IL4	1266 ± 1107	112 ± 25	0.419
P05113	IL-5	IL5	4168 ± 788	477 ± 116	**0.004**
Q01344	IL-5 Rα	IL5RA	648 ± 142	560 ± 183	0.716
P05231	IL-6	IL6	882821 ± 284453	421784 ± 164289	0.210
P08887	IL-6R	IL6R	10350 ± 5159	3724 ± 1962	0.275
P13232	IL-7	IL7	805 ± 545	447 ± 218	0.565
P10145	IL-8	IL8	272132 ± 88962	136061 ± 75582	0.288
P15248	IL-9	IL9	462 ± 63	508 ± 232	0.856
P01579	INFg	IFNG	142 ± 43	381 ± 148	0.173
P02778	IP-10	CXCL10	2297 ± 501	1432 ± 376	0.217
O14625	I-TAC	CXCL11	243 ± 68	286 ± 74	0.680
P17676	LAP	CEBPB	1100 ± 211	701 ± 167	0.188
P41159	Leptin	LEP	6676 ± 5713	50476 ± 29321	0.193
P48357	Leptin R	LEPR	557 ± 88	401 ± 132	0.363
P15018	LIF	LIF	1047 ± 209	689 ± 102	0.175
O43557	LIGHT	TNFSF14	839 ± 159	1463 ± 555	0.321
P14151	L-Selectin	SELL	372 ± 77	415 ± 122	0.772
P47992	Lymphotactin	XCL1	231 ± 73	179 ± 82	0.659
P13500	MCP-1	CCL2	269756 ± 70361	243230 ± 88360	0.822
P80075	MCP-2	CCL8	33432 ± 13170	5828 ± 4041	0.092
P80098	MCP-3	CCL7	756 ± 516	84 ± 58	0.322
Q99616	MCP-4	CCL13	64 ± 19	282 ± 141	0.130
P09603	M-CSF	CSF1	526 ± 80	761 ± 262	0.423
P07333	M-CSF R	CSF1R	706 ± 111	562 ± 214	0.571
O00626	MDC	CCL22	272 ± 88	463 ± 182	0.383
P14174	MIF	MIF	1146 ± 130	1269 ± 387	0.774
Q07325	MIG	CXCL9	253 ± 105	94 ± 37	0.267
P10147	MIP-1a	CCL3	43463 ± 6153	6964 ± 4973	**0.004**
P13236	MIP-1b	CCL4	65757 ± 13625	19409 ± 13510	0.052
Q16663	MIP-1d	CCL15	245 ± 76	559 ± 197	0.188
P78556	MIP-3a	CCL20	334 ± 141	227 ± 127	0.614
Q99731	MIP-3b	CCL19	533 ± 107	298 ± 74	0.120
P03956	MMP-1	MMP1	5533 ± 789	4022 ± 484	0.154

P45452	MMP-13	MMP13	767 ± 146	617 ± 264	0.637
P08254	MMP-3	MMP3	11168 ± 4837	8236 ± 1689	0.588
P14780	MMP-9	MMP9	2884 ± 463	1631 ± 640	0.164
P55773	MPIF-1	CCL23	552 ± 47	439 ± 244	0.618
P26927	MSPa	MST1	829 ± 128	837 ± 140	0.968
P02775	NAP-2	PPBP	4860 ± 659	11159 ± 4175	0.187
P08138	NGF R	NGFR	346 ± 108	490 ± 322	0.686
P20783	NT-3	NTF3	572 ± 220	622 ± 55	0.857
P34130	NT-4	NTF4	372 ± 78	556 ± 101	0.201
P13725	Oncostatin	OSM	303 ± 57	369 ± 63	0.467
O00300	Osteoprotegerin	TNFRSF11B	714 ± 225	1340 ± 477	0.280
P55774	PARC	CCL18	166 ± 25	260 ± 114	0.453
P04085	PDGF AA	PDGFA	1515 ± 225	758 ± 106	**0.023**
P16234	PDGF Rα	PDGFRA	324 ± 50	342 ± 81	0.851
P09619	PDGF Rβ	PDGFRB	177 ± 26	236 ± 67	0.448
na	PDGF-AB		631 ± 56	412 ± 130	0.173
P01127	PDGF-BB	PDGFB	402 ± 124	545 ± 314	0.686
P16284	PECAM-1	PECAM1	210 ± 33	192 ± 91	0.847
Q07326	PIGF	PIGF	934 ± 246	720 ± 237	0.554
P01236	Prolactin	PRL	589 ± 83	548 ± 266	0.887
P13501	RANTES	CCL5	2321 ± 453	1922 ± 587	0.610
P21583	SCF	KITLG	405 ± 56	321 ± 46	0.291
P10721	SCF R	KIT	609 ± 90	508 ± 171	0.622
P48061	SDF-1	CXCL12	72 ± 15	165 ± 50	0.126
P48061	SDF-1β	CXCL12	478 ± 75	443 ± 254	0.882
P40189	sgp 130	IL6ST	686 ± 174	685 ± 167	0.997
O15389	Siglec-5	SIGLEC5	1129 ± 435	610 ± 162	0.306
P19438	sTNFR I	TNFRSF1A	723 ± 200	725 ± 188	0.994
P20333	sTNFR II	TNFRSF1B	2774 ± 333	1055 ± 306	**0.009**
Q92583	TARC	CCL17	107 ± 50	159 ± 41	0.483
O15444	TECK	CCL25	276 ± 58	490 ± 231	0.405
P01135	TGFα	TGFA	732 ± 168	609 ± 239	0.690
P01137	TGFβ1	TGFB1	252 ± 68	334 ± 82	0.471
P61812	TGFβ2	TGFB2	105 ± 26	192 ± 40	0.117
P10600	TGFβ3	TGFB3	106 ± 53	163 ± 42	0.435
P40225	Thrombopoeitin	THPO	338 ± 34	519 ± 76	0.071
P35590	Tie-1	TIE1	648 ± 41	597 ± 286	0.865
Q02763	Tie-2	TEK	845 ± 65	906 ± 382	0.881
P01033	TIMP-1	TIMP1	37022 ± 8654	20310 ± 8608	0.220
P16035	TIMP-2	TIMP2	9406 ± 3244	6152 ± 1949	0.423
Q99727	TIMP-4	TIMP4	1365 ± 257	1000 ± 357	0.439
P01375	TNFα	TNF	187 ± 68	244 ± 54	0.563

P01374	TNFβ	LTA	404 ± 123	929 ± 291	0.148
O14798	TRAIL R3	TNFRSF10C	157 ± 37	245 ± 105	0.454
Q9UBN6	TRAIL R4	TNFRSF10D	270 ± 51	494 ± 96	0.076
Q03405	uPAR	PLAUR	1396 ± 287	1097 ± 269	0.476
P33151	VE-Cadherin	CDH5	380 ± 69	255 ± 83	0.289
P15692	VEGF	VEGFA	492 ± 112	750 ± 224	0.344
P35968	VEGF R2	KDR	636 ± 39	461 ± 117	0.208
P35916	VEGF R3	FLT4	1497 ± 127	1115 ± 356	0.351
O43915	VEGF-D	FIGF	314 ± 62	608 ± 109	0.057

Data are expressed as mean ± standard error of the mean. *P*-values were calculated using an independent student's t-test.

Supplementary Table 4. Immunoreactivity in conditioned media generated from pericardial adipose tissue explants from patients without (ND) and with type 2 diabetes (DM2)

Swiss Prot	*Name on array*	*Symbol*	*ND (n=4)*	*DM2 (n=4)*	*P*
Q15848	Acrp30	ADIPOQ	130014 ± 10123	168694 ± 22025	0.175
P08476	Activin A	INHBA	276 ± 151	260 ± 46	0.443
O00253	AgRP	AGRP	1171 ± 269	1138 ± 345	0.800
Q13740	ALCAM	ALCAM	118 ± 77	286 ± 154	0.572
P15514	Amphiregulin	AREG	372 ± 135	687 ± 181	0.149
P03950	Angiogenin	ANG	1472 ± 447	4058 ± 1713	0.195
O15123	Angiopoietin-2	ANGPT2	5147 ± 3218	8654 ± 802	0.701
P30530	Axl	AXL	484 ± 135	654 ± 344	0.587
P33681	B7-1 (CD80)	CD80	252 ± 216	459 ± 156	0.979
P23560	BDNF	BDNF	2471 ± 776	2608 ± 621	0.898
P09038	bFGF	FGF2	425 ± 127	270 ± 43	0.621
O43927	BLC	CXCL13	531 ± 252	390 ± 108	0.994
P12644	BMP-4	BMP4	1401 ± 656	266 ± 53	0.307
P22003	BMP-5	BMP5	123 ± 102	152 ± 53	0.665
P22004	BMP-6	BMP6	143 ± 140	368 ± 11	0.480
P18075	BMP-7	BMP7	39 ± 44	202 ± 28	0.080
P01138	b-NGF	NGF	650 ± 254	410 ± 135	0.726
P35070	BTC	BTC	709 ± 91	461 ± 154	0.311
Q16619	Cardiotrophin-1	CTF1	122 ± 103	249 ± 95	0.854
Q9NRJ3	CCL-28	CCL28	396 ± 262	305 ± 25	0.746
P08571	CD14	CD14	1044 ± 713	1657 ± 262	0.944
P55773	CK b 8-1	CCL23	1769 ± 381	2119 ± 372	0.733
P26441	CNTF	CNTF	440 ± 106	184 ± 24	0.187
Q9Y4X3	CTACK	CCL27	334 ± 75	313 ± 81	0.700
Q9H2A7	CXCL-16	CXCL16	345 ± 115	389 ± 44	0.768
O75509	DR6 (TNFRSF21)	TNFRSF21	120 ± 59	235 ± 82	0.550
Q06418	Dtk	TYRO3	360 ± 61	179 ± 95	0.200
P01133	EGF	EGF	4529 ± 1113	5890 ± 1659	0.664
P00533	EGF-R	EGFR	238 ± 87	793 ± 524	0.238
P42830	ENA-78	CXCL5	33743 ± 3333	39505 ± 14325	0.761
P17813	Endoglin	ENG	108 ± 47	236 ± 105	0.388
P51671	Eotaxin	CCL11	205 ± 20	403 ± 138	0.192
O00175	Eotaxin-2	CCL24	227 ± 87	503 ± 64	0.106
Q9Y258	Eotaxin-3	CCL26	223 ± 121	149 ± 64	0.602
P21860	ErbB3	ERBB3	302 ± 72	241 ± 88	0.534
P16581	E-Selectin	SELE	360 ± 187	79 ± 43	0.094
P48023	Fas Ligand	FASLG	155 ± 63	150 ± 34	0.726
Q549F0	Fas/TNFRSF6	FAF1	1127 ± 215	1747 ± 276	0.098
P08620	FGF-4	FGF4	3646 ± 590	6395 ± 762	0.063
P10767	FGF-6	FGF6	343 ± 154	108 ± 36	0.424
P21781	FGF-7	FGF7	309 ± 53	385 ± 158	0.671

P31371	FGF-9	FGF9	586 ± 117	884 ± 171	0.347
P49771	Fit-3 Ligand	FLT3LG	505 ± 282	303 ± 113	0.562
P78423	Fractalkine	CX3CL1	345 ± 176	204 ± 96	0.532
P80162	GCP-2	CXCL6	7401 ± 4171	6232 ± 2523	0.703
P09919	GCSF	CSF3	722 ± 186	332 ± 108	0.113
P39905	GDNF	GDNF	272 ± 115	317 ± 91	0.723
Q9Y5U5	GITR	TNFRSF18	1130 ± 233	2068 ± 643	0.316
Q9UNG2	GITR-Ligand	TNFSF18	170 ± 57	142 ± 41	0.785
P04141	GM-CSF	CSF2	61819 ± 24038	1225 ± 569	**0.027**
P09341	GRO	CXCL1	336964 ± 31090	486758 ± 110849	0.267
P09341	GROα	CXCL1	170027 ± 5231	179718 ± 58710	0.907
O15467	HCC-4	CCL16	666 ± 162	941 ± 416	0.487
P14210	HGF	HGF	5942 ± 1506	14079 ± 3337	0.117
P22362	I-309	CCL1	1228 ± 441	396 ± 173	0.164
P05362	ICAM-1	ICAM1	11840 ± 2404	25776 ± 9727	0.282
P13598	ICAM-2	ICAM2	4468 ± 1893	10211 ± 5120	0.457
P32942	ICAM-3	ICAM3	227 ± 50	336 ± 188	0.460
P01343	IGF-1	IGF1	787 ± 308	425 ± 211	0.259
P08833	IGFBP-1	IGFBP1	555 ± 154	140 ± 41	0.193
P18065	IGFBP-2	IGFBP2	1313 ± 688	547 ± 174	0.461
P17936	IGFBP-3	IGFBP3	322 ± 47	251 ± 78	0.658
P22692	IGFBP-4	IGFBP4	3310 ± 511	6531 ± 4166	0.450
P24592	IGFBP-6	IGFBP6	292 ± 38	1079 ± 321	0.055
P08069	IGF-I SR	IGF1R	229 ± 82	231 ± 94	0.849
P01344	IGF-II	IGF2	993 ± 515	1510 ± 525	0.919
P27930	IL-1 R II	IL1R2	13212 ± 7813	23770 ± 10652	0.637
Q01638	IL-1 RA/ST2	IL1RL1	303 ± 72	1333 ± 714	0.202
P14778	IL-1 RI	IL1R1	225 ± 41	nd	nd
P22301	IL-10	IL10	8393 ± 4870	3325 ± 3094	0.316
Q08334	IL-10 Rβ	IL10RB	135 ± 75	208 ± 65	0.964
P20809	IL-11	IL11	390 ± 49	376 ± 142	0.896
P29459	IL-12 p40	IL12A	797 ± 247	1044 ± 194	0.977
P29460	IL-12 p70	IL12B	381 ± 118	311 ± 190	0.737
P35225	IL-13	IL13	413 ± 112	201 ± 30	0.396
Q14627	IL-13 Rα2	IL13RA2	260 ± 136	419 ± 85	0.824
P40933	IL-15	IL15	180 ± 54	166 ± 39	0.607
Q14005	IL-16	IL16	157 ± 49	1363 ± 1178	0.381
Q16552	IL-17	IL17A	278 ± 42	539 ± 266	0.331
Q13478	IL-18 BPα	IL18R1	42 ± 126	168 ± 25	0.993
O95256	IL-18 Rβ	IL18RAP	280 ± 158	351 ± 45	0.787
P01583	IL-1a	IL1A	1209 ± 621	312 ± 53	0.101
P01584	IL-1b	IL1B	1534 ± 750	Nd	nd

P18510	IL-1ra	IL1RN	811 ± 156	404 ± 228	0.280
P60568	IL-2	IL2	447 ± 168	339 ± 144	0.878
P31785	IL-2 Rg	IL2RG	222 ± 128	353 ± 134	0.906
P01589	IL-2 Rα	IL2RA	124 ± 85	247 ± 50	0.627
P14784	IL-2 Rβ	IL2RB	514 ±88	841 ± 305	0.348
Q9HBE5	IL-21R	IL21R	218 ± 29	157 ± 68	0.461
P08700	IL-3	IL3	449 ± 134	288 ± 36	0.377
P05112	IL-4	IL4	353 ± 52	373 ± 31	0.720
P05113	IL-5	IL5	22659 ± 2526	19019 ± 4834	0.386
Q01344	IL-5 Rα	IL5RA	110 ± 72	153 ± 39	0.789
P05231	IL-6	IL6	1686235 ± 15412	1327246 ± 184207	0.086
P08887	IL-6R	IL6R	1459 ± 227	2502 ± 363	0.061
P13232	IL-7	IL7	5277 ± 1764	1336 ± 436	0.200
P10145	IL-8	IL8	125902 ± 17399	154422 ± 28483	0.346
P15248	IL-9	IL9	177 ± 102	223 ± 17	0.808
P01579	INFg	IFNG	102 ± 92	357 ± 119	0.310
P02778	IP-10	CXCL10	1328 ± 434	1010 ± 196	0.530
O14625	I-TAC	CXCL11	376 ± 140	353 ± 116	0.892
P17676	LAP	CEBPB	393 ± 196	790 ± 410	0.622
P41159	Leptin	LEP	312 ± 191	25095 ± 16215	0.177
P48357	Leptin R	LEPR	151 ± 78	234 ± 101	0.858
P15018	LIF	LIF	489 ± 191	521 ± 106	0.737
O43557	LIGHT	TNFSF14	1038 ± 577	1118 ± 274	0.534
P14151	L-Selectin	SELL	192 ± 56	281 ± 104	0.615
P47992	Lymphotactin	XCL1	469 ± 91	650 ± 225	0.372
P13500	MCP-1	CCL2	189247 ± 30326	151622 ± 31512	0.279
P80075	MCP-2	CCL8	105751 ± 27042	60654 ± 36043	0.423
P80098	MCP-3	CCL7	2415 ± 837	3517 ± 2020	0.472
Q99616	MCP-4	CCL13	320 ± 128	820 ± 434	0.393
P09603	M-CSF	CSF1	578 ± 115	320 ± 114	0.091
P07333	M-CSF R	CSF1R	163 ± 80	269 ± 69	0.668
O00626	MDC	CCL22	419 ± 165	514 ± 209	0.966
P14174	MIF	MIF	2137 ± 487	3057 ± 403	0.471
Q07325	MIG	CXCL9	691 ± 239	733 ± 398	0.718
P10147	MIP-1a	CCL3	93873 ± 18907	52233 ± 18051	0.102
P13236	MIP-1b	CCL4	92230 ± 29163	99980 ± 41775	0.746
Q16663	MIP-1d	CCL15	902 ± 426	209 ± 69	0.332
P78556	MIP-3a	CCL20	1983 ± 664	2111 ± 1191	0.929
Q99731	MIP-3b	CCL19	1715 ± 1243	4313 ± 2706	0.579
P03956	MMP-1	MMP1	3437 ± 1593	5001 ± 2555	0.948
P45452	MMP-13	MMP13	325 ± 108	464 ± 110	0.780
P08254	MMP-3	MMP3	3032 ± 1843	4921 ± 2493	0.925

P14780	MMP-9	MMP9	644 ± 573	1159 ± 341	0.967
P55773	MPIF-1	CCL23	149 ± 62	270 ± 119	0.639
P26927	MSPa	MST1	1063 ± 548	551 ± 119	0.534
P02775	NAP-2	PPBP	8060 ± 3152	6685 ± 1445	0.827
P08138	NGF R	NGFR	139 ± 35	214 ± 28	0.375
P20783	NT-3	NTF3	679 ± 216	438 ± 112	0.573
P34130	NT-4	NTF4	192 ± 83	415 ± 73	0.194
P13725	Oncostatin	OSM	416 ± 89	504 ± 75	0.683
O00300	Osteoprotegerin	TNFRSF11B	518 ± 137	2067 ± 697	0.082
P55774	PARC	CCL18	690 ± 437	284 ± 120	0.185
P04085	PDGF AA	PDGFA	506 ± 154	638 ± 59	0.691
P16234	PDGF Rα	PDGFRA	143 ± 49	111 ± 43	0.566
P09619	PDGF Rβ	PDGFRB	95 ± 51	114 ± 44	0.684
na	PDGF-AB		201 ± 107	342 ± 73	0.385
P01127	PDGF-BB	PDGFB	1405 ± 443	705 ± 208	0.089
P16284	PECAM-1	PECAM1	110 ± 10	150 ± 31	0.518
Q07326	PIGF	PIGF	2447 ± 1027	1641 ± 473	0.479
P01236	Prolactin	PRL	273 ± 122	417 ± 101	0.482
P13501	RANTES	CCL5	17286 ± 8828	13157 ± 11784	0.974
P21583	SCF	KITLG	302 ± 87	294 ± 42	0.897
P10721	SCF R	KIT	318 ± 42	171 ± 60	0.169
P48061	SDF-1	CXCL12	252 ± 70	339 ± 99	0.314
P48061	SDF-1β	CXCL12	182 ± 76	143 ± 40	0.604
P40189	sgp 130	IL6ST	1094 ± 157	1422 ± 134	0.149
O15389	Siglec-5	SIGLEC5	742 ± 328	1878 ± 827	0.294
P19438	sTNFR I	TNFRSF1A	1326 ± 146	2055 ± 449	0.196
P20333	sTNFR II	TNFRSF1B	7474 ± 549	8944 ± 3008	0.681
Q92583	TARC	CCL17	490 ± 272	302 ± 88	0.477
O15444	TECK	CCL25	467 ± 109	274 ±81	0.458
P01135	TGFα	TGFA	295 ± 161	382 ± 83	0.912
P01137	TGFβ1	TGFB1	205 ± 57	262 ± 94	0.655
P61812	TGFβ2	TGFB2	329 ± 150	30 ± 3	0.285
P10600	TGFβ3	TGFB3	259 ± 118	130 ± 58	0.368
P40225	Thrombopoeitin	THPO	208 ± 74	159 ± 48	0.996
P35590	Tie-1	TIE1	222 ± 106	367 ± 105	0.764
Q02763	Tie-2	TEK	245 ± 174	416 ± 151	0.941
P01033	TIMP-1	TIMP1	20898 ± 4738	32175 ± 8635	0.503
P16035	TIMP-2	TIMP2	4575 ± 1545	11044 ± 3768	0.185
Q99727	TIMP-4	TIMP4	776 ± 303	1014 ± 364	0.874
P01375	TNFα	TNF	422 ± 222	332 ± 111	0.995
P01374	TNFβ	LTA	732 ± 330	183 ± 23	0.163
O14798	TRAIL R3	TNFRSF10C	399 ± 51	370 ± 91	0.891

Q9UBN6	TRAIL R4	TNFRSF10D	210 ± 38	503 ± 248	0.343
Q03405	uPAR	PLAUR	1938 ± 766	3118 ± 659	0.525
P33151	VE-Cadherin	CDH5	117 ± 54	203 ± 46	0.309
P15692	VEGF	VEGFA	286 ± 37	412 ± 140	0.402
P35968	VEGF R2	KDR	332 ± 137	415 ± 58	0.445
P35916	VEGF R3	FLT4	654 ± 251	779 ± 48	0.634
O43915	VEGF-D	FIGF	670 ± 97	893 ± 156	0.488

Data are expressed as mean ± standard error of the mean. *P*-values were calculated using an independent student's t-test.

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4

Omentin a novel cardioprotective adipokine

Manuscript submitted for publication

Chapter 4

Study 3

Omentin a novel cardioprotective adipokine

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Abstract

Secretory products from adipose tissue have been implicated in the development of cardiovascular dysfunction in type 2 diabetes mellitus (DM2). In humans, plasma levels of omentin, an adipokine preferentially expressed in epicardial adipose tissue, are decreased in DM2. Here, we examined whether decreased omentin-1 levels could contribute to cardiac dysfunction in DM2. We determined whether omentin-1 levels in serum and adipose tissue derived conditioned media are altered in DM2. Furthermore, we evaluated whether omentin-1 levels associated with (cardio-) metabolic parameters in males with uncomplicated DM2. Finally, we examined whether omentin-1 could reverse the induction of insulin resistance and contractile dysfunction by adipose tissue-derived factors in primary rat cardiomyocytes.

Omentin-1 is preferentially expressed and secreted by epicardial adipose tissue relative to pericardial and subcutaneous adipose tissue. DM2 significantly lowers expression and secretion by these depots. Circulating levels of omentin-1 are lower in DM2 versus controls, and positively correlated with whole body insulin sensitivity and the diastolic parameters early peak filling rate, early deceleration peak and early deceleration mean (all $P< 0.05$). Treating DM2 men with pioglitazone, which improves left ventricular diastolic function, resulted in higher omentin-1 levels. *In vitro*, incubation of cardiomyocytes with adipose tissue-derived conditioned media lowered insulin-stimulated Akt-phosphorylation and induced contractile dysfunction. These cardiosuppressive effects were prevented in the presence of physiological levels of omentin-1 in the conditioned media.

We have identified omentin as a cardioprotective adipokine, and decreases in omentin levels in DM2 could contribute to the induction of cardiovascular dysfunction.

Introduction

Cardiac contractile dysfunction and myocardial insulin resistance are common in patients with type 2 diabetes mellitus (DM2).[1,2] Recent studies indicate a role for adipocyte-derived factors in the pathogenesis of these cardiac alterations.[3,4] Specifically, conditioned media derived from either adipocytes or adipose tissue have been found to suppress insulin action and contractile function in isolated cardiomyocytes and hearts under Langendorff perfusion.[4-8] These cardiosuppressive effects of adipose tissue have been ascribed to adipokines like activin A, fatty acid binding protein 4 (FABP4), interleukins (IL) 1β and 6, and tumor necrosis factor α (TNFα).[4,6,9-12]

Omentin-1, also known as intelectin-1 or endothelian lectin HL-1, is an adipokine with decreased plasma levels in syndromes associated with insulin resistance, like obesity, DM2 and the polycystic ovary syndrome.[13-15] Plasma omentin-1 levels were found to correlate negatively with body mass index (BMI), body weight, fat mass, leptin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, fasting insulin, and positively with insulin sensitivity, adiponectin and high density lipoprotein (HDL)-cholesterol.[13-16] Accordingly, increases in circulating levels of omentin-1 have been reported in response to interventions that improve insulin sensitivity, such as weight loss and metformin treatment of women with the polycystic ovary syndrome.[16,17] *In vitro*, omentin-1 has been found to enhance insulin-mediated stimulation of Akt-phosphorylation and glucose uptake in primary human adipocytes.[18] These findings suggest that omentin-1 acts as a protective adipokine.

Omentin-1 mRNA is preferentially expressed in the stromal vascular fraction of visceral adipose tissue as compared to subcutaneous adipose tissue (SAT).[18,19] Significant, omentin-1 expression is also demonstrated in epicardial adipose tissue (EAT), a visceral fat depot located around the heart and coronary arteries.[3,19] Since EAT is not separated by a fascia from the myocardium, factors secreted from EAT may directly affect cardiac function.[3,4] Here, we examined whether omentin-1 expression and secretion in various adipose tissue depots, including EAT, pericardial (PAT), and SAT, differs between patients without (ND) or with DM2. Furthermore, we evaluated whether omentin-1 levels associated with (cardio-) metabolic

parameters in males with uncomplicated DM2. Finally, we examined the effect of omentin-1 on the induction of insulin resistance and contractile dysfunction by adipose tissue-derived factors in primary rat cardiomyocytes. Our data identify omentin-1 as a cardioprotective adipokine, and suggest that decreases in omentin-1 levels could participate in the induction of cardiac dysfunction in DM2.

Research design and methods

Patient characteristics

Adipose tissue biopsies

Biopsies from epicardial (EAT, obtained from above the right ventricle), pericardial (PAT) and intrathoracal subcutaneous adipose tissue (SAT) were collected from the same patients, i.e. males of Caucasian origin undergoing open heart surgery (coronary artery bypass or valve replacement) at the University Clinic in Duesseldorf, after written informed consent. The procedure to obtain adipose tissue samples was approved by the medical ethical committee of the Heinrich-Heine-University (Duesseldorf, Germany). Patients of other ethnic origins, diagnosed as having HIV infection, lipodystrophy or chronic coexistent inflammatory disease were excluded from participation. Participants were distributed into two groups, non-diabetes (ND) or type 2 diabetes (DM2), on the basis of the diagnosis DM2 in the status of the patient. Anthropomorphic characteristics and medication use are listed in Table 1. Biopsies were either used to generate conditioned media (CM), or snap-frozen in liquid nitrogen and stored at -80^{o}C for protein extraction.

Serum analysis

Plasma omentin-1 levels were determined in participants from the previously described PIRAMID (Pioglitazone Influence on tRiglyceride Accumulation in the Myocardium in Diabetes) study.[20] Healthy males, aged 45-65 years, with normal glucose metabolism as determined by a 75 g oral glucose tolerance test, served as control subjects [21]. Participating healthy controls and

males with uncomplicated DM2 had a BMI between 25-32 kg/m^2, and a blood pressure lower than 150/85 mm Hg. Furthermore, only DM2-patients with glycosylated hemoglobin between 6.5-8.5 % were eligible for inclusion. DM2-patients, diagnosed as having any symptoms or history of diabetes-related complications, or cardiovascular or liver disease, as well as prior use of thiazolidinediones or insulin, were excluded from participation. Exclusion criteria for healthy controls were a history or current cardiovascular disease, dyslipidemia, and the use of any prescribed medication. The clinical studies were conducted at two university medical hospitals in the Netherlands (Leiden University Medical Center, Leiden, and Vrije University Medical Center, Amsterdam), were approved by the medical ethics committee of both centers, and performed in full compliance with the Declaration of Helsinki. Eligible DM2-participants ceased their regular blood glucose lowering agents before entering a 10-week run-in period in which they were transferred to glimepiride monotheraphy and titrated until a stable dose was reached 2 weeks before randomization. Then, DM2-participants were randomized to pioglitazone (15 mg once daily, titrated to 30 mg once daily after 2 weeks) or metformin (500 mg twice daily, titrated to 1000 mg twice daily) in addition to glimepiride throughout the study. Details on cardiac function, myocardial metabolism, and PAT volume in the study groups have been described elsewhere.[20] Fasting blood samples for determination of omentin-1 levels were collected before randomization and in the case of DM2-participants after 24 weeks of pioglitazone or metformin therapy.

Generation of conditioned media (CM)

Conditioned media (CM) were generated as described previously.[4,22,23] Briefly, freshly collected adipose tissue biopsies were washed 3 times with PBS, supplemented with antibiotic-antimycotic (Invitrogen, Carlsbad, CA, USA) at 37°C, and cut into small 10 mg pieces. Subsequently, the explants were washed 3 times with PBS, centrifuged for 1 min at 1200 rpm at room temperature, and cultured overnight in adipocyte medium (AM) (containing DMEM F12 containing 10 % fetal calf serum, 33 µmol/l biotin, 17 µmol/l panthothenate (all from Invitrogen, Carlsbad, CA, USA), and antibiotic-antimycotic, in a humidified atmosphere (95 % air and 5 % CO_2) at 37°C. CM was generated by subsequent 24 h culture of the explants in serum-free AM

(100 mg explants/ml), whereafter the CM was collected and stored as aliquots at -80°C until further use.

Omentin-1 levels

Omentin-1 levels in human plasma and CM were determined using an omentin-1 Elisa (USCN Life Science Inc, Cologne, Germany). For protein expression, biopsies were homogenized in 50 mM Tris.HCl [pH 7.5], 150 mM NaCl, 0.5 % Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 2 mM $MgCl_2$, 1 mM DTT, and protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany). Then, homogenates were cleared by centrifugation (15 min; 12.000 rpm; 4°C), and protein content was determined using Bradford reagent (Biorad Laboratories, München, Germany). Protein expression was determined by Western blot analysis of ten microgram of protein using omentin-1 antibody (R&D systems). Immunoblots were quantified using a LUMI Imager system (Roche Diagnostics, Mannheim, Germany), and normalized by reprobing the stripped filters with GAPDH antibody (Abcam plc, Cambridge, UK).

Animal experiments

Abdominal adipose tissue for the generation of CM was collected from female guinea pigs (Crl:HA, Dunkin Hartley) purchased from Charles River (Sulzfeld, Germany), housed under standard conditions at a temperature of 18-20°C and a day-night rhythm of 12 h, and fed a high-fat diet (HFD, Altromin, Lage, Germany) for 30 weeks as described.[4] Animal experiments were performed in accordance with the "Principle of laboratory animal care" (NIH publication No. 85-23, revised 1996) and the current version of the German Law on the protection of animals.

Preparation of adult rat cardiomyocytes

Primary adult cardiomyocytes were prepared from male Lewis rats (LEW/Crl, Charles River, Sulzfeld, Germany) weighing 250-350 gram using a Langendorff perfusion system as described.[24] Briefly, rats were sacrificed following anaesthesia with ketamine (100mg/kg) (Ratiopharm, Ulm, Germany) and xylazine (Rompun, 5 mg/kg) (Bayer Healthcare, Leverkusen, Germany). The

anaesthetic further contained heparin (666 µl/kg) (Biochrom AG, Berlin, Germany). Isolated hearts were retrograde perfused through the aorta for 5 min with Ca^{2+}-free Krebs-Ringer bicarbonate buffer (KRBB) (composition: NaCl, 35 mM; KCl, 4.75 mM; KH_2PO_4, 1.19 mM; Na_2HPO_4, 16 mM; $NaHCO_3$; 25 mM; sucrose, 134 mM; Hepes, 10 mM; glucose, 10 mM) gassed with 95 % O_2 and 5 % CO_2. After the addition of 0.1 mmol/L $CaCl_2$, the isolated heart was perfused for 5 min with Krebs-Ringer bicarbonate buffer (KRBB), followed by KRBB, containing 1.25 g/l bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), 0.7 g/l collagenase (Worthington, Lakewood, NJ, USA) and 0.1 g/l hyaluronidase (Applichem, Darmstadt, Germany). Perfusion medium was gassed with O_2. After 20 min, the softened heart was minced and incubated for 5 min at 37°. Then, the dispersion was filtered through a nylon mesh, and centrifuged for 5 min at 500 rpm. After centrifugation, cell pellet was washed with HEPES-buffer (composition: NaCl, 130 mM; KCl, 4.7mM; KH_2PO_4, 1.2 mM; Hepes, 25 mM; glucose, 5 mM, equilibrated with O_2), containing 3 g/l bovine serum albumin (Carl Roth GmbH, Karlsruhe, Germany). Subsequently, cells were incubated for 7 min at 37°C in HEPES-buffer containing 0.059 units/ml trypsin and treated as described.[24] Isolated cells were seeded on laminin-coated dishes ($1x10^5$ cells per 35 mm plate) in Medium 199 with Hank's salts, supplemented with ITS (insulin, transferrin, selenium), 100 U/ml penicillin, 100 mg/ml streptomycin and 5 % fetal calf serum (all from PAA laboratories, Pasching, Austria) on laminin-coated 35 mm culture dishes (for signaling experiments: Greiner Bio-One GmbH, Solingen, Germany; for fluorescence analysis: ibidi GmbH, Martinsried, Germany). The medium was renewed after 4 h, and culture was continued overnight.

Analysis of contractile function

Cardiomyocytes were cultured overnight on laminin-coated dishes before exposure to CM from guinea pig abdominal adipose tissue to induce cardiomyocyte dysfunction. For analysis of the effects on contractile function, cells were preloaded with Fura-2-AM (Merck chemicals, Darmstadt, Germany) for 25 min at room temperature, washed twice with AM and then incubated for 30 min with CM or AM in the presence or absence of 300 ng/ml recombinant human omentin (Cell systems GmbH, Germany; CS-C1212). Subsequently, sarcomere shortening

and Ca^{2+}-transients were analyzed. Before measurement was started, cells were electrically pre-stimulated for 5 min with 1 Hz to reach a steady-state level for sarcomere shortening and Fura-2 fluorescence. Then, cells were paced with bipolar pulses of 5 ms duration at 1 Hz. The cytosolic Ca^{2+}-concentration was monitored as a ratio of the fluorescence emission peaks at 340 and 380 nm. In each experimental condition, data files were recorded of 10 consecutive beats for at least 8 different cells. Sarcomere shortening and Ca^{2+}-transients were calculated using IonWizard (IonOptix).

Analysis of insulin signaling

Cultured cardiomyocytes were incubated for 24 h with CM (diluted 1:2 with AM) or AM in the presence or absence of omentin. Then, cells were stimulated for 10 min with insulin (100 nM), washed twice with ice-cold PBS and lysed for 2 h at 4°C in Triton X-100 lysis buffer, containing 50 mM Tris.HCl [pH 7.5]; 150 mM NaCl; 0.5 % Triton X-100; 1 mM NaF; 1 mM Na_3VO_4; 2 mM $MgCl_2$, 1 mM DTT; and protease inhibitors (Complete, Roche Diagnostics, Mannheim, Germany) under gentle rotation. Lysates were cleared by centrifugation (15 min; 12.000 rpm; 4^oC), and protein content was determined using Bradford reagent (Biorad Laboratories, Muenchen, Germany). Ten microgram of protein was loaded onto 10 % SDS-Page gels, and transferred to polyvinylidene difluoride (PVDF) membranes. After blotting, membranes were blocked with Tris-buffered saline (TBS), containing 0.1 % Tween 20 and 5 % non fat dry milk for 2 h at room temperature and then incubated overnight at 4^oC with antibodies recognizing Akt phosphorylated at Ser473 (Cell Signalling Technology, Danvers, MA, USA). Immunoblots were quantified using a LUMI Imager system, and normalized by reprobing the stripped filters with α-tubulin antibody (Calbiochem, Darmstadt, Germany).

Statistical analysis

Data are presented as mean ± standard error of the mean. Significant differences between experimental conditions were evaluated by one-way ANOVA or unpaired Student´s *t* test using SPSS 17.0 (SPPS Inc, Chicago, IL). A value of $P< 0.05$ was considered as statistically significant.

Results

Expression and secretion of omentin-1 in intrathoracal adipose tissue depots

Omentin-1 protein expression was determined in biopsies from EAT, PAT and SAT from males of Caucasian origin undergoing open heart surgery. In patients without DM2, omentin-1 expression in EAT was higher when compared to PAT and SAT, respectively (Figure 1A/B). Omentin-1 expression in EAT and SAT from patients with DM2 were markedly reduced. The decrease in omentin-1 expression in PAT from patients with DM2 failed to reach significance (*P*=0.26). Subsequently, we examined whether the alterations in omentin-1 expression are reflected by changes in omentin-1 secretion. To assess the release of omentin-1 from adipose tissue, CM generated from paired EAT, PAT and SAT biopsies were analyzed using Elisa. In line with the expression data, omentin-1 levels in CM generated from patients without DM2 were highest in EAT (310±38 ng/ml) as compared to PAT and SAT, and were markedly decreased in CM generated from patients with DM2 (Figure 1C).

Table 1. Characteristics of patients from which adipose tissue biopsies were collected

	Control	**Type 2 diabetes**	***p*-value**
Anthropomorphic parameters:			
n	12	13	
Age (years)	69.6±2.1	69.4±2.9	0.957
BMI (kg/m2)	28.9±1.2	31.9±1.8	0.186
Fasting plasma glucose (mg/dl)	98.5±3.6	164±13.6	0.001
Medication use:			
statins	3	9	
diuretics	4	6	
anti-hypertension	5	9	

Omentin-1 plasma levels in DM2 and controls

We determined plasma omentin-1 levels in 14 healthy control males and 78 males with uncomplicated DM2. The anthropomorphic and cardiometabolic parameters of the participants have been described previously.[20, 21] Briefly, as summarized in Table 2, DM2-patients had a

slightly higher BMI, increased systolic blood pressure, visceral fat and SAT volumes, as well as elevated HbA1c and fasting plasma glucose and insulin levels (all *P*< 0.05). Furthermore, DM2-patients versus controls had impaired left ventricular diastolic function, altered myocardial substrate metabolism, as illustrated by reduced myocardial glucose uptake and increased fatty acid uptake and oxidation, and a lower M-value for insulin sensitivity (all *P*< 0.05) (Table 2). As compared to controls, plasma omentin-1 levels were lower in patients with DM2 (313 vs. 426 ng/ml; *P*=0.0078) (Table 2).

Table 2. Correlations between plasma omentin-1 levels and anthropomorphic, hemodynamic and plasma parameters.

	all subjects (n=92)	***controls (n=14)***	***DM2 (n=78)***
Age, years	-0.025	-0.061	0.005
BMI, kg/m^2	-0.219*	-0.069	-0.184
Systolic blood pressure, mm Hg	-0.130	0.709**	-0.108
Diastolic blood pressure, mm Hg	-0.037	0.476	-0.113
Heart rate, beats per min	-0.221*	-0.210	-0.153
Rate pressure product, (beats/min).mm Hg	-0.243*	0.117	-0.184
Fasting plasma glucose, mmol/l	-0.227*	0.059	-0.090
Fasting insulin, pmol/l	-0.378***	0.117	-0.379***
HbA1c, %	-0.259*	-0.589#	-0.146
Total cholesterol, mmol/l	0.235*	0.703*	0.152
HDL-cholesterol, mmol/l	0.280**	0.187	0.230*
Triglycerides, mmol/l	-0.173	0.474	-0.131
Free fatty acids, mmol/l	-0.050	-0.345	0.015
M-value, mg/kg.min	0.379***	0.502	0.322**
Visceral fat volume, ml	-0.269**	-0.363	-0.205#
Subcutaneous fat volume, ml	-0.176#	0.037	-0.149
Pericardial fat volume, ml	-0.180#	-0.288	-0.138

Data are Pearson's r. In case of non-Gaussian distributions, parameters were log-transformed for correlation analysis. BMI, body mass index; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein. #, *P*< 0.1; *, *P*< 0.05; **, *P*< 0.01; ***, *P*< 0.001.

Regression analysis identified positive associations between omentin-1 levels and the diastolic parameters early peak filling rate, early deceleration peak and early deceleration mean, M-value for insulin sensitivity, adiponectin and HDL-cholesterol (Table 2/3, Figure 2). Significant negative correlations were observed with BMI, fasting plasma glucose, insulin and Hb1Ac, visceral fat volume, heart rate, and heart rate pressure product (Table 2/3, Figure 2). Treating DM2-patients

with metformin and pioglitazone led to similar improvement of glycemic control, but only pioglitazone treatment resulted in improved left ventricular diastolic function.[20] Figure 2E shows that plasma omentin-1 levels were increased in DM2-patients following pioglitazone, but not metformin, treatment ($P< 0.0001$).

Table 3. Correlations between plasma omentin-1 levels and cardiometabolic parameters.

	all subjects (n=92)	***controls (n=14)***	***DM2 (n=78)***
LV mass, gram	-0.071	0.164	-0.147
LV mass/volume ratio, g/ml	-0.167	0.181	-0.144
LV end diastolic volume, ml	0.076	0.007	-0.009
LV end systolic volume, ml	0.098	-0.053	0.019
Stroke volume, ml	0.039	0.104	-0.024
Ejection fraction, %	-0.074	0.179	-0.032
Cardiac output, l/min	-0.098	0.020	-0.125
Pulse wave velocity, m/s	0.031	0.067	0.060
Aorta ascendens distensibility, 10^{-3}mm Hg^{-1}	0.175#	0.006	0.132
E acceleration peak, $ml/s^2.10^{-3}$	0.148	0.316	0.122
E acceleration mean, $ml/s^2.10^{-3}$	0.146	0.207	0.114
E peak flow rate, ml/s	0.246*	0.325	0.182
E deceleration peak, $ml/s^2.10^{-3}$	0.221*	0.232	0.185
E deceleration mean, $ml/s^2.10^{-3}$	-0.218*	-0.278	-0.179
E/A peak ratio	0.139	0.419	0.040
E/A volume ratio	0.087	0.323	0.007
MMRGlu, nmol/ml/min	0.142	-0.106	0.100
Myocardial TG content, %	-0.156	-0.439	-0.087

Data are Pearson's r. In case of non-Gaussian distributions, parameters were log-transformed for correlation analysis. LV, left ventricular. #, $P< 0.1$; *, $P< 0.05$.

Effect of omentin on contractile function in primary adult rat cardiomyocytes

The above findings suggest that decreases in omentin-1 levels could participate in the induction of cardiac dysfunction in DM2. This raises the possibility that omentin-1 could prevent the induction of cardiomyocyte dysfunction by adipose tissue-derived CM. Figure 3 shows that exposure of primary adult rat cardiomyocytes to CM reduced peak sarcomere shortening and the departure and return velocity of contraction in the absence of morphological changes (Figure 3A-C). Omentin-1 alone had no effect on cardiomyocyte contractile function, but completely prevented the cardiodepressant activity induced by CM (Figure 3A-C). Also, the CM-induced abrogation in cytosolic Ca^{2+}-transients, as illustrated by reductions in departure and

return velocities and a lower peak Fura-2 fluorescence signal, were not observed when omentin-1 was present in the CM (Figure 3D-E).

Effect of omentin-1 on insulin action in primary adult rat cardiomyocytes

Finally, we determined the effect of recombinant omentin-1 in the presence and absence of CM on insulin-induced Akt-Ser473-phosphorylation. Omentin-1 alone enhanced insulin-stimulated Akt-phosphorylation in primary adult rat cardiomyocytes (Figure 4). In contrast, CM generated from abdominal adipose tissue from HFD-fed guinea pigs had an inhibitory effect on insulin-stimulated Akt-phosphorylation, but this inhibitory effect was not observed when omentin-1 was present in the CM (Figure 4).

Discussion

The key finding of this study is that omentin-1 plasma levels associate with left ventricular diastolic function. In DM2-patients, plasma omentin-1 levels were decreased versus controls and increased following treatment with pioglitazone, which was found to improve left ventricular diastolic function.[20] Furthermore, we show that omentin-1 is preferentially expressed and secreted by EAT as compared to PAT and SAT. Finally, in cardiomyocytes, the presence of omentin-1 prevented the induction of contractile dysfunction and insulin resistance by adipose tissue-derived factors. Collectively, these findings provide evidence for a cardioprotective function of omentin-1, and suggest that decreases in omentin-1 levels may contribute to the pathogenesis of cardiac dysfunction in DM2.

In line with previous reports, we confirmed that plasma omentin-1 levels are lower in patients with DM2 versus controls, as well as positive associations with insulin sensitivity, adiponectin, and HDL-cholesterol, and negative associations with obesity indices like BMI, visceral fat volume, fasting plasma insulin and glucose levels.[13-16,25,26] Plasma omentin-1 levels have also been linked to endothelial function in males with normal and impaired glucose tolerance.[26] In our study cohort, we observed a weak tendency that plasma omentin-1 levels

could associate with aorta distensibility, but not with pulse wave velocity. Furthermore, both pioglitazone and metformin were found to improve glycemic control in our study population.[20] It has been demonstrated that omentin-1 levels are increased following a 6–month metformin intervention in German women with the polycystic ovary syndrome.[17] Also a study on Chinese DM2-patients with poor glycemic control reported increases in omentin-1 levels following intervention with metformin together with liraglutide.[27] In contrast to these studies, metformin had no effect on omentin-1 levels in our study population consisting of males with well-controlled DM2. Because of the differences among the study cohorts with respect to gender, disease status, ethnicity, and the lack of a metformin only group in the Chinese study, further studies are clearly required to clarify why metformin did not affect plasma omentin-1 levels in our study.

A major finding in the present study is the association of plasma omentin-1 levels with left ventricular diastolic function. Because of the strong positive association with adiponectin levels, it has been suggested that omentin-1 levels may be regulated by adiponectin.[25] Like omentin, adiponectin was found to positively associate with insulin sensitivity and HDL-cholesterol and negatively with BMI, and waist circumference, but not with parameters of left ventricular diastolic function (Chen et al., submitted for publication). Although this does not exclude a role for adiponectin in the regulation of omentin-1, our findings suggest a direct cardioprotective effect of omentin-1 rather than adiponectin.

In vitro, studies have indeed ascribed a signaling function to omentin-1 because of its ability to promote Akt-phosphorylation in isolated rat blood vessels, vascular smooth muscle cells, and human microvascular endothelial cells, and to enhance insulin-mediated Akt-phosphorylation and glucose uptake in adipocytes. [17,18,28,29] Furthermore, omentin-1 has an anti-inflammatory action as illustrated by its ability to reduce c-reactive protein (CRP)- and vascular endothelial growth factor (VEGF)-induced migration, angiogenesis, and CRP- and TNF-α induced nuclear factor kappa B (NF-kB) activation in human microvascular endothelial cells.[17] Therefore, to obtain insight how omentin affects cardiac function, we studied omentin-1 action in primary adult rat cardiomyocytes. Interestingly, recombinant omentin-1 alone had no effect on sarcomere shortening, cytosolic Ca^{2+}-fluxes and basal Akt-phosphorylation in cardiomyocytes. Rather, omentin was found to slightly enhance insulin-mediated Akt-

phosphorylation and to protect against the induction of cardiomyocyte contractile dysfunction and insulin resistance by adipose tissue-derived factors. These observations suggest that omentin could exert its cardioprotective effects by acting as a scavenger for detrimental (pro-inflammatory) factors secreted by adipose tissue.

Like most organs in the body, the human heart is surrounded by adipose tissue. Epicardial adipose tissue (EAT) is a visceral depot located inside the pericardium, and factors secreted from this fat depot can directly affect the function of the underlying myocardium because of the absence of fascial boundaries.[3] In line with gene expression studies, omentin-1 protein expression and secretion was higher in EAT relative to PAT and SAT from the same patients.[19] Furthermore, omentin-1 expression and secretion in EAT was markedly reduced in DM2. A lower omentin-1 content could also be demonstrated in CM from EAT of HFD-fed versus standard diet-fed guinea pigs (data nor shown). Recently, we have found that the secretory products of EAT from patients with DM2 versus controls (Greulich et al., submitted for publication) and HFD-fed guinea pigs induce cardiomyocyte contractile dysfunction and insulin resistance.[4] These detrimental effects could be ascribed in part to accumulation of activin A in CM from EAT. Here we extent these findings in that a loss of a protective action of omentin-1 could also contribute to the detrimental effects of these CM on cardiomyocyte function.

Although our data strongly suggest a cardioprotective function of omentin-1, several issues remain to be addressed. For example, like adiponectin, omentin-1 is synthesized in a trimeric form.[30] The immunoassays used in this study recognize both forms, and the *in vitro* data are based on the monomeric form. Because of the current unavailability of appropriate commercial reagents, it remains unclear whether the monomeric form has the same biological activity as the trimeric form, and which form mediates the effects of omentin-1 *in vivo*. In addition, it is unclear what underlies the decrease in omentin-1 synthesis in DM2. In adipose tissue, omentin-1 synthesis has been ascribed to the stromal vascular fraction.[18] This fraction contains the wide variety of non-adipose cells in adipose tissue, including pre-adipocytes, endothelial cells, stem cells, fibroblasts, and immune cells. Adipose tissue in DM2 is characterized by infiltration of immune cells, which is paralleled by an enhanced secretion of pro-inflammatory adipokines. These factors have been implicated various toxic responses, like induction of apoptosis and endoplasmic reticulum (ER)-stress, which results in inhibition of the

synthesis of abundantly expressed proteins. Given the abundant amounts of omentin-1 in the circulation, it is plausible that omentin-1 synthesis is affected by ER-stress. However, because of the complex cellular composition of adipose tissue, it may be preferable to specify the cell type(s) in which omentin-1 is produced first, before studying a contribution of stress responses on omentin-1 synthesis.

In conclusion, the present study indicates that omentin-1 levels in plasma and EAT are decreased in plasma level in DM2-patients. Furthermore, the positive association of omentin-1 levels with left ventricular diastolic function and the *in vitro* experiments in isolated rat cardiomyocytes suggest that omentin-1 has a cardioprotective function. Based on these findings, we suggest that a lack of omentin-1 expression in EAT might contribute to the induction of cardiac dysfunction in DM2-patients.

Acknowledgements

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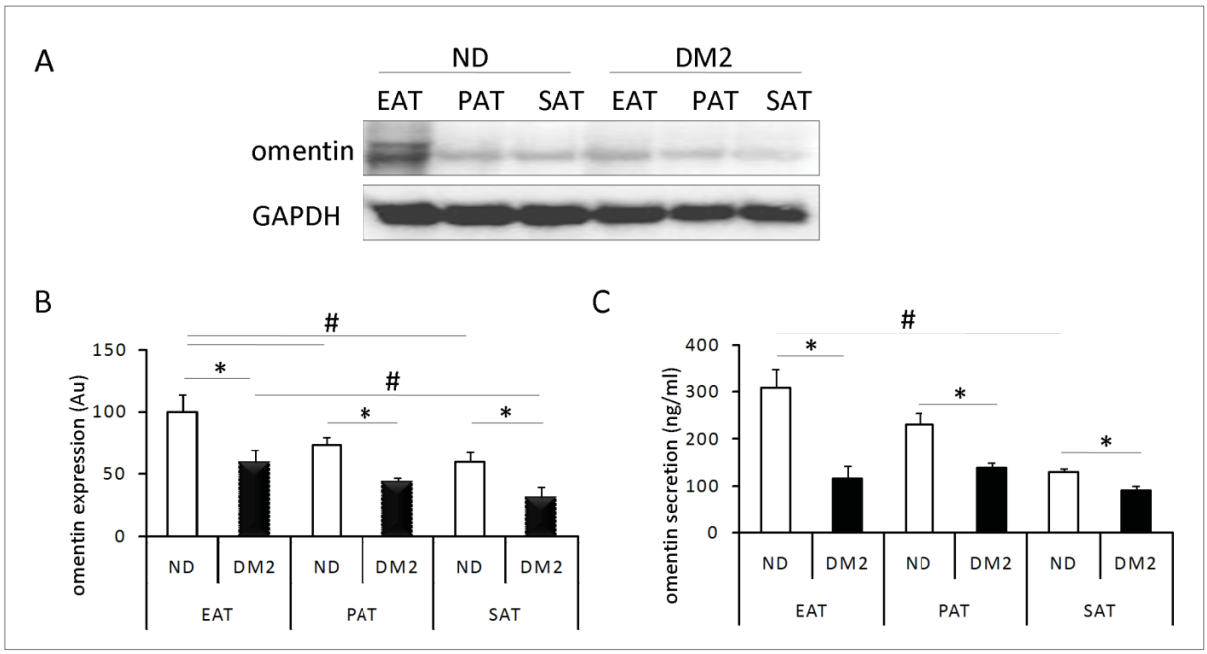
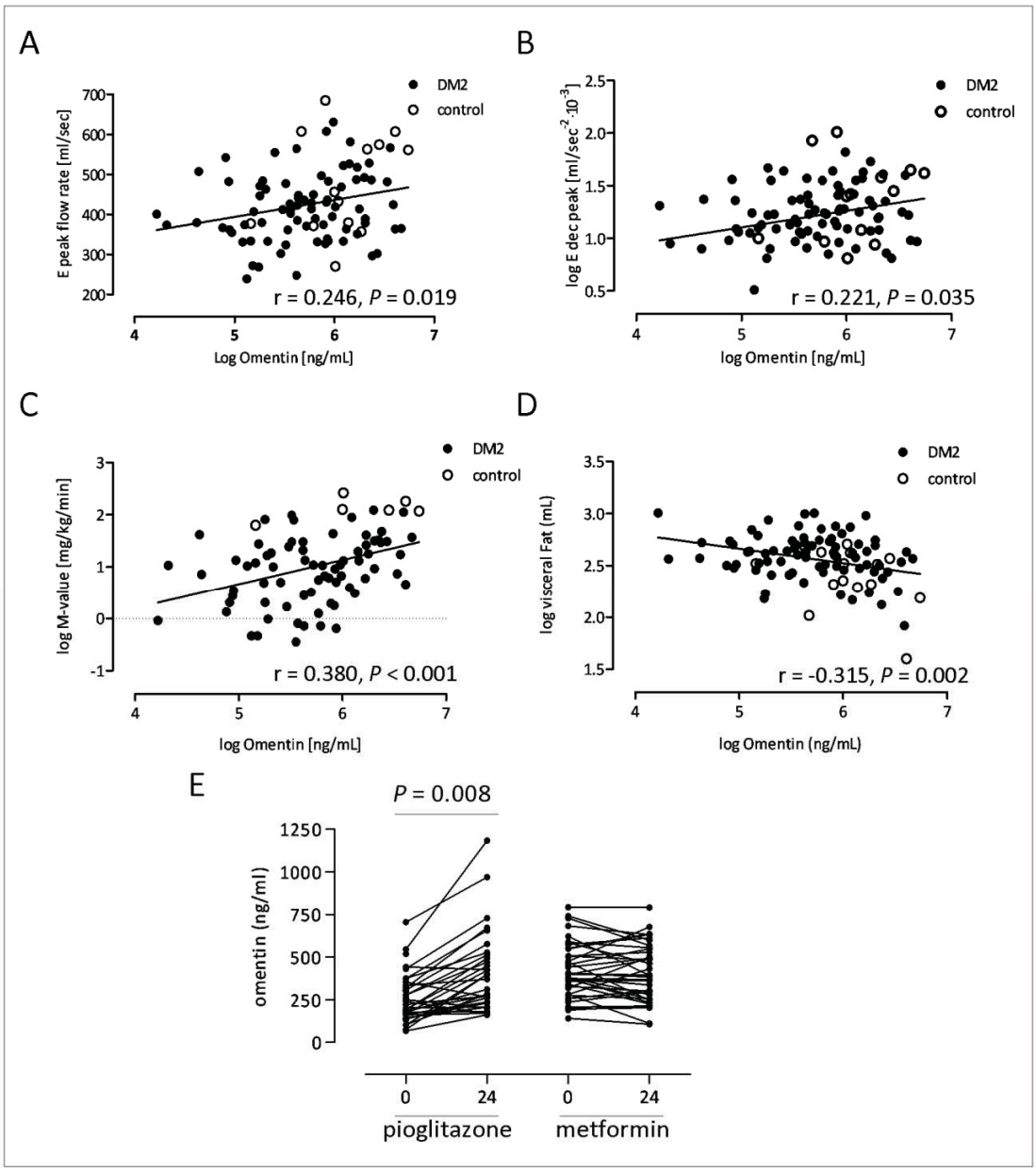
Figure 1. Expression and secretion of omentin in intrathoracal adipose tissue depots.



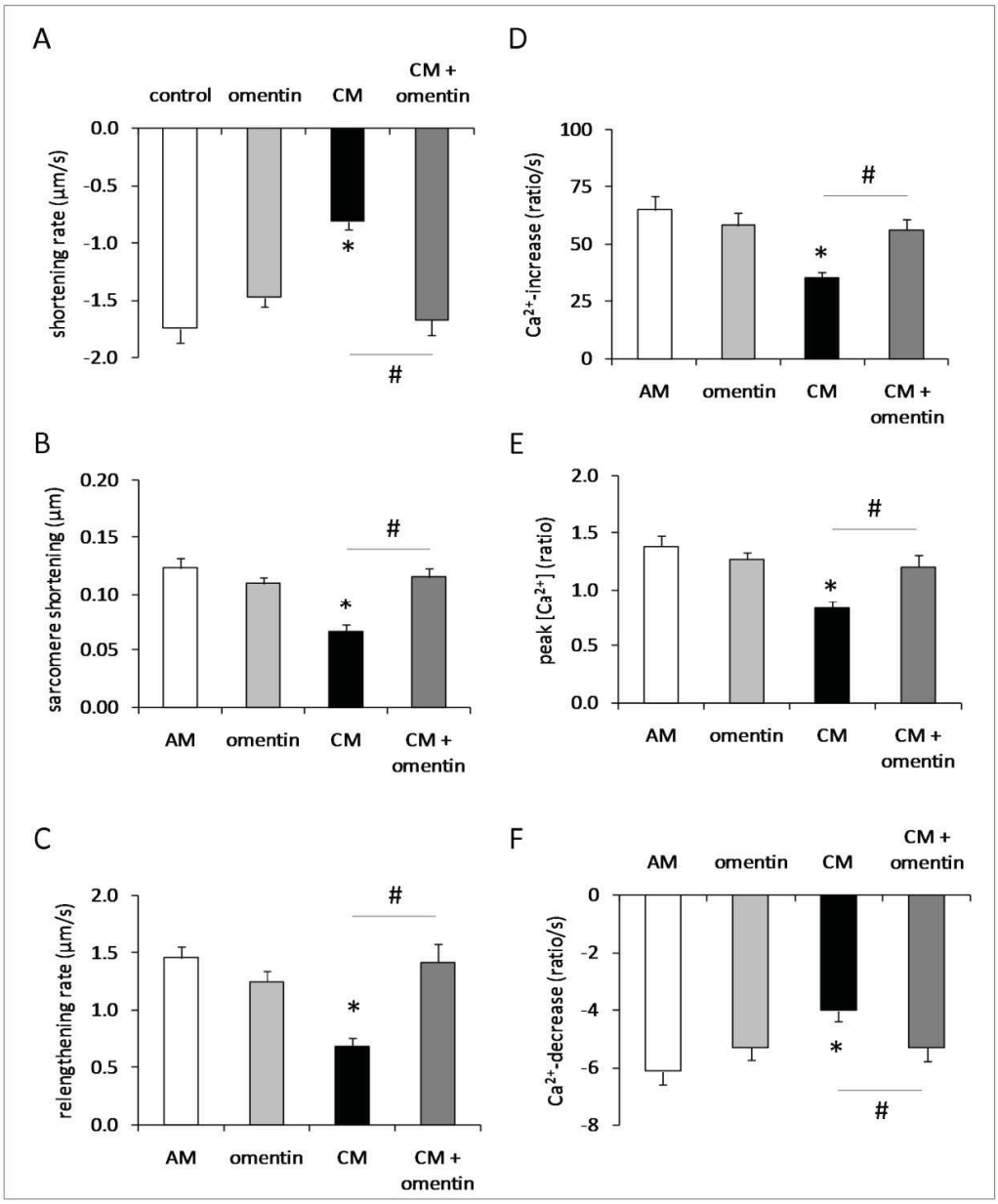
Representative Western blot (A) and quantification (B) of omentin expression in paired epicardial (EAT), pericardial (PAT), and subcutaneous (SAT) adipose tissue biopsies of patients with (DM2, n=7) and without (ND, n=6) type 2 diabetes. Equal loading was verified by probing the immunoblots with GAPDH-antibody. Data are expressed as mean ± standard error of the mean. (C) Quantification of omentin levels in conditioned media generated from paired EAT, PAT and SAT explants from patients with and without DM2. Data are expressed as mean ± SEM (n=6 patients per group). * indicates a $P<0.05$ for differences between ND and DM2 (independent *t*-test); # indicates $P< 0.05$ for differences between the various fat depots (paired *t*-test).

Figure 2. Plasma omentin-1 levels in males with uncomplicated type 2 diabetes.



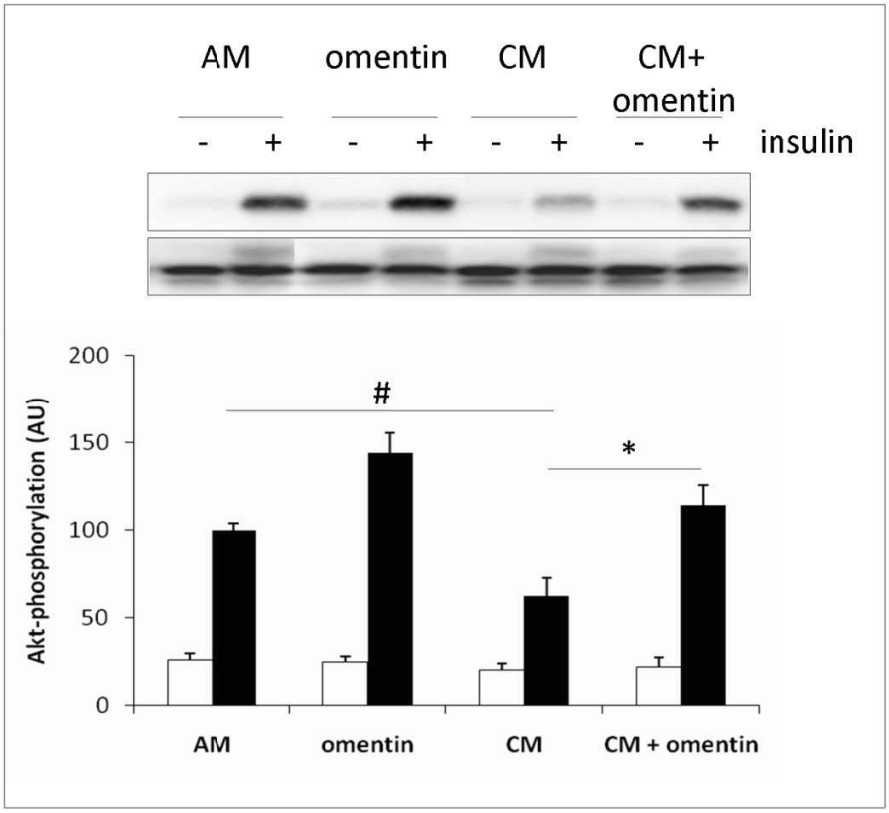
Plasma omentin-1 levels, fat distribution, insulin sensitivity and diastolic parameters were determined in participants from healthy controls and patients with type 2 diabetes (DM2). Regression analysis identified significant correlations between baseline omentin-1 plasma levels and E peak flow rate (A), early deceleration peak (B), M-value (C), and visceral fat mass (D). (E) Plasma omentin levels before (0) and after 24 weeks of treating males with uncomplicated DM2 with metformin or pioglitazone. *P*-values for treatment-effects were calculated using a paired *t*-test. *, *P*< 0.05 versus healthy control

Figure 3. Effect of recombinant omentin-1 on sarcomere shortening and calcium fluxes in primary adult rat cardiomyocytes.



Primary rat cardiomyocytes were incubated with control adipocyte medium (AM) or conditioned media (CM) for 30 min before analysis of contractile function in the absence or presence of 300 ng/ml recombinant omentin-1. Effect of exposure of cardiomyocytes to CM and omentin on departure velocity of contraction (A), peak sarcomere shortening (B), return velocity of contraction (C), departure velocity of cytosolic [Ca^{2+}] (D), peak fura-2 fluorescence (E) and departure velocity of cytosolic [Ca^{2+}] (F). Data are expressed as mean ± standard error of the mean of at least 3 independent experiments in which at least 10 cardiomyocytes were analyzed. Differences between the experimental groups were calculated by one-way ANOVA. *, $P< 0.05$ versus AM; #, $P< 0.05$ versus omentin.

Figure 4. Effect of recombinant omentin-1 on insulin action in primary adult rat cardiomyocytes.



Western blot (A) and quantification (B) of recombinant omentin-1 on insulin action. Lysates from primary adult rat cardiomyocytes exposed for 24 h to control adipocytes medium (AM) or recombinant omentin-1 (100 ng/ml) in the absence or presence of conditioned media (CM), generated from abdominal adipose tissue from HFD-fed guinea pigs, were analyzed for insulin-induced Akt-Ser473-phosphorylation. Data are presented as mean ± S.E.M. (n=4 per group). Open bars, basal; filled bars, insulin-stimulated cells (10 min; 100 nM). *, $P< 0.05$ versus AM.

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5

Discussion

Chapter 5

General Discussion

Multiple epidemiological studies have demonstrated that expansion of EAT represents a risk factor for the development of cardiovascular dysfunction in obesity, DM2, and the metabolic syndrome. It is generally accepted that expansion of adipose tissue in obesity is closely linked to the development of a state of low-grade inflammation. In this condition, immune cells infiltrate into the adipose tissue, thereby altering the adipokine secretory profile to a pro-inflammatory one. Using the explant technology to generate conditioned media from adipose tissue biopsies, we could demonstrate for the first time that secreted products from EAT affect cardiomyocyte function. Specifically, conditioned media generated from EAT of high-fat diet fed guinea pigs [chapter 2] and patients with DM2 [chapter 3] were found to induce insulin resistance and reduce sarcomere shortening and cytosolic Ca^{2+}-fluxes in primary adult rat cardiomyocytes. Protein profiling of the conditioned media showed that the detrimental effects of EAT on cardiomyocyte function could at least in part be ascribed to accumulation of activin A and reductions in omentin content. Collectively, these findings show that alterations in the secretory profile of EAT can contribute to the development of cardiac dysfunction in DM2.

5.1 Crosstalk between epicardial adipose tissue and the heart

5.1.1 Effects on cardiomyocyte contractile function

A key finding in the present study is that secretory products from EAT of high-fat diet fed guinea pigs and DM2-patients abrogate sarcomere shortening and intracellular Ca^{2+}-fluxes in primary adult rat cardiomyocytes. This observation extends a previous study, in which has been demonstrated that conditioned media from EAT of patients with coronary artery disease show an enhanced potential to induce atherogenic changes in monocytes and endothelial cells.[182]

The detrimental effect of conditioned media from EAT of either high-fat diet fed guinea pigs or DM2-patients on sarcomere shortening and intracellular Ca^{2+}-fluxes in rat cardiomyocytes was dose-dependent, and occurred within minutes after the addition of the conditioned media. Furthermore, the effect could be reversed by replacing conditioned media by control media. However, the mechanism(s) underlying this rapid response are largely unknown. In this respect, it may be interesting to assess changes in the activity of the SERCA2a-phosholamban complex. Serca2a resides at the sarcolemmal membrane, and transfers Ca^{2+} from the cytosol to the lumen of the sarcoplasmic reticulum during relaxation.[228] The activity of SERCA2a is amongst others regulated by phospholamban. When bound to SERCA2a, phospholamban inhibits the activity of SERCA2a.[229] However, upon phosphorylation of phospholamban, the complex dissociates, leading to enhanced activity of SERCA2a. Generally, changes in the phosphorylation state are rapidly induced in response to extracellular stimuli. It may therefore be worthwhile to assess whether exposure of rat cardiomyocytes to conditioned media from EAT induces changes in phospholamban phosphorylation, which can be linked to the rapid induction of contractile function.

Alterations in myocardial SERCA2a and phospholamban expression are a common characteristic of cardio-pathological states in humans, but have also been reported in animal models for diabetes-related heart disease.[35,204,230] In guinea pigs, we observed that a 6-month exposure to a high-fat diet induced cardiac contractile function. At the molecular level, this associated with tendency for a decreased SERCA2a expression and decreased phosphorylation of phospholamban.[chapter 2] Furthermore, protein expression of calreticulin was increased in

the hearts from HFD-fed versus standard diet-fed guinea pigs.[chapter 2] Calreticulin, is an endoplasmic reticulum luminal Ca^{2+}-buffering chaperone, which is involved in regulation of intracellular Ca^{2+} homeostasis and endoplasmic reticulum Ca^{2+} capacity.[231] Furthermore, calreticulin binds misfolded proteins and prevents them from being exported from the endoplasmic reticulum to the Golgi apparatus. Therefore, the increased expression of calreticulin could be an indicator of endoplasmic reticulum stress.[232] While calreticulin has been shown to be critical for cardiac development, its role in cardiac pathology is still unclear.[233] However, animal studies revealed that overexpression of calreticulin enhances the inactivation and degradation of SERCA2a in myocardiac H9c2 cells under oxidative stress, suggesting some pathophysiological functions of calreticulin in Ca^{2+} homeostasis of myocardiac disease.[234]

It is unclear whether all these changes in the heart of HFD-fed guinea pigs can be ascribed to alterations in the secretory profile of EAT. However, chronic exposure of primary cardiomyocytes to conditioned media, generated from EAT of HFD-fed guinea pigs, induced a decrease in the protein expression of SERCA2a.[chapter 2] Similar findings were observed in cardiomyocytes exposed to conditioned media, generated from EAT of DM2-patients.[chapter 3] Therefore, it is tempting to speculate that the alterations in the secretory profile of EAT could contribute to the decreases in SERCA2a expression found in cardio-pathological states, such as diabetes.[235] Whether the induction of ER-stress, oxidative stress, or increases in calreticulin expression participate in the decrease in SERCA2a expression remains to be investigated.

The detrimental effect of adipocytes-derived factors is not unique to EAT. In previous studies, it has been shown that SAT, which also secretes a variety of bioactive mediators, exerts a highly potent cardiodepressant activity with an acute effect directly on cardiomyocyte contraction.[194,203,236] In support of these studies, we observed that cardiomyocyte dysfunction could also be induced by conditioned media from SAT of HFD-fed animals and DM2-patients. However, the dose required to induce these effects was markedly higher when compared to conditioned media from EAT. Notably, compared to the conditioned media from EAT, the effects on parameters of sarcomere shortening were lower and effects on cytosolic Ca^{2+}-transients and SERCA2a expression were absent. Furthermore, the physiological relevance of the cardiodepressant factor(s) secreted by other fat depots remains to be elucidated since secreted adipokines may only affect cardiomyocyte function, via the systemic circulation. In

contrast, factors secreted from the EAT can directly affect the function of the underlying myocardium, because to the absence of fascial boundaries.

5.1.2 Effects on insulin signaling

Multiple studies have shown that secretory products from adipose tissue interfere with insulin action, amongst others by reducing Akt-phosphorylation and glucose uptake, in skeletal muscle cells, adipocytes, and cardiomyocytes.[207,237] However, as indicated above, EAT can directly communicate with the myocardium because of the lack of fascial boundaries. Here, we show that that besides inhibition of contractile function, exposure of cardiomyocytes to secretory products from EAT of either HFD-fed animals or DM2-patients abrogates insulin-mediated phosphorylation of Akt. Phosphorylation of Akt is critical for the regulation of glucose uptake. Alterations in myocardial energy substrate metabolism, i.e. decreases in glucose metabolism and increases in fatty acid metabolism due to myocardial insulin resistance characterize diabetic cardiomyopathy in patients with DM2.[205,206,238] The effects of EAT on energy substrate metabolism have not been examined in cardiomyocytes. However, abrogation of insulin-mediated Akt-phosphorylation in cardiomyocytes exposed to conditioned media from epididymal adipose tissue from diabetic rats was paralleled by a reduced ability of insulin to stimulate glucose uptake.[207] Furthermore, in obese Zucker diabetic rats, a decrease in insulin-mediated Akt-phosphorylation could be linked to a decrease in insulin-mediated myocardial glucose utilization *in vivo* as determined by positron emission tomography under hyperinsulinaemic euglycaemic clamp conditions.[208] Collectively, these findings strongly suggest that secretory products of EAT from HFD-fed animals and DM2-patients could contribute to the induction of insulin resistance at the level of the Akt-pathway regulating myocardial glucose uptake.

In contrast to the effects on contractile function, the detrimental effects on insulin action were only demonstrated upon prolonged incubation of the cardiomyocytes with conditioned media. Therefore, it seems likely that the induction of insulin resistance by conditioned media from EAT of DM2-patients and HFD-fed animals is established through

distinct mechanisms. Possible mechanisms include the initiation of the ER-stress response.[232] This leads to activation of kinases, such as Jun N-terminal kinase (JNK) and IKK, which on their turn induce inhibitory serine phosphorylation on IRS1.[232] As a result, IRS1 cannot be effectively phosphorylated on tyrosine residues, thereby abrogating the activation of more downstream components, like PI3K and Akt. Alternatively, accumulation of pro-inflammatory cytokines in conditioned media from EAT itself may trigger activation of the kinases exerting an inhibitory action of IRS1. Finally, preliminary experiments indicate that exposure of cardiomyocytes to conditioned medium from EAT increases the expression of an inhibitor of insulin action SOCS3.[239] Interestingly, SOCS3 expression also tended to be increased in the hearts of HFD-fed guinea pigs. [chapter 4] Yet, further investigations are required to assess whether induction of SOCS3 is indeed essential for the observed induction of insulin resistance.

5.2 Adipokine profiling of epicardial adipose tissue

Profiling of the conditioned media using antibody arrays indicates that the secretory profile of EAT differs from that of other fat depots. Table 5.1 lists the factors that were selectively altered in conditioned media from EAT of both standard diet-fed guinea pigs and ND-patients as compared to conditioned media derived from SAT and PAT. In both species, adiponectin and GRO release were decreased in EAT versus the other depots in guinea pigs, while secretion for activin A, cardiotrophin-1, endoglin, MCP-1, omentin and TIMP-2 were increased in EAT compared to the other depots (all $P< 0.05$). A limitation of applying the antibody array approach on conditioned media from guinea pigs is that some factors in guinea pig conditioned media may been incorrectly or not recognized by the human antibody array. Furthermore, we have not analyzed conditioned media generated from guinea pig PAT. Finally, the array used in our studies contains only 174 factors, while the list of adipokines is continuously expanding and comprises between 500-1000 adipokines. Therefore, further characterization of the secretory profile of EAT has the potential to identify additional factors that are selectively secreted by this depot.

Table 5.1 Alterations in the secretion of mediators from EAT compared to other fat depots

Adipokine	**Guinea pig EAT vs. SAT**	**Human EAT vs. SAT**	**Human EAT vs. PAT**
adiponectin	↓	=	↓
activin A	↑	↑	↑
angiopoietin-2	↑	↑	↑
cardiotrophin-1	↑	=	↑
endoglin	↑	=	↑
E-selectin	↑	↓	↓
GRO	↓	↓	↓
omentin	↑	↑	↑
MCP-1	↑	↑	↑
TIMP-2	↑	↑	↑

The reduced secretion of adiponectin by EAT versus the other fat depots is in line with a reduced expression of adiponectin mRNA expression in EAT versus SAT.[240] The physiological significance of the finding is unclear. It should be noted however that EAT is highly enriched in another protective factor, omentin-1. This protein appears to have a cardioprotective function rather than adiponectin.[chapter 4] Activin A was found to be highly secreted by EAT compared to the other depots studied. Recently, activin A was identified as an adipokine in SAT, where its expression was increased by macrophage infiltration.[221] Angiopoietin-2 is an antagonist of the angiopoietin-1/Tie-2 signaling pathway, which regulates vascular maturation.[241] Defects in myocardial vascular maturation and angiogenesis to abrogation of angiopoietin-1/Tie-2 signaling through increased expression of angiopoietin-2.[242] Others have shown that angiopoietin-2 it is expressed in adipocytes from SAT and visceral adipose tissue.[243,244] Cardiotrophin-1 also showed an increased release from EAT as compared to SAT. Cardiotrophin-1 is a member of the IL-6 family involved in cardiac growth and dysfunction, which acts both by paracrine and autocrine mechanisms.[245] Interestingly cardiotrophin-1 was first described in cardiomyocytes, but the expression of this molecule have also been found in a variety of tissues outside the cardiac compartment such as liver, kidney, skeletal muscle, pancreas, and particularly adipose tissue.[246] To the best of our knowledge, our studies are the first to demonstrate that cardiotrophin-1 is not only expressed but also released by adipose tissue. Expression of endoglin, a component of the TGF-ß receptor complex, has been described in adipose tissue. Here, we observed that endoglin is also released in adipose tissue and in particular in EAT. Moreover, an enhanced secretion of E-selectin from EAT of guinea pigs can be found. However, the secretion of E-selectin from EAT from ND-patients compared to SAT or PAT did not show any alterations. E-selectin, also known as CD62 antigen-like family member E (CD62E), is a cell adhesion molecule, which is expressed on endothelial cells. Adhesion molecules are involved not only in cell adhesion per se, but also in signal transduction, mediating activation and proliferation.[247] High levels of pro-inflammatory factors such as TNF-α and IL-1α, mediate the expression and synthesis of E-selectin on vascular endothelium, which together with chemokines contribute to the recruitment of circulating leukocytes to the site of infection or inflammation.[248,249] In addition, we found that the secretion of E-selectin is higher from EAT than from SAT. In support of previous studies, which have revealed that omentin,

GRO, MCP-1 and TIMP-2 are expressed by EAT, we showed that these factors are also secreted by EAT.[130,171,182,197] Furthermore, the release of omentin, MCP-1 and TIMP-2 were higher from EAT as compared to SAT or PAT.

The reason by the release of adipokines from EAT differs from that of SAT or PAT, is unclear. In this respect, it remains to be investigated whether these differences could be ascribed to differences in the cellular composition or, for example, to myokines, which are factors released by muscle cells such as cardiomyocytes. As mentioned earlier, EAT is not separated by fascial boundaries from the heart, thereby allowing a direct cross-talk between the heart and EAT.

5.2.1 Secretory profile of epicardial adipose tissue is altered in high fat diet and type 2 diabetes mellitus

The detrimental activity of conditioned medium on rat cardiomyocytes was sensitive to boiling. Therefore, it is likely to ascribe this activity to alterations in adipokine release. Antibody profiling of the conditioned media indeed showed that both HFD-feeding of guinea pigs and DM2 in humans affect adipokine secretion by EAT (Table 5.2). A previous report demonstrated alterations in EAT release that can be ascribed to obesity.[182] Because the human subjects in our study had comparable BMI [chapter 3], our data indicate that also DM2 itself can affect adipokine release by EAT. In line with this, the alterations induced by HFD feeding were also found independent of alterations in body weight [chapter 2].

Table 5.2 Alteration in the release of factors from EAT of HFD-fed animals and DM2-patients

Adipokine	HFD-fed animals	DM2-patients
adiponectin	↓	↓
activin A	↑	↑
angiopoietin-2	=	↑
AgRP	↓	↑
angiogenin	↑	↑
cardiotrophin-1	↑	↑
CD14	↑	↑
eotaxin-2	↑	↑
ICAM-1	↑	↑
leptin	↑	↑
omentin	↓	↓
RANTES	↑	↑
TRAILR4	=	↑

Most of the factors listed in Table 5.2 were found to be affected by both HFD-feeding in guinea pigs and DM2-patients in humans as compared to the corresponding controls. However, some of these factors showed no consensus, like for example AgRP and TRAILR4. As discussed

earlier, no specific adipokine arrays are available for guinea pigs. Thus, the use of a human antibody arrays could have contributed to these differences. Alternatively, although HFD feeding of guinea pigs led to impaired glucose tolerance and cardiac dysfunction, the animals do not develop DM2. Therefore, not all alterations present in humans may be found in EAT from HFD-fed guinea pigs. However, an important question that remains to be addressed is what underlies the alterations in the secretory profile of EAT from patients with DM2 and HFD-fed animals. In addition to adipocytes, adipose tissue also contains other cell types, like macrophages, endothelial cells and lymphocytes, which can release a variety of chemo-, cytokines and other factors.[218,219] Hypertrophy of adipose tissue in obesity and DM2 is closely linked to low-grade inflammation, which can be ascribed to accumulation of immune cells in the adipose tissue, and secretion of pro-inflammatory cytokines. A study performed on humans with coronary artery disease reports the infiltration of immune cells in EAT. Although in the present study, the cellular composition of the EAT biopsies were not assessed, two lines of evidence indicate that also the accumulation of activin A in conditioned media from EAT of DM2-patients and HFD-fed guinea pigs can be ascribed to infiltration of immune cells in EAT. First, CD14 is mainly expressed in macrophages and secreted in its soluble form by monocytes. In addition, it has been demonstrated that activin A expression is elevated in adipose tissue from obese subjects, and dramatically increased by factors secreted by macrophages isolated from obese adipose tissue.[221] Furthermore, proteomic analysis observed that oxidative stress for example is higher in EAT than in SAT from patients with cardiovascular disease.[183] In particular, production of inflammatory ROS, which are known to be associated with cardiovascular disease, was higher in EAT than in SAT.[183,184] And thus may also contribute to the alterations of the secretory profile of adipose tissue-derived factors from EAT. However, the relevant mechanisms which contribute to these alterations are not fully understood, and remains to be further investigated.

5.3 Role of epicardial adipose tissue-derived factors in cardiac function

5.3.1 Activin a is a cardiodepressant factor

Accumulation of activin A was consistently found in EAT from both HFD-fed guinea pigs and DM2-patients.[chapters 2 and 3] Activin A is a member of the TGF-β superfamily, which consists of at least 23 members, including TGF-βs, inhibins, activin B and C, anti-Müllerian hormones (AMH), bone morphogenetic proteins (BMPs), myostatin and growth differentiation factor 15 (GDF15).[250] While, activins were originally identified as endocrine regulators of pituitary function and independently as inducers of mesoderm in frogs.[251] Nowadays, the members of the TGF-ß family have been linked to a wide range of biological effects on a large variety of cell types, including the regulation of cell growth, differentiation, matrix production and apoptosis. TGF-β ligands transmit their signals through heteromeric receptors consisting of type I and type II serine/ threonine kinase subunits (Figure 5.1).[252] Seven type I and five type II receptors, also termed activin receptor-like kinases (ALKs), are present in mammals.[253] Because these receptors are shared by a number of different TGF-ß family proteins, the activities of TGF-ß ligands are redundant with those of activins. In general, ligand binding of activin A for example induces the formation of a heterotetracomplex, in which two type II receptors transphosphorylate two type I receptors.[254] The activated type I receptors specifically phosphorylate the Smad (mothers against decapentaplegic) subgroup known as receptor-activated Smads (R-Smads), including Smad2 and 3. The Smad proteins are a family of transcription factors, found in vertebrates, insects and nematodes. Following TGF-ß receptor-mediated phosphorylation, the Smad proteins Smad2 and 3 are phosphorylated and associate with the common signaling mediator Smad4, to form a complex. Then, this Smad-complex translocates into the nucleus and associates with various Smad partners to regulate gene expression.

Apart from the Smad signaling pathway, TGF-ß ligands can also activate non-Smad signaling pathways, including TGF-ß activated kinase-1 (TAK-1), a serine/threonine kinase of the MAPK kinase kinase family, extracellular signal-regulated kinases (ERK)-1 and -2, the stress-activated

protein MAP kinase (SAPK)/JNK, and members of the Ras or Rac families of small GTP-binding proteins.[255-259] However, the developmental relevance of many of these non-Smad proteins awaits further analysis.

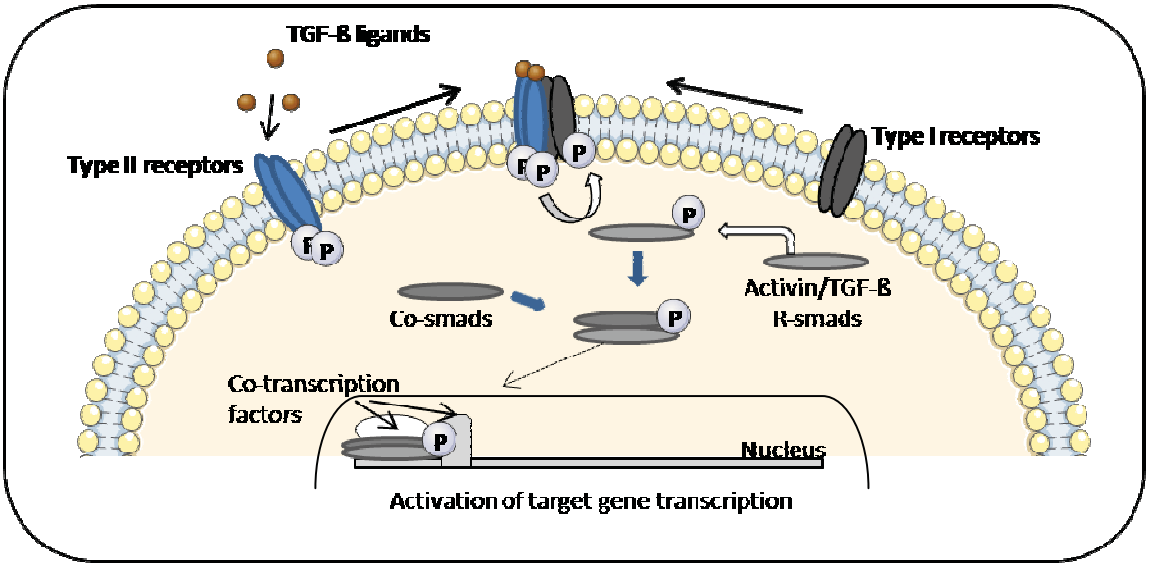

Figure 5.1 Assembly of the smad signaling pathway (from the "Cell membrane slide kit" from Servier). P, phosphate; TGF, tumor necrosis factor.

In our studies, we showed that activin A in the conditioned media from EAT of both HFD-fed guinea pigs and patients with DM2, is biologically active as illustrated by the induction of SMAD2-phosphorylation in cardiomyocytes incubated with these conditioned media. [chapter 2 and 3] Several lines of evidence suggest that activin A could indeed contribute to the development of cardiomyocyte dysfunction induced by conditioned media from EAT of DM2-patients and HFD-fed animals. First, incubation of primary adult rat cardiomyocytes with recombinant activin A, at a concentration comparable to that found in conditioned media from EAT of DM2-patients, reduced cardiomyocyte contractile parameters up to 30-50 % as compared to the inhibition caused by conditioned media from EAT of DM2-patients or HFD-fed animals. Second, the cardiosuppressive effects of conditioned media from EAT of DM2-patients could be partially reversed by pharmacological inhibition of the activin A receptor by using SB431542.

Important further support for a pathophysiological role for activin A comes from our subsequent analysis of plasma samples obtained from patients with uncomplicated DM2, in which cardiac function and myocardial substrate metabolism was characterized through magnetic resonance imaging and positron emission tomography.[205] Although plasma activin A levels in DM2-patients were found to associate with the left ventricular mass over volume ratio (LVM/V), suggesting that increases in activin A levels could contribute to left ventricular hypertrophy (Figure 5.2).

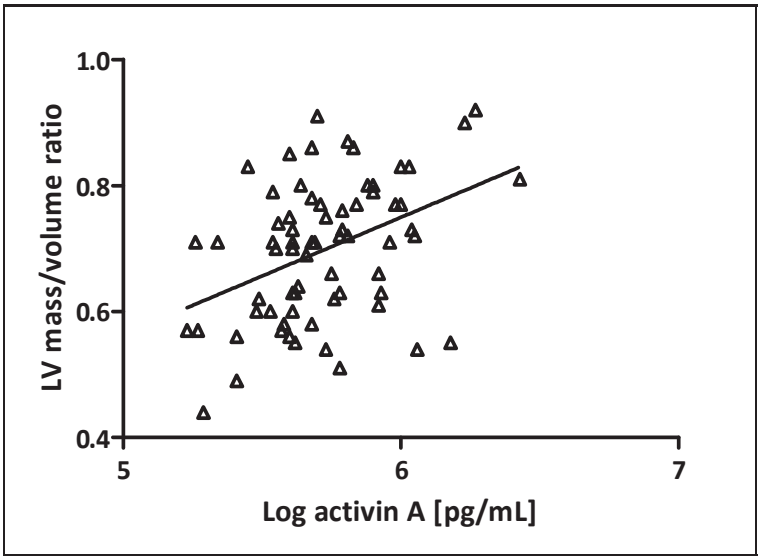

Figure 5.2 Plasma activin A levels associate with the left ventricular mass over volume ratio in DM2-patients. LV left ventricular

In support of these findings, activin A was found to increase the expression of genes involved in myocardial remodeling, like ANP, BNP, MMP-9, TIMP-1, and TGF-ß1 in neonatal rat cardiomyocytes.[117] We are currently examining whether these pathways are also affected in an activin A dependent manner in adult rat cardiomyocytes, exposed to conditioned media from EAT. However, preliminary data indicate that activin A increases the expression of a regulator of cardiac hypertrophy, myosin heavy chain α in adult rat cardiomyocytes.

The analysis of the clinical samples further revealed a negative association between serum activin A levels and insulin-mediated myocardial metabolic glucose uptake (MMRGlu) in DM2-patients compared to ND-patients (Figure 5.3).

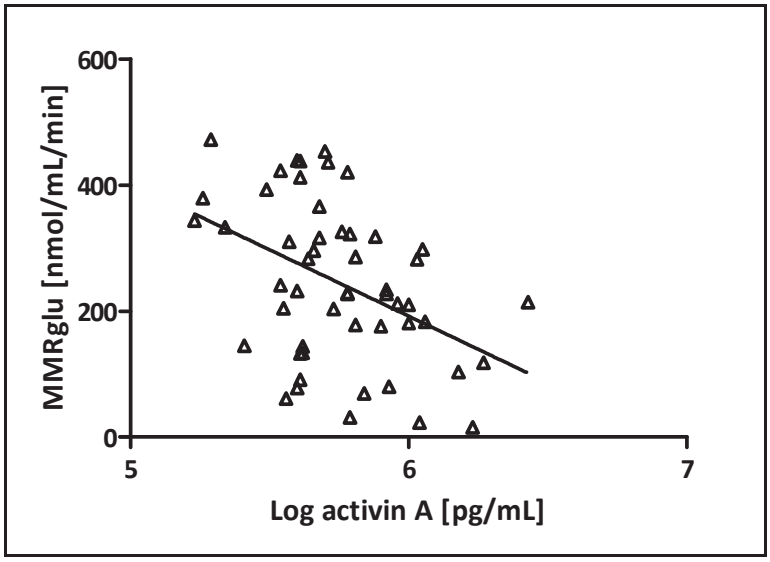

Figure 5.3 Plasma activin A level is associated with myocardial metabolic rate for glucose. MMRGlu, myocardial metabolic glucose uptake.

In line with this, recombinant activin A was able to abrogate insulin-mediated Akt-Ser473-phosphorylation *in vitro* in cardiomyocytes. Also the related activin-like TGF-ß ligand myostatin has been found to reduce Akt-phosphorylation in mouse C2C12 and human myotubes.[260,261] In 2009, it has been shown that inhibition of the Akt/mTOR pathway, using small interfering RNA to regulatory-associated protein of mTOR (RAPTOR), enhances myostatin-induced phosphorylation of Smad2 proteins, and establishing a myostatin signaling-amplification role for inhibition of Akt.[260] Thus has been assumed that this effect may depends on Smad2 and 3 proteins. However, the mechanism by which TGF-ß ligands, such as myostatin or activin A, interferes with Akt-activation remains unclear. Alternatively, in neonatal cardiomyocytes, activin A has been linked to the induction of SOCS3, which abrogates insulin action through inhibition of the insulin receptor and IRS1. Moreover, it has been shown that the effect of activin A depends on the Smad2/3-signaling pathway since inhibition of the phosphorylation of Smad 2/3 proteins prevented the activin A-induced induction of SOCS3 gene expression.[117] As mentioned earlier, exposure of cardiomyocytes to conditioned media from EAT of DM2-patients was found to enhance the expression of SOCS3 in adult rat cardiomyocytes, but it remains to be clarified whether this effect can be ascribed to activin A.

Although these data provide evidence that activin A could participate in the induction of insulin resistance, it should be noted that the abrogation of insulin-mediated Akt-phosphorylation occurred at a higher concentration of activin A than was found in conditioned media from EAT of DM2-patients. Furthermore, SB431542 only partially prevented the detrimental effects induced by conditioned media from EAT of DM2-patients on cardiomyocytes. Therefore, it cannot be excluded that other factors, dysregulated in conditioned media from EAT of DM2-patients, participate in this process. Interestingly, a recent study identified follistatin-like 1 (Fstl1), a physiological antagonist of activin A, as novel adipokine.[262,263] Fstl1, also referred to as TSC36, is an extracellular glycoprotein that belongs to the Fstl family of proteins.[264] Previously, Fstl1 has been reported to be secreted from cardiac muscle under conditions of Akt-mediated hypertrophy and injury, and can thus also be considered as cardiomyokine.[265] Overexpression of Fstl1 was found to promote Akt and ERK 1/2 signaling in cardiomyocytes and protect these cells from hypoxia/reoxygenation-induced apoptosis through activation of these pathways.[266] Furthermore, Fstl1 has been linked to tissue regeneration after injury in skeletal muscle and liver, suggesting that Fstl1 could be a potential targets for myocardial repair.[267,268] Finally, in endothelial cells, it has been demonstrated that Fstl1 activation of PI3K/Akt signaling leads to the activation of phosphorylation of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) production.[269] Based on these findings, we hypothesized that Fstl1 could protect the detrimental effects induced by conditioned media in primary adult rat cardiomyocytes. Figure 5.3 shows that addition of Fstl1 enhanced insulin-induced phosphorylation of Akt by 30 % ($P< 0.01$), and completely prevented the abrogation of insulin-mediated Akt-phosphorylation by conditioned media ($P< 0.001$). Preliminary experiments further show that Fstl1 has no effect on parameters of contractile function. However, Fstl1 restored the reductions in sarcomere shortening and cytosolic Ca^{2+}-transients to 75 % and 90 %, respectively, of the response induced by control medium (both $P< 0.001$) (data not shown).

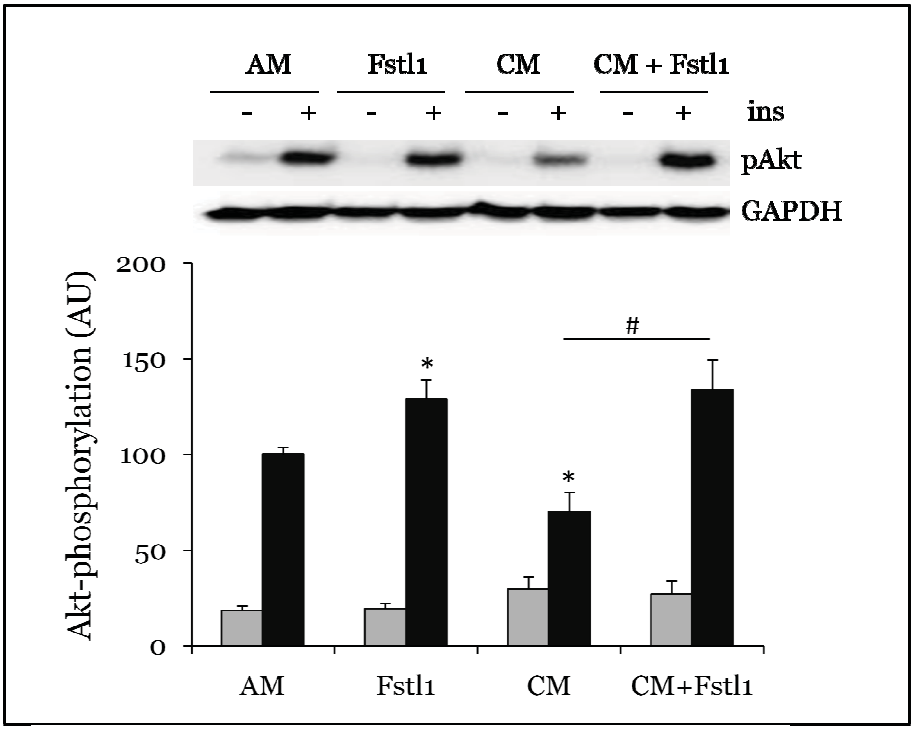

Figure 5.3 Reversal of the detrimental effect of conditioned media by Fstl1 in cardiomyocytes. AM, control adipocyte media; CM, conditioned Media; Fstl1, follstatin-like 1, ins, insulin.

Finally, expression of Fstl1 was reduced by 50 % in EAT from DM2-patients compared to ND-patients (*P*= 0.07) (Figure 5.4). It remains to be clarified whether these alterations in expression are reflected by alterations in secretion and whether plasma levels of Fstl1 can be linked to parameters of cardiometabolic function. Yet, these preliminary findings suggest that a loss of Fstl1 in EAT from DM2-patients could participate in the detrimental effects of conditioned media generated from EAT of DM2-patients on cardiomyocyte function.

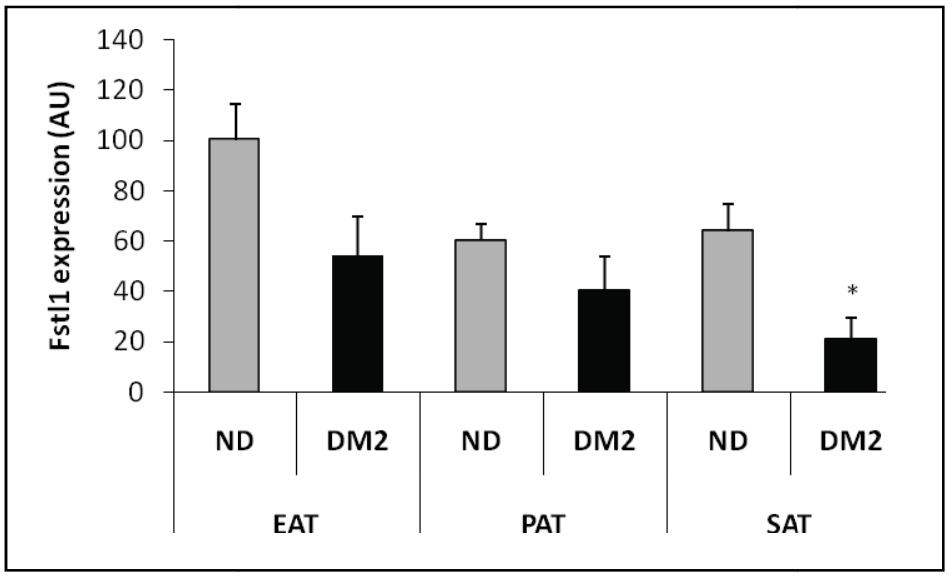

Figure 5.4 Fstl1 expression in adipose tissue from patients with and without DM2. DM2, type 2 diabetes, EAT, epicardial adipose tissue; Fstl1. follstatin-like 1; ND, non-diabetics; PAT, pericardial adipose tissue; SAT, subcutaneous adipose tissue.

5.3.2 Omentin-1, a novel cardioprotective adipokine?

As described in chapter 4, also a loss of omentin-1 expression in EAT could participate in the detrimental effects of conditioned media generated from EAT of DM2-patients and HFD-fed guinea pigs on cardiomyocyte function. Multiple studies have reported a beneficial function for omentin-1. In addition to the association of plasma omentin-1 levels with left ventricular diastolic function, these include anti-inflammatory actions on primary human microvascular endothelial cells, and a vasodilating effect in isolated blood vessels, which is mediated through endothelium-derived NO.[270,271] Based on its strong association with insulin sensitivity and adiponectin, omentin-1 could also have an insulin-sensitizing effect. The reversal of insulin resistance, induced by adipose tissue derived conditioned medium in cardiomyocytes, supports this.[chapter 4]

Despite these observations, little is known about the mechanism of omentin-1 action. For example, omentin-1 exists in a monomeric and trimeric form, and it is unknown whether the biological activity of the two forms is similar. Another question that remains to be addressed is whether ometin-1 acts as a scavenger for anti-inflammatory molecules or through binding to a cell surface receptor. Some studies have reported direct effects of omentin-1 on endothelial cells and adipocytes, suggesting that a cell surface receptor should exist.[271,272] Yet, in our studies we failed to observe direct effects. Rather, omentin-1 prevented the toxic effects induced by conditioned media, thereby supporting the notion that omentin-1 could also act as a scavenger molecule for anti-inflammatory factors. In support of this, omentin-1 was found to reduce CRP- and vascular endothelial growth factor (VEGF)-induced migration, angiogenesis, and CRP- and TNF-α induced NF-kB activation in human microvascular endothelial cells.[273] Finally, the regulation of omentin-1 expression under both physiological and pathophysiological conditions, remains to be clarified. In adipose tissue, omentin-1 synthesis has been ascribed to the stromal vascular fraction.[274] This fraction contains the wide variety of non-adipose cells in adipose tissue, including pre-adipocytes, endothelial cells, stem cells, fibroblasts, and immune cells. Adipose tissue in DM2 is characterized by infiltration of immune cells, which is paralleled by an enhanced secretion of pro-inflammatory adipokines. These factors have been implicated various toxic responses, like induction of apoptosis and endoplasmic reticulum stress, which results in

inhibition of the synthesis of abundantly expressed proteins. Given the abundant amounts of omentin-1 in the circulation, it is plausible that omentin-1 synthesis is affected by endoplasmic reticulum stress. Furthermore, multiple studies demonstrate a strong association between adiponectin and omentin-1 levels, suggesting that adiponectin may regulate omentin expression.[275] Thus, a decrease of adiponectin expression in obesity may result in a lowering of adiponectin levels. Yet, direct evidence supporting this suggestion, such as examination of omentin-1 levels in adiponectin knock-out mouse models, is still lacking. It would be worth to examine the unclarities in our knowledge on omentin-1 action and regulation because of its emerging function as (cardio-) protective adipokine, and thus therapeutically target.

5.3.3 Potential functions of other factors showing a dysregulated release by epicardial adipose tissue

Apart from activin A, Fstl1 and omentin-1, a variety of other factors including adiponectin, AgRP, cardiotrophin-1, CD14, ICAM-1, leptin and RANTES were identified as EAT-derived factors, which are dysregulated in either DM2-patients or HFD-fed guinea pigs (Table 5.2). These factors have not been validated using ELISA assays and with recombinant proteins in our studies. Below a brief description of their potential relevance in cardiac function is provided.

In contrast to most factors synthesized by adipose tissue, adiponectin has anti-inflammatory, anti-diabetic, and anti-atherogenic properties, and it is downregulated in obesity.[276,277] Apart from negative correlations with measures of obesity, adiponectin levels are also reduced in association with insulin resistance, DM2, coronary artery disease, and the metabolic syndrome.[278,279] Recently, it has been shown that adiponectin gene expression is reversibly downregulated by IL-6 and supports the concept of adiponectin being an important selectively controlled modulator of insulin sensitivity.[280-282] It should, however, be noted that adiponectin levels secreted by EAT are lower as compared to these secreted by SAT and PAT. Therefore, the function of adiponectin in EAT requires further studies.

In patients with metabolic syndrome decreased levels of adiponectin associate with increased levels of leptin.[283] In line with this, we observed that leptin release from EAT of DM2-patients and HFD-fed animals was increased as compared to the corresponding controls. Leptin, a key regulator of body weight, was first discovered by Zhang and Friedman in 1994 and effectively lowers blood glucose and insulin levels in DM2-patients.[284,285] In addition, leptin has also been implicated in cardiac hypertrophy and the development of diabetic cardiomyopathy.[286] Thus, apart from activin A, leptin could be another factor derived from EAT of DM2-patients or HFD-fed animals, which could contribute to cardiac dysfunction in primary adult rat cardiomyocytes.

Another factor accumulating in conditioned media from EAT of DM2-patients and HFD-fed guinea pigs is cardiotrophin-1. Cardiotrophin-1 is a member of the IL-6 family involved in cardiac growth and dysfunction which acts both by paracrine and autocrine mechanisms.[245] Through a unique receptor system, consisting of the IL-6 receptor and a common signal transducer

glycoprotein 130 (gp130), a pleiotropic set of growth and differentiation activities are mediated. The expression pattern of this 21.5 kDa protein and its range of activities in the hematopoietic, neuronal, and developmental assays suggest that cardiotrophin-1 may also play an important role in several other organ systems and has a great number of functions that sometimes have opposite results. In a recent study, it has been demonstrated that there is an increase in circulating cardiotrophin-1 in patients with cardiovascular disease and metabolic syndrome.[287] Chronic treatment with cardiotrophin-1 resulted in an increase in SOCS3 (suppressor of cytokine signaling 3) mRNA expression in adipocytes, and the development of insulin resistance as judged by a decrease in insulin-stimulated glucose uptake.[288] Interestingly, both beneficial and pathological effects have been ascribed to cardiotrophin-1. For example, cardiotrophin-1 phosphorylates Akt, and may thus participate in cell survival via the PI3K-dependent pathway.[289] Furthermore, it has been shown that, via the activation of an anti-apoptotic signaling pathway that requires MAP kinases, cardiotrophin-1 also plays an important cardioprotective role on myocardial damage.[290] In contrast, chronic exposure of cardiomyocytes to cardiotrophin-1 depressed basal force of contraction and the inotropic response to Ca^{2+}.[291] Furthermore, cardiotrophin-1 induced myocyte hypertrophy and collagen synthesis, thereby participating in the progression of ventricular remodelling, which results in cardiac muscle failure.[292-294] In particular, the JAK/STAT (Janus Kinase-Signal Transducer and Activator of Transcription) and MAP kinase/c-Jun NH2-terminal protein kinase have been implicated in cardiotrophin-1-induced hypertrophy.[295] The hypertrophic effect of cardiotrophin-1 was found to be mediated by STAT3. However, ERK1/2-phosphorylation of STAT3 has an inhibitory effect on hypertrophy.[296] A recent study further demonstrated that STAT3 not only transduces a hypertrophic signal, but also a protective signal against, for instance, doxorubicine-induced cardiomyopathy by inhibiting reduction of cardiac contractile genes and inducing cardiac protective factors.[297] Other reports further demonstrated that cardiotrophin-1 induced also ERK5-overactivation in cardiomyocytes from spontaneously hypertensive rats, and cardiac-specific overexpression of activated ERK5 has been shown to result in contractile heart failure.[298] Interestingly, it has finally been observed that cardiotrophin-1 may be able to induce MCP-1, which might be responsible for progression of heart failure, either by recruiting inflammatory cells within the myocardium, or by a direct modulation of myocyte function.[299] Due to the opposite results of cardiotrophin-1, it

is not possible to classify cardiotrophin-1 as a favourable or an adverse molecule, and thus further studies seem required.

CD14 is preferentially expressed on macrophages, and acts along with the Toll-like receptor 4, as a co-receptor for signalling mediated by lipopolysaccharide (LPS). It is well known that activation of macrophages by LPS via membrane, CD14 triggers the release of a number of different pro-inflammatory, immunoregulatory and cytotoxic molecules such as TNF-α, IL-1, IL-6 and ROS. Interestingly, some of these factors also exert a negative inotropic action on cardiomyocytes.[194,211] In addition, these factors may negatively interfere with insulin action. It seems however likely that the immunoreactivity that we observed in the conditioned media could be ascribed to the soluble form of CD14 (sCD14), which is released from monocytes. Interestingly, monocyte expression of CD14 and serum levels of sCD14 are elevated in patients with chronic heart failure.[300,301] Although little is known about the action of sCD14 in cardiomyocytes, these findings are in favour of a role of sCD14 in the pathogenesis of cardiac dysfunction in DM2. Examination whether the detrimental effects of conditioned media could be prevented by the addition of neutralizing antibodies may further address this issue.

RANTES is an inflammatory chemokine that recruits and activates different subtypes of leukocytes such as T cells, monocytes, basophils, eosinophils, or mast cells.[302] Mainly, RANTES is expressed by adipocytes and has been hypothesized to mediate leukocyte infiltration of adipose tissue in obesity. Moreover, it has been reported that circulating concentrations of RANTES are increased in patients with obesity, impaired glucose tolerance, and DM2.[303,304] Furthermore, coronary artery disease, independently of obesity, was associated with increased RANTES release by EAT, which contributes to atherosclerotic plaque formation.[182] Although, a recent study provides evidence that inhibition of RANTES exerts cardioprotective effects, specifically during early reperfusion through anti-inflammatory and anti-oxidant properties, the role of this factor in relation to myocardial function and insulin sensitivity is still unclear.

The soluble intercellular adhesion molecule-1 (sICAM-1 also called CD54), an adhesion molecule which play an important role in the immune system and during leukocyte emigration from blood vessels, was also higher secreted by EAT from DM2-patients and HFD-fed animals compared to ND-patients and control animals, respectively. In line with this, the working group of Mohamed-Ali also reported that sICAM-1 was secreted by EAT. Moreover, the release of this

adhesion molecule was higher in EAT from patients with coronary artery disease compared to patients without coronary artery disease.[182] Interestingly, sICAM-1 level in patients with obesity, DM1, dilated cardiomyopathy or patients with diffuse coronary artery disease and DM2, is elevated.[305-308] Several other studies assumed that sICAM-1 is regulated by NF-kB and the transcription of ICAM-1 can be induced by a variety of inflammatory factors, such as IL-1, TNF-α and angiotensin II.[309,310] Moreover, it has been reported that elevation in sICAM-1 levels in obesity is related to the activation of the TNF-α-system and insulin resistance.[311] Thus, it has been hypothesised that sICAM-1 can be a potential factor linking obesity and diabetes with cardiovascular disease. However, the mechanism of the increase in sICAM-1 secretion from EAT in DM2-patients and the role of this adhesion molecule in the development of diabetic cardiomyopathy, remains unclear.

Finally, elevated secretion level of angiogenin, AgRP, Eotaxin-2 and TRAILR4 from EAT could be seen in patients with DM2 or HFD-fed animals. Angiogenin, which belongs to the family of pro-angiogenic factors, is expressed in adipocytes and cells from the stromal vascular fraction.[312] Angiogenin not only induces angiogenesis, but also triggers a wide range of cellular responses including cell migration, invasion, proliferation, and formation of tubular structures.[313] Interestingly, some studies have been reported that angiogenin levels are increased in patients with chronic heart failure and DM1, and that angiogenin could also play a role in the development of peripheral vascular disease and diabetes.[314,315] Surprisingly, we identified agouti-related protein, AgRP, among the factors secreted by EAT. AgRP is a powerful appetite-stimulating peptide produced by the orexigenic neurons in the hypothalamus.[316] The enhanced immunoreactivity in conditioned media generated from EAT of DM2-patients suggests that AgRP is also expressed in adipose tissue, and supports other reports describing synthesis of AgRP in peripheral tissues.[317] Furthermore, plasma levels of AgRP are elevated in obese males versus males with normal body weight.[318] Finally, the identification of AgRP as epicardial adipokine is substantiated by the observation that AgRP expression can be regulated by a key regulator of adipogenesis, the Kruppel like transcription factor KLF4.[319] Eotaxin-2, which belongs to the CC chemokine family, is expressed in adipose tissue, contribute to eosinophil migration and play important roles in the process of inflammation.[320] Hashimoto *et al.* has suggested that eotaxin-2 is linked to the metabolic syndrome since eotaxin-2 levels significantly

correlates with the triglyceride levels which is one of the diagnostic criteria of the metabolic syndrome.[320] We and other revealed that soluble TNF-related apoptosis inducing ligand (TRAIL)R4, one of the four TRAIL receptors, is secreted by adipose tissue.[321] In 1997, Degli-Esposti *et al.* reported that TRAILR4 does not signal apoptosis like TRAILR1 or R2 for example, but it induces NF-kB activation.[321] However, the effect of the EAT-released factors angiogenin, AgRP, eotaxin-2 or TRAILR4 on cardiac muscle, and the mechanism of the increase in TRAILR4 secretion from EAT in DM2-patients and HFD-fed animals, remains to be elucidated.

5.4 Conclusion and Perspectives

Over the last decades, it has been demonstrated that patients with DM2 often develop diabetic cardiomyopathy. Here we have shown that alterations in the secretory profile of EAT from patients with DM2 might substantially contribute to changes in myocardial function. Because of the close relationship to the heart, adipose tissue-derived factors from EAT can directly affect the underlying myocardium, and thus, it has been assumed that EAT may play a role in the pathogenesis of this specific heart muscle disease such as diabetic cardiomyopathy.[103] Efforts to understand the complex biological signaling underlying the development and evolution of diabetic cardiomyopathy, have resulted in the identification of a multiplicity of circulating and adipose tissue-derived factors. Therefore, a number of different mediators derived from EAT could be identified, including FABP-4, IL-6, RANTES, CD-14, activin A and omentin. Moreover changes in the secretory profile of adipose tissue induce by DM2 or HFD has been found and several studies indicate that DM2 and cardiovascular disease are closely linked to low-grade inflammation, which can be ascribed to accumulation of immune cells such as macrophages in the adipose tissue, and secretion of pro-inflammatory cytokines.[180,219] Further studies should reveal whether the alterations in the production of factors secreted by EAT can be explained by mechanisms such as infiltration of inflammatory factors, mediators produced by the myocardium, or increased generation of ROS in response to regional ischemia and depressed myocardial function, which could activate oxidant-sensitive inflammatory signals. Although in the present study, it has been shown that factors like CD14, which is mainly expressed in macrophages and secreted in its soluble form by monocytes, is secreted by EAT, the infiltrations of immune cells in EAT, induced by pathological states such as DM2, remain to be investigated.

In 2011 a working group of Toda established a culture model to study the interaction between adipose tissue and the heart.[322,323] In that study, mouse HL-1 cardiomyocytes which display a differentiated adult cardiac phenotype were co-cultured with adipose tissue fragments obtained from visceral adipose tissue and SAT of mice, and EAT of human. Interestingly, this system revealed that adipose tissue fragments enhance the expression of the fatty acid transporter proteins FATP4 and CD36 on the one hand, and lipid accumulation in

cardiomyocytes on the other. However, this model does not clarify the crosstalk between primary cardiomyocytes and EAT, and the effect of adipose tissue from different pathological states on cardiac function and metabolism.

In the present study, we established a co-culture model to analyse the interaction between adipose tissue, especially EAT, and the myocardium. By using conditioned media generated from adipose tissue it has been observed that EAT communicates with the myocardium by the release of factors, which have been detrimental to isolated primary cardiomyocytes since these exposed cells are characterized by an impaired insulin signaling and contractile function. We identified activin A, a member of the TGF-ß superfamily, as a major cardiodepressant factor. Because the cardiodepressant effect of conditioned media could be reversed only partly by using the TGF-ß-signaling inhibitor SB431542, it has been assumed that other EAT-derived factors, apart from activin A, can affect myocardium and lead to cardiac dysfunction. Interestingly, for activin A it has also been observed that it induces the expression of SOCS3, a key repressor of insulin action in neonatal cardiomyocytes.[177,214] Moreover, in 2007 it has been reported that increased SOCS3 expression induced by pro-inflammatory cytokines including IL-6 contributes to the induction of hepatic insulin resistance, since the expression of SOCS3 in the liver is elevated when wild-type mice were fed a HFD and prevented by JNK1 ablation in adipocytes.[324] In general, it has been shown that the upregulation of SOCS3 expression in the liver is induced by activation of STAT and NF κB-mediated pathways.[325] Interestingly, in preliminary experiments we could show that SOCS3 mRNA expression is upregulated in cardiomyocytes exposed to conditioned media generated from EAT of DM2-patients. Another important factor released by EAT of DM2-patients is cardiotrophin-1. Because cardiotrophin-1 is obviously released by EAT, and due to the opposite effect of cardiotrophin-1 in the cardiovascular system, further experiments are needed to analyse the role of cardiotrophin-1 in the development of cardiovascular disease. Thus, future work should be aimed to elaborate the mechanism by which activin A and other feasible detrimental factors derived from EAT, affect cardiac myocyte function and metabolism.

Apart from adipokines, EAT is also a rich source of free fatty acids, which additionally can affect the myocardium because of the absence of fascial boundaries. Interestingly, altered cardiac energy metabolism can have direct consequence on cardiac function and this is a

prominent feature of cardiac dysfunction.[326] However, the molecular mechanism that link altered mechanism with cardiac pathology, is poorly understood. Nevertheless, recently it has been reported that an acute exposure of elevated saturated fatty acids such as palmitate, as occurs in diabetes, impaired myocardial function, and thus lipid metabolism plays a critical role in cardiac electrical and mechanical function.[327] Interestingly, the rate of fatty acids release by EAT are in part slightly higher than in other fat depots, such as pericardial and perirenal depots.[136] Moreover, unsaturated fatty acids, including palmitoleic acid, oleic acid, and linoleic acid were higher, and the saturated fatty acids such as myristic acid, palmitic acid, and stearic acid were lower in EAT compared SAT.[328] Thus, regional differences in fatty acid composition, suggesting a depot specific impact of adipose tissue based fatty acids on the adjacent tissue. In the present study, we observed that glycerol and free fatty acids levels were similar in the conditioned media generate from adipose tissue of DM2-patients and HFD-fed guinea pigs compared to the respective control groups. Therefore, it seems unlikely that the free fatty acid release, which can affect myocardial performance, contributes to the observed effects caused by conditioned media generated from EAT of HFD-fed animals and DM2-patients. Consistent with these observations, we observed that the cardiodepressant effect of conditioned media was heat sensitive, since the conditioned media-derived effects could be completely blunted by boiling the media, and thus indicating the involvement of protein(s) and not fatty acid(s).

By using quantitative real time PCR, very recent preliminary data indicates that detrimental effects induced by EAT-derived factors on cardiomyocytes can be ascribed to alterations in miRNA expression. Predicted target genes for these miRNAs, include the SERCA2a, which is downregulated in cardiomyocytes exposed to conditioned media generated from EAT of DM2-patients. In line with this, preliminary validation experiments suggest that silencing of let7c, an miRNA important in cell growth, increases the abundance of SERCA2a.[329] Collectively, these data suggest that alterations in miRNA expression in cardiac myocytes induced by factors secreted from EAT, could contribute to the pathogenesis of diabetes-related heart disease.

In general, in the mammalian heart normal cardiac function is regulated by interaction of the two major cell types, cardiomyocytes and cardiac fibroblasts.[330] Cardiomyocytes are the contractile cells in the myocardium that provide mechanical force, whereas the cardiac fibroblasts, which surround cardiomyocytes, are a key source of components of the extracellular

matrix that regulates the structure of the heart and hence mechanical, chemical and electrical signals between the cellular and non-cellular components. While a lot is known about structure and function of cardiomyocytes, much less is known about cardiac fibroblasts. However, studies revealed that cardiac fibroblasts, which account for 60-70 % of the cells in the human heart, respond to environmental stimuli such as adipose tissue-derived factors in multiple ways, including proliferation, secretion of cytokines, and other factors, migration and particularly altering extracellular matrix turnover through changes in matrix protein synthesis and degradation.[331] However, prolonged alterations in mechanical and chemical properties of the myocardial environment lead to cardiac fibroblasts activation and proliferation, their differentiation into myofibroblasts, and abnormal release of extracellular matrix proteins, which contribute to cardiac disease. It is well known that both hyperplasia and fibrosis, a result of increased production and reduced degradation of collagen of cardiac fibroblasts, play a key role in the pathogenesis of diabetic cardiomyopathy.[40] Moreover, as already mentioned, EAT can also contributes to this metabolic state, but it is still unknown whether EAT has effects on proliferation and collagen synthesis in cardiac fibroblasts. Finally, further studies are needed to analyze signaling pathways of these effects in this specific cell type in order to explore whether EAT-derived factors might play a potential role in the processes of myocardial fibrosis, which might contribute to the progress of heart failure. Interestingly, in rats it has been reported that PI3K or ERK 1/2 and p38MAPK signaling pathways, rather than JNK pathways, are involved in proliferation and collagen synthesis.[332] However, this needs to be further explored.

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Contribution to chapter 2-4

The published results in this thesis involve contribution of my own and contributions from colleagues and members of the laboratory of Prof. Dr. Eckel and Dr. Ouwens. In the following, my specific contribution to each publication is outlined.

Chapter 2

- Generation of all CM together with Daniella Herzfeld de Wiza
- Preparation and isolation of rat cardiomyocytes together with Daniella Herzfeld de Wiza
- Analysis of protein expression in tissue biopsies together with Heidi Mueller
- Effect of CM on SERCA2a expression together with Daniella Herzfeld de Wiza
- Cytokine arrays of CM
- Quantification of cytokines in CM
- Effect of CM on insulin and Smad signaling together with Daniella Herzfeld de Wiza
- Contraction measurements of CM on cardiomyocytes
- Preparation of manuscript together with Dr. Ouwens and Prof. Dr. Eckel

Chapter 3

- Generation of all CM together with Daniella Herzfeld de Wiza
- Preparation and isolation of rat cardiomyocytes together with Daniella Herzfeld de Wiza
- Effect of CM on insulin and Smad signaling together with Daniella Herzfeld de Wiza
- Effect of CM on SERCA2a expression together with Daniella Herzfeld de Wiza
- Dose-dependent effect of activin A on insulin signaling in cardiomyocytes together with Daniella Herzfeld de Wiza
- Dose-dependent effect of activin A on contraction in cardiomyocytes
- Contraction measurements of CM on cardiomyocytes
- Cytokine arrays of CM
- Quantification of cytokines in CM

- Preparation of manuscript together with Dr. Ouwens and Prof. Dr. Eckel

Chapter 4

- Generation of all CM together with Daniella Herzfeld de Wiza
- Preparation and isolation of rat cardiomyocytes together with Daniella Herzfeld de Wiza
- Analysis of protein expression in tissue biopsies together with Heidi Mueller
- Effect of Omentin on Insulin signaling together with Heidi Mueller
- Contraction measurements of CM in the presence or in the absence of Omentin on cardiomyocytes
- Preparation of manuscript together with Dr. Ouwens and Prof. Dr. Eckel

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Eidesstattliche Erklärung

Die vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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Sabrina Greulich