IGF-IR signaling attenuates the age-related decline of diastolic cardiac function

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IGF-IR signaling attenuates the age-related decline of diastolic cardiac function. Am J Physiol Endocrinol Metab 303: E213–E222, 2012. First published May 15, 2012; doi:10.1152/ajpendo.00538.2011.—Insulin-like growth factor (IGF-I) signaling has been implicated to play an important role in regulation of cardiac growth, hypertrophy, and contractile function and has been linked to the development of age-related congestive heart failure. Here, we address the question to what extent cardiomyocyte-specific IGF-I signaling is essential for maintenance of the structural and functional integrity of the adult murine heart. To investigate the effects of IGF-I signaling in the adult heart without confounding effects due to IGF-I overexpression or adaptation during embryonic and early postnatal development, we inactivated the IGF-I receptor (IGF-IR) by a 4-hydroxytamoxifen-inducible Cre recombinase in adult cardiac myocytes. Efficient inactivation of the IGF-IR (iCMIGF-IRKO) as assessed by Western analysis and real-time PCR went along with reduced IGF-I-dependent Akt and GSK3β phosphorylation. Functional analysis by conductance manometry and MRI revealed no functional alterations in young adult iCMIGF-IRKO mice (age 3 mo). However, when induced in aging mice (11 mo) diastolic cardiac function was depressed. To address the question whether insulin signaling might compensate for the defective IGF-IR signaling, we inactivated β-cells by streptozotocin. However, the diabetes-associated functional depression was similar in control and iCMIGF-IRKO mice. Similarly, analysis of the cardiac gene expression profile on 44K microarrays did not reveal activation of overt adaptive processes. Endogenous IGF-IR signaling is required for conservation of cardiac function of the aging heart, but not for the integrity of cardiac structure and function of young hearts.

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INSULIN AND INSULIN-LIKE GROWTH FACTOR I (IGF-I) transmit signals via the closely related insulin and IGF-I receptors (IGF-IR), respectively. Upon ligand binding, both receptors activate the PI 3-kinase pathway leading, a.o., to stimulation of Akt kinases, which in turn play a central role in cellular growth, metabolism, and survival. The crucial function of IGF-I in mammalian development is illustrated best by the finding that most of constitutive IGF-I knockout mice die at birth and exhibit substantial growth reduction as well as defects in muscular and lung tissues (3, 22). Similarly to IGF-I-deficient mice, IGF-IR knockout mice display a growth retardation phenotype and are characterized by perinatal death due to the inability of the homozygous mutants to deflate their lungs and start to breath after birth. Moreover, muscle hypoplasia and impaired skin development were associated with IGF-IR deficiency. In the adult, a major source for IGF-I is the liver, where it is produced in response to growth hormone stimulation. Likewise, hepatocyte-specific IGF-I knockout mice display IGF-I plasma levels that are reduced by 75% (21). These data indicate that, besides the hepatic formation, IGF-I is also synthesized locally and may exert paracrine effects at the site of production. Thus, organ-specific effects of IGF-I may be influenced by both the circulating and the locally released IGF-I.

Experimental and clinical studies suggest that IGF-I may play a critical role in the regulation of cardiac growth, contractile force, and metabolism. Large epidemiological studies such as the Rotterdam and Framingham studies revealed that the risk to develop heart failure was inversely correlated with the circulating IGF-I levels (4, 38). Moreover, a polymorphism in the IGF-I promoter has been identified, which is associated with reduced IGF-I expression and elevated mortality after myocardial infarction (5). These data suggest that IGF-I is an important factor conserving the integrity of cardiac structure and function. On the other hand, a more recent prospective clinical study revealed a decrease of IGF-I levels with age but no differences in patients with congestive heart failure versus controls were identified (1). Thus, the influence of circulating IGF-I on heart failure development remains elusive.

Cardiac hypertrophy is the result of increased growth of cardiomyocytes. A rough classification distinguishes an adaptive/physiological versus maladaptive/pathological hypertrophy (11). IGF-I signaling via its activation of the PI3Kα pathway has been thought to mediate an adaptive hypertrophy that occurs in response to physical exercise or during pregnancy. Transgenic mice with cardiomyocyte-specific overexpression of IGF-I are characterized by the development of cardiac hypertrophy, which may be the result of the growth-promoting effects of IGF-I. However, there are still discrepant results as to whether hypertrophy is the result of elevated cell size (32) or cell number (31). In further studies, IGF-I overexpression protected the heart from ischemic injury in vivo and led to enhanced repair upon myocardial infarction (32, 40). This function was ascribed to an anti-inflammatory effect of IGF-I with a decrease in proinflammatory and an increase in anti-inflammatory cytokines in the heart. Along the same line, mice with reduced plasma levels of IGF-I due to heterozygous deletion of the IGF-I gene, displayed a reduced adaptation to myocardial infarction. Thus, IGF-I-dependent signaling not only contributes to the development of physiological hypertrophy but also appears to stimulate cardiac repair processes under pathological conditions.
Whereas overexpression models suggest an important protective role of IGF-I signaling in the heart, it remains unclear to what extent the endogenous signaling via the IGF-IR might modulate cardiac structure and function. Due to the perinatal mortality of constitutive IGF-IR knockout mice, this problem cannot be addressed in classical knockout mice. With the development of conditional knockout models (21, 35) for the IGF-IR gene, it is now feasible to analyze the cardiomyocyte-specific functions of IGF-I. In this study, we used a tamoxifen-inducible, cardiomyocyte-specific knockout of the IGF-IR to circumvent possible developmentally associated adaptation and to study the functional role of IGF-IR-dependent signaling in adult mice at various postnatal ages.

**EXPERIMENTAL PROCEDURES**

**Animals.** Mice were bred at the Tierversuchsanlage of the Heinrich-Heine-Universität, Düsseldorf, Germany. They were fed a standard chow diet and received tap water ad libitum. The study conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and was performed in accordance with the national guidelines on animal care and approved by the Bezirksregierung Düsseldorf.

Generation of IGF-IR floxed mice is described by Stuchelscheid et al. (35). To generate an inducible, cardiomyocyte-specific knockout of the IGF-IR (iCMIGF-IRKO), those mice were crossed with mer-Cremer mice (34), expressing 4-OH-tamoxifen (4-OHTX)-regulated Cre recombinase (39) under the control of the α-MHC promoter. Double-transgenic mice (merCremer + IGFloxP/IGFloxP) served as the experimental group. Siblings that were positive for Cre recombinase but negative for the floxed IGF-IR served as control animals. Also, mice with only the floxed IGF-IR (IGFloxP/IGFloxP) were used as control animals. Mice with endothelial cell-specific IGF-IR knockout were generated by breeding IGFloxP mice with Tie2-Cre animals (16) (ecIGF-IRKO).

**Induction of IGF-IR exon 3 excision with 4-OHTX experimental protocol.** Double-transgenic mice and control animals were treated with 4-OHTX (500 μg dissolved in 100 μl peanut oil, ip injection) on 10 consecutive days starting on day 1. Animals were allowed to recover after the injection period, and functional measurements were performed after 6 wk (i.e., between day 52 and day 57 after start of OHTX injection). The age given in the paper refers to the age at the beginning of the protocol (3 mo, 11 mo). Effective excision of IGF-IR exon 3 was tested by PCR (forward primer 5'-TTCACCAGTACCATGGGCCTC, reverse primer 5'-CTTCCAGCTTTGCAGGTT-CACG).

In vivo measurement of cardiac function. Magnetic resonance imaging (MRI) was performed using a vertical Bruker DRX 9.4 Tesla Wide Bore NMR spectrometer equipped with an actively shielded 57-mm gradient set and a 30-mm birdcage resonator. Mice were anesthetized with 1.5% isoflurane and kept at body temperature during the whole experiment. Acquisition and analysis of data were performed as described by Jacoby et al. (14). Hemodynamic parameters were obtained by introduction of a Millar 1.4 French pressure-volume catheter into the left ventricle, as described previously (9, 30).

**Histology; immunohistochemistry; and western blotting.** For histological examination, hearts were excised, the blood was washed out with ice-cold saline, embedded in tissue-freezing medium (Leica Microsystems Nussloch, Germany), and immediately shock-frozen in liquid nitrogen. Ten-micrometer sections from the midcavity were cut with a cryostat and stained with hematoxylin and eosin. For detection of fibrosis, sections were fixed with formaldehyde and stained with Sirius red (0.1% in saturated aqueous picric acid). To measure cardiomyocyte diameters, membranes were stained with FITC-conjugated wheat germ agglutinin (WGA) using Zamboni fixed slices (Zamboni fixative: 4% paraformaldehyde and 15% picric acid in 0.1 M Na2PO4). Per heart the diameters of 100 cells from two different slices were measured using the Cell-F software (Soft Imaging Systems, Olympus, Germany).

For detection of apoptosis, heart sections were stained with a cleaved caspase-3 antibody (1:200; Cell Signaling Technologies). As a second method, we used the TUNEL method (ApoAlert DNA Fragmentation Assay kit; Clontech), which was carried out according to the manufacturer’s protocol. Western blot analysis was performed as described before (42). Primary antibodies were from Cell Signaling Technologies: IGF-IR (no. 3027), Akt1 (no. 2967), Akt2 (no. 5239), P-Ser373-Akt (no. 9271), GSK3β (no. 9315), and P-Ser473-GSK3β (no. 9336). Detection of proteins was performed by near-infrared detection using IRDye-labeled secondary antibodies and a LICOR Odyssey scanner (Licor Biosciences, Lincoln, NE).

**Isolation of cardiomyocytes and real-time PCR.** Ventricular cardiomyocytes were isolated from mouse hearts by retrograde perfusion with a collagenase-containing calcium-free buffer equilibrated at 37°C, pH 7.4, in a Langendorff apparatus. The basal culture medium (CTT) was modified medium 199 including Earl’s salts, 5 mMol/l creatine, 2 mMol/l t-carnitine, 5 mMol/l taurine, 100,000 IU/l penicillin, 100 μg/ml streptomycin, and 10 μMol/l cytosine-β-D-arabinofuranoside (pH 7.4). After separation, cardiomyocytes were readjusted to a physiological calcium concentration. The adjustment of calcium concentration to the culture before plating of the cells was carried out in four centrifugation steps with 125, 250, 500, and 1,000 μM CaCl2. Then, cells were suspended in basal culture medium on culture dishes, which were preincubated for 2 h with 7.5 mg/l laminin in CTT medium. After 90 min, cardiomyocytes were washed twice with CTT medium. This resulted in cultures of ~90% quiescent rod-shaped cells on average.

For preparation of RNA, cells were harvested with a cell scraper and transferred with medium into a tube and centrifuged by 1,000 g for 5 min. Cardiomyocytes were washed once with 1 ml of PBS and centrifuged again at 1,000 g for 5 min. Cell pellets were frozen in liquid nitrogen. Commercially available Taqman assays (Applied Biosystems) were used to measure gene expression in total RNA from 4-OHTX-treated control and iCMIGF-IRKO mice. The following premade assays were used to analyze expression levels of IGF-IR (no. Mm00802841_m1), CD31 (no. Mm00476708_m1), DDR2 (no. Mm01281890_m1). Samples were normalized to HPRT (no. Mm00446968_m1).

**Induction of diabetes/implantation of osmotic minipumps.** Mice at 3 mo of age were injected with OHTX on 10 consecutive days (see above). After recovering for 5 wk, mice were injected with streptozotocin (STZ, 45 mg/kg ip for 5 days) and maintained for 3–4 wk with free access to standard laboratory chow and tap water. Blood glucose levels were measured weekly using a glucose monitor (Contour Set Plasma mg/dl, Bayer Vital, Germany). For a detailed review of the diabetes development, we also performed glucose tolerance tests 2–3 days before and 2 and 4 wk after diabetes induction by STZ. Therefore, the animals were starved overnight. In the morning, we measured the fasting blood glucose levels, and subsequently the animals were injected with glucose (2 g/kg body wt). Blood glucose levels were measured repeatedly over a period of 2 h.

Implantation of osmotic minipumps was performed as described elsewhere (24). Briefly, 10-mo-old mice were treated with OHTX as described above. Six weeks later, mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (2.5 mg/kg) diluted in phosphate-buffered saline (final volume 200 μl). An incision was made on the back of each animal between the shoulder blades, and a microosmotic pump (Alzet Type 1002; Durect) containing isoproterenol dissolved in saline solution (0.9% NaCl, 5 mM ascorbic acid) was inserted into the infrascapular subcutaneous tissue. Isoproterenol was applied at a dose of 20 mg·kg·day−1 over a period of 14 days. Cardiac function was then analyzed by MRI.

**IGF-I, IGFBP1, and IGFBP3 plasma levels.** To determine IGF-I and IGFBP plasma levels, heparinized blood was directly collected.
Characterization of iCMIGF-IRKO mice. Taking into account that cardiomyocytes represent only 30–40% of the cell number in whole hearts (41), functionality of the knockout was tested by real-time RT-PCR on total RNA isolated from cardiomyocyte preparations of control and iCMIGF-IRKO mice. As shown in Fig. 1A, IGF-IR expression levels dropped to 20% of that of control cells. Whereas DDR2 expression as a marker for cardiac fibroblast contamination was not detectable, we still found CD31 expression, which revealed the presence of endothelial cells in our preparations. Thus, the residual IGF-IR expression detected in our preparations might have been due to endothelial cell contamination. To analyze whether cardiac endothelial cells represent a relevant source of IGF-IR expression, we compared IGF-IR expression in control hearts, iCMIGF-IRKO hearts, and endothelial cell-specific IGF-IR knockout hearts (Tie2 Cre × IGF-IRloxP). Western blot analysis revealed a substantial reduction of IGF-IR protein in iCMIGF-IRKO animals (Fig. 1B). In ecIGF-IRKO hearts, IGF-IR expression was reduced to a similar extent, demonstrating that, besides cardiomyocytes, the cardiac endothelium represents a major source of IGF-IR expression.

Therefore, we concluded that IGF-IR expression was downregulated almost completely in cardiomyocytes after induction of Cre recombinase activity in iCMIGF-IRKO mice. Inactivation of the insulin receptor, downstream targets of IGF-IR signaling. Representative blots of 4 control (C) and 4 iCMIGF-IRKO (IGF) hearts. GSK3β, subunit of IR; p-GSK3β, Ser9 phosphorylation level of GSK3β, and p-ACC (Ser79) phosphorylation level of ACC. As a control, we performed additionally a cleanup step with the RNeasy Kit (Qiagen, Hilden, Germany) as recommended by the supplier. Total RNA was isolated from heart tissue using the TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturers' protocols. All analyses and calibrations were carried out in triplicate according to the manufacturers’ protocols.

Total RNA isolation, cDNA synthesis, and microarray analysis. Total RNA was isolated from heart tissue using the TRIZol reagent (Invitrogen, Karlsruhe, Germany) as recommended by the supplier. Additionally, a cleanup step with the RNeasy Kit (Qiagen, Hilden, Germany) was performed. RNA was quantified by A260 measurement (NanoDrop, Kisker, Steinfurt, Germany). The integrity of total RNA was checked by capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA). Analysis of cardiac gene expression in control and iCMIGF-IRKO mice was performed using the Agilent Mouse GE 4 × 44K v2 arrays following the manufacturer’s instructions. Briefly, for each sample (4 controls, 4 KO) 100 ng of total RNA was reverse transcribed, linearly amplified, and labeled using the One Color Low Input Quick Amp Labeling Kit (Agilent). After fragmentation (fragmentation buffer, Agilent), the labeled cDNA was hybridized on the array in Hi-RPM Hybridization Buffer (Agilent) at 65°C for 17 h.

The arrays were washed in wash buffer A and wash buffer B (Agilent) following the manufacturer’s protocol. The arrays were scanned with the High Resolution C Scanner, and the fluorescence signal intensities were calculated by Feature Extraction Software version 10.7 (Agilent). All further data analyses were performed using GeneSpring V.11 (Agilent). The data were normalized to the quantile of the signal intensities, and a baseline transformation to the median of all samples was performed. Significantly regulated genes (P < 0.05) were identified by performing Student’s t-test. Data have been deposited at GEO under accession no. GSE32936.

Statistical methods. All results are presented as means ± SD. Results were analyzed by either Student’s t-test or two-way ANOVA as appropriate, followed by Bonferroni post hoc tests. Statistical analyses were performed using GraphPad Prism 4 software. Differences were considered to be statistically significant at a value of P < 0.05.

RESULTS

Characterization of iCMIGF-IRKO mice. Taking into account that cardiomyocytes represent only 30–40% of the cell number in whole hearts (41), functionality of the knockout was tested by real-time RT-PCR on total RNA isolated from cardiomyocyte preparations of control and iCMIGF-IRKO mice. As shown in Fig. 1A, IGF-IR expression levels dropped to 20% of that of control cells. Whereas DDR2 expression as a marker for cardiac fibroblast contamination was not detectable, we still found CD31 expression, which revealed the presence of endothelial cells in our preparations. Thus, the residual IGF-IR expression detected in our preparations might have been due to endothelial cell contamination. To analyze whether cardiac endothelial cells represent a relevant source of IGF-IR expression, we compared IGF-IR expression in control hearts, iCMIGF-IRKO hearts, and endothelial cell-specific IGF-IR knockout hearts (Tie2 Cre × IGF-IRloxP). Western blot analysis revealed a substantial reduction of IGF-IR protein in iCMIGF-IRKO animals (Fig. 1B). In ecIGF-IRKO hearts, IGF-IR expression was reduced to a similar extent, demonstrating that, besides cardiomyocytes, the cardiac endothelium represents a major source of IGF-IR expression.

Therefore, we concluded that IGF-IR expression was downregulated almost completely in cardiomyocytes after induction of Cre recombinase activity in iCMIGF-IRKO mice. Insulin receptor levels were unaffected by the induction of the IGF-IR knockout (Fig. 1C).

In another experimental series, we analyzed to what extent downstream signaling was affected by the deletion of the IGF-IR. Figure 1C shows representative Western blots of extracts from WT and iCMIGF-IRKO hearts after stimulation of mice with IGF-I (1.5 μg/g body wt ip). Akt1 and Akt2 expression was not affected by deletion of the IGF-IR. However, measurement of activated Akt (P-Ser473-Akt and P-Thr308-Akt) revealed a decrease of the phosphorylation level by 75% (P < 0.05, n = 4) in iCMIGF-IRKO hearts. GSK3β and ACC1, downstream targets of Akt, were also analyzed.

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Again, no differences were observed on the protein level, but Ser9 phosphorylation of GSK3β as well as Ser79 phosphorylation of ACC were substantially reduced in iCMIGF-IRKO mice upon IGF-I stimulation.

We further investigated whether cardiomyocyte-specific deletion of IGF-IR led to altered expression of IGF-I, IGFFBP1, and IGFFBP3 plasma levels, respectively. However, neither IGF-I concentrations nor insulin or IGFFBP1 or IGFFBP3 levels in plasma were changed in iCMIGF-IRKO animals (Table 1).

Functional consequences of iCMIGF-IRKO in young adult mice. To determine the influence of IGF-IR signaling on cardiac structure and function in adult hearts, we first induced knockout at the age of 3 mo. Cardiac function was analyzed in vivo by high-resolution MRI 3 mo after induction of the knockout. End-systolic (ESV) and end-diastolic volumes (EDV) were not different in iCMIGF-IRKO compared with WT mice. Similarly, ejection fraction (EF), wall thickness, and calculated cardiac mass were not altered (Table 2). After assessment by MRI, we also collected cardiac pressure data from the same animals using a left ventricular (LV) catheter. In accordance with MRI data, pressure-related parameters were not significantly different after 3 mo of IGF-IR inactivation (Table 3).

Despite the lack of functional differences, we analyzed cardiac structure by means of histological examination. Cardiomyocyte diameters of iCMIGF-IRKO mice did not differ from control values (control 17.0 ± 1.47 μm, n = 10; KO 16.76 ± 0.84 μm, n = 7). Also, Sirius red staining, used to detect collagen deposition, did not reveal any fibrotic changes due to the iCMIGF-IRKO (data not shown). Since an antipapoptotic action of IGF-I was demonstrated in several studies (for overview see Ref. 36), we were interested in whether the iCMIGF-IRKO led to an increase of apoptotic cells in cardiac tissue. Using two different methods for apoptosis detection (cleaved caspase-3; and TUNEL), we could not detect a noteworthy amount of apoptotic cells either in control or in iCMIGF-IRKO animals.

In line with the aforementioned functional data, no signs of lung edema were observed, as the wet/dry weight index of the lung showed no difference between the groups (control 4.17 ± 0.5, n = 6; KO 4.29 ± 0.2, n = 6). IGF-IR KO in cardiomyocytes of aging mice. Several experimental and clinical studies (4, 38) correlated an aging-associated drop of IGF-I levels with an elevated risk of developing heart failure. To investigate the impact of age dependence on cardiomyocyte-specific IGF-I signaling, iCMIGF-IRKO mice were generated in aged adult mice (11 mo at induction of KO). We first assessed to what extent aging altered cardiac performance and compared data measured in young and aged mice. LV pressure data revealed an impairment of cardiac function in older mice compared with 3-mo-old mice. This finding is illustrated by a rise in Pead (control 3 mo: 0.3 ± 1.5 mmHg; control 11 mo: 2.7 ± 1.8 mmHg, P = 0.01) and Tau (control 3 mo: 2.5 ± 1.2 ms; control 11 mo: 4.5 ± 1.7 ms, P = 0.02) and a decline of dP/dt max (control 3 mo: 9,806 ± 2,502 mmHg/s; control 11 mo: 7,759 ± 1,352 mmHg/s, P = 0.03; Fig. 2).

When we compared control and iCMIGF-IRKO mice, induction of the IGF-IR-deletion led to functional differences between the two groups (Fig. 3). End-systolic pressure (Pead) dropped from 73.3 ± 8 mmHg in control animals to 68.4 ± 6.27 mmHg in iCMIGF-IRKO. Whereas this difference was only minor, dP/dt min was reduced by 25% from −6,090 ± 1,310 mmHg/s in controls to −4,618 ± 438 mmHg/s; n = 8–13, P = 0.01 in iCMIGF-IRKO. Cardiac function of old control and iCMIGF-IRKO mice was also analyzed by MRI (Fig. 4). The analysis did not reveal any differences with respect to LV volumes and wall thickness.

Histological studies, which were performed in young adults as before, displayed no changes in CM diameter (control 18.6 ± 2.5 μm, n = 8; KO 17.6 ± 1.4 μm, n = 6), the content of collagen fibers (Sirius red staining), or the rate of apoptosis (activated caspase-3, TUNEL) in the hearts of old iCMIGF-IRKO mice.

Insulin receptor signaling as a compensator of a defect IGF-IR signaling? The unexpected finding of a low impact of IGF-IR signaling in the adult murine heart in iCMIGF-IRKO mice led us to consider that the IGF-IR signaling might be compensated for by another signaling pathway. As insulin receptor and IGF-IR share a high degree of homology (37) and are known to form even hybrid receptors in the heart (2), the insulin receptor signaling pathway was a likely candidate to compensate for a defect of IGF-IR signaling.

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**Table 1. Plasma levels of IGF-I and IGFBP1 and -3 and insulin in WT and iCMIGF1RKO under basal conditions and STZ treatment**

<table>
<thead>
<tr>
<th>Condition</th>
<th>IGF-1 (pg/ml)</th>
<th>IGFBP1 (pg/ml)</th>
<th>IGFBP3 (pg/ml)</th>
<th>Insulin (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Basal</td>
<td>268 ± 44</td>
<td>863 ± 424</td>
<td>301 ± 169</td>
<td>635 ± 353</td>
</tr>
<tr>
<td>iCMIGF-IRKO Basal</td>
<td>246 ± 28</td>
<td>887 ± 469</td>
<td>326 ± 86</td>
<td>548 ± 422</td>
</tr>
<tr>
<td>WT STZ</td>
<td>247 ± 61</td>
<td>2199 ± 1396</td>
<td>176 ± 244</td>
<td>242 ± 157</td>
</tr>
<tr>
<td>iCMIGF-IRKO STZ</td>
<td>310 ± 40</td>
<td>2779 ± 1266*</td>
<td>473 ± 292</td>
<td>209 ± 163</td>
</tr>
</tbody>
</table>

Data represent means ± SD in pg/ml; n = 6 samples each. iCMIGF-IRKO, inducible, cardiomyocyte-specific IGF-I receptor (IGF-IR) knockout (iCMIGF-IRKO) mice; STZ, streptozotocin. *P < 0.05 vs. basal.

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**Table 2. MRI measurements in young adult animals 3 mo after KO induction**

<table>
<thead>
<tr>
<th>Condition</th>
<th>EDV, μl</th>
<th>ESV, μl</th>
<th>EF, %</th>
<th>SV, μl</th>
<th>LV weight index, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48 ± 11</td>
<td>14 ± 4</td>
<td>71 ± 5</td>
<td>34 ± 8</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>iCMIGF-IRKO</td>
<td>47 ± 7</td>
<td>15 ± 4</td>
<td>68 ± 3</td>
<td>32 ± 4</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

Data are means ± SD; n = 8. NS, not significant; SV, stroke volume; EDV, end-diastolic volume; ESV, end-systolic volume; LV, left ventricular.

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**Table 3. LV in vivo pressure measurements in young adult animals 3 mo after KO induction**

<table>
<thead>
<tr>
<th>Condition</th>
<th>n</th>
<th>dP/dt max, mmHg/s</th>
<th>dP/dt min, mmHg/s</th>
<th>Pead, mmHg</th>
<th>Tau, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>6,899 ± 1,929</td>
<td>9,806 ± 2,502</td>
<td>81 ± 13</td>
<td>2.5 ± 1.2</td>
</tr>
<tr>
<td>iCMIGF-IRKO</td>
<td>7</td>
<td>6,798 ± 1,348</td>
<td>9,437 ± 1,646</td>
<td>81 ± 9</td>
<td>3 ± 2</td>
</tr>
</tbody>
</table>

Data are means ± SD. Pead, end-systolic pressure; Pmax, end-diastolic pressure; Tau, relaxation time constant; SV, stroke volume; HR, heart rate.
To investigate a possible compensation, we inactivated pancreatic β-cells by repeated STZ injection, aiming at extinction of insulin signaling. After the STZ injection, blood glucose levels increased substantially over the basal control levels, and mice developed glucose intolerance. However, since blood glucose levels varied between 200 and 600 mg/dl after the treatment, animals were subdivided into a “low” (≤200 mg/dl) and a “high” blood glucose group (≥300 mg/dl) as measured 125 min after glucose challenge. Low-glucose animals served as controls. Despite the very early stage of diabetes, the catheter measurements already revealed the development of a diastolic dysfunction in the high-blood-glucose group (low- vs. high-blood-glucose: control Ped 1.57 ± 0.65 vs. 4.33 ± 0.91 mmHg, P < 0.01; control Tau 4.65 ± 1.02 vs. 5.77 ± 0.64 ms, P = 0.08; KO Ped 1.36 ± 0.19 vs. 3.93 ± 1.54 mmHg, P < 0.05; KO Tau 3.16 ± 2 vs. 5.94 ± 0.79 ms, P = 0.05). Despite the depressive effects of high glucose levels on cardiac function, no differences between control and iCMIGF-IRKO animals were observed. MRI data were also collected 4 wk after induction of diabetes; however, no differences in cardiac mass, EDV, ESV, EF, and SV were detected.

Histological studies, which were performed as before, displayed no changes in CM diameter due to diabetes (control 16.9 ± 1.1 μm, n = 7; KO 17.5 ± 2.6 μm, n = 9), the content of collagen fibers (Sirius red staining), or the rate of apoptosis (activated caspase-3, TUNEL) in the hearts of diabetic iCMIGF-IRKO mice.

Isoproterenol-induced cardiac hypertrophy. Since cardiomegaly-specific overexpression of IGF-I in mice had a remarkable cardiac protective effect, we further investigated whether iCMIGF-IR might aggravate the cardiac dysfunction after induction of pathological hypertrophy. Sequential MRI measurements (Fig. 5) immediately before (day 0) and ~8 wk after (day 55) 4-OHTX treatment were performed. Then we induced cardiac hypertrophy by chronic isoproterenol (ISO) treatment (20 mg·kg⁻¹·day⁻¹) and measured again 14 days later (day 70). As shown in the representative MRI measurements of an iCMIGF-IRKO mouse (Fig. 5F), taken before (basal, day 55) and after 14 days of ISO treatment (ISO, day 70), chronic β-adrenergic stimulation induced cardiac hypertrophy and LV dilatation that was evident in systole and diastole. In control mice (n = 9), LV mass index increased by
The GeneSpring V.11 software (Agilent). Figure 6 shows that altered genes were distributed among many GO terms major clusters belonging to specific gene ontology (GO) terms. Annotation of the differentially expressed genes revealed no functional profile. As is evident from the color code, most of the alterations were only modest and below a factor of 2. Functional analysis led to the identification of two major clusters, being comprised of WT and iCMIGF-IRKO arrays; 348 differentially expressed genes (348K v2) were found to be enriched among the altered transcripts. With low levels of significance. Except for a downregulation of Acsm3, encoding medium-chain acyl-CoA synthetase, no other genes directly involved in fatty acid or glucose metabolism were found to be altered. Similarly, network analysis did not uncover major networks of coregulated genes that were affected by the receptor inactivation.

**DISCUSSION**

Numerous studies in worms, flies, and mice have demonstrated that IGF-I/IGF-IR signaling plays an important role in the regulation of cellular function, organ growth, and aging. The importance of this signaling pathway is underlined by the smaller size of newborn constitutive IGF-I/IGF-IR knockout mice and their perinatal death (3, 22, 29). It is well known that constitutive KO mice may activate compensatory mechanisms (8, 10) that might hide a phenotypic alteration. To study the function of IGF-IR-mediated signal transduction in the adult heart, we generated a tamoxifen-inducible knockout model that allows the inactivation of the IGF-IR gene at chosen time points in the adult heart. The combination of αMHCmerCremer mice (34) and exon 3-floxed IGF-IR (35) mice enabled precise manipulation of the cardiac IGF-IR gene. Using a sensitive PCR for the excision of exon 3, we were unable to detect recombination events in the hearts of αMHCmerCremer/loxP/loxP mice in the absence of tamoxifen, confirming that the merCremer fusion protein was silent. However, repetitive injection of 4-OHTX resulted in an efficient excision of exon 3 in the IGF-IR gene as shown on the protein and RNA levels. Moreover, the substantial reduction of Akt and GSK3β phosphorylation in response to an IGF-I challenge clearly showed that also on the functional level the knockout induction protocol was successful.

Despite the efficient inactivation of the IGF-IR gene, young adult mice did not develop cardiac structural or functional alterations, as assessed by MRI and LV P/V catheter measurements. This finding was surprising, because many cardiac functions of IGF-I had been proposed: inhibition of apoptosis, regeneration after myocardial infarction (28, 32), modulation of cardiac contractility (19), regulation of cardiac growth (22, 29), and hypertrophy in response to exercise (15). If these functions also operated in response to endogenous IGF-I levels through cardiomyocyte IGF-IRs, then one or several of the following phenotypic alterations would have to be expected in iCMIGF-IRKO hearts: atrophy of cardiomyocytes, exacerbated cardiac dysfunction and remodeling under pathological conditions, decreased cardiac contractility, and enhanced apoptosis. At least in young adult mice, none of these effects were observed when the IGF-IR was inactivated. However, IGF-I signaling has also been associated with an age-dependent decline in cardiac function and an elevated risk of developing heart failure with increasing age (4, 5). When we inactivated the IGF-IR gene in older mice, we found a more pronounced depression of cardiac function in iCMIGF-IRKO mice than in their age-matched controls. In general, hearts of control and iCMIGF-IRKO mice performed worse than those of young mice. However, the diastolic parameters dP/dt and Tau were more compromised in the old iCMIGF-IRKO group, indicating a reduced relaxation in iCMIGF-IRKO hearts exceeding that of old control mice. Since the observed phenotype involved a reduced diastolic function, we conclude that IGF-IR signaling...
appears to sustain contractile function in the heart of aging mice. A hallmark of diastolic dysfunction is increased myocardial stiffness, which may be caused by alterations of the extracellular matrix or by intrinsic factors residing in cardiomyocytes. Among the latter, reduced Ca\textsuperscript{2+} removal, slower detachment during the cross-bridge cycle (7), and modulation of titin’s structure (25) or phosphorylation (17) may have a major impact on LV relaxation. It is unclear which of these mechanisms might be involved in the age-related decline associated with defective IGF-IR-dependent signaling. However, it is interesting to note that cardiomyocyte-specific overexpression of IGF-I was able to suppress the age-related retardation of cytoplasmic Ca\textsuperscript{2+} clearance in old mice (20).

Interestingly, this effect was associated with a lower decrease of Serca2a expression levels in the hearts of IGF-I-overexpressing mice (20), which occurred with aging.

IGF-I signaling is usually linked to the development of adaptive hypertrophy, but overexpression of IGF-I was highly protective in models of cardiac damage. We therefore further investigated whether endogenous IGF-IR-dependent signaling in cardiomyocytes might affect the development of hypertrophy. Chronic ISO application via osmotic minipumps is characterized by a rapid development of hypertrophy associated with substantial cardiac remodeling (27), focal necrosis, and LV dilatation (24) and therefore induces a pathological hypertrophy. When applied to controls and iCMIGF-IRKO mice, ISO increased cardiac mass along with LV dilatation. However, WT and iCMIGF-IRKO mice responded to the same extent to ISO treatment. Thus, loss of endogenous IGF-IR-mediated signaling in cardiomyocytes did not exacerbate the development of a pathological hypertrophic response, or, reciprocally, endogenous IGF-IR signaling did not attenuate the development of a pathological cardiac hypertrophy. In contrast to the cardiac overexpression of IGF-I (32, 40), the endogenously released IGF-I, acting via receptors on the cardiomyocytes, was not sufficient to attenuate myocardial remodeling in the ISO model.

The relatively mild phenotype of iCMIGF-IRKO mice was surprising and prompted us to look for compensatory mechanisms that might have been activated in the hearts after IGF-IR deletion. On the basis of the high degree of similarity between insulin and IGF-I signaling, we hypothesized that signaling via the IR might be a likely candidate to take over functions of the defective IGF-IR signaling. Inactivation of pancreatic β-cells
by STZ led to reduced plasma levels of insulin and the development of glucose intolerance in WT and iCMIGF-IRKO mice. Moreover, we found an increase in the plasma levels of IGFBP1, which had been described before to be associated with type 1 diabetes in STZ-treated rats (23, 26). On the functional level, STZ treatment led to an increase in end-diastolic pressure, indicating a stiffening of the myocardium. This effect was demonstrated in both WT and iCMIGF-IRKO mice and was quantitatively not different. Thus, the removal of insulin signaling elicited an effect on cardiac function that was not augmented by the concomitant loss of the IGF-IR. Therefore, we conclude that IR-dependent signaling appears to be more critical for the functional integrity of the adult heart than IGF-IR signaling. A similar conclusion was drawn by Laustsen et al. (18). Those authors used muscle-specific IR (mIRKO) and IGF-IR (mIGF-IRKO) as well as (mIRKO/mIGF-IRKO) double-knockout mice to analyze the impact of both receptors on cardiac structure and function. When inactivated by MCK-

Fig. 6. Hierarchical cluster analysis of genes differentially expressed in control and iCMIGF-IRKO mice. Cluster analysis separated experimental groups according to genotype (control vs. iCMIGF-IRKO). Left: overview over entire heat map of significantly altered genes. Right: magnification of lower part (33 genes). Color coding reaches from ≤4-fold downregulated (green) to ≥4-fold upregulated (red) in iCMIGF-IRKO hearts.
driven Cre recombinase, the muscle-specific double knockouts died within 4 wk after birth. The miRKO mice had smaller hearts, whereas the miGF-IRKO mice displayed no size reduction. Thus, even in the newborn mouse, insulin appears to be the more potent growth factor than IGF-I. Although the growth effects observed by Laustsen et al. and our functional data obtained in diabetic mice with an induced KO in adult mice differ substantially, both studies indicate a less critical role of the IGF-IR than IR signaling in the heart.

The mild cardiac phenotype of iCMIGF-IRKO mice was also observed on the molecular level. In the search for possible adaptive mechanisms involving alteration of growth-related genes or metabolic remodeling, array analysis led to a clear distinction of the gene expression patterns of control and iCMIGF-IRKO hearts. Whereas the number of significantly altered genes amounted to ~350, the differences in expression levels were rather small for the majority of the identified genes. This number was reduced to 159 when we considered only genes above the low threshold of ≥1.5-fold change to be differentially expressed. A more detailed analysis of gene ontology (12, 13) to identify possible pathways that might be preferentially affected by the iCMIGF-IRKO failed to detect major functionally related groups. Also, the pathway analysis package in Genespring software did not identify substantially altered gene networks. With the exception of Acsm3, genes encoding enzymes and regulators of glucose and fatty acid metabolism, which are frequently found to be coregulated in myocardial metabolic remodeling, such as PGC-1α, PPARG, PPARG, GLUT4, PDK-4, etc., were not among the altered genes, suggesting that loss of IGF-IR-associated signaling did not lead to a major rearrangement of cardiac metabolism. However, our findings on the gene expression level do not exclude that IGF-IR deletion resulted in altered metabolic profiles that might arise in IGF-IRKO hearts, e.g., due to modulation of key metabolic enzymes on the posttranslational level. Thus, metabolic profiling, which was at the time not feasible due to limited resources in age-matched mice, will be needed to unambiguously resolve this question.

Among the altered transcripts that gave rise to a specific molecular pattern after IGF-IR deletion, there were many unassigned transcripts belonging to the group of RIKEN sequenced transcripts (n = 17), encoding hypothetical proteins (n = 7) and predicted genes (n = 16). Surprisingly, the largest homogeneous group of transcripts encoded olfactory receptors (n = 7). These receptors are also expressed in the heart, but their cardiac function remains elusive. Inactivation of IGF-IR signaling in cardiomyocytes, therefore, induced characteristic but rather dispersed changes in gene regulation. Most importantly, the identified alterations did not indicate a substantial rearrangement of the cardiac gene expression profile that obviously could support the idea of compensatory mechanisms that were activated to preserve cardiac functions including growth, metabolism, or antiapoptotic effects.

On the basis of the findings described in this paper, we conclude that the major function of endogenous IGF-IR signaling in cardiomyocytes of the adult heart is the retardation of an aging-related decline of cardiac function. However, the question remains why IGF-I exerts a substantial cardioprotective and growth-promoting effect when overexpressed but appears to be almost dispensable for the preservation of cardiac structure when the endogenous signaling is inactivated. Most likely, quantitative aspects have to be considered. Assuming that the endogenous basal levels of IGF-I result in a weak activation of the cardiac IGF-I signaling, it could be expected that inactivation of IGF-IR in cardiomyocytes would have little effect, which indeed was observed in this study. On the contrary, the cardiac-specific overexpression models generated so far displayed, in part, even elevated IGF-I plasma levels. Taking into account that under basal conditions the main IGF-I production site is the liver, contributing 75% to the total IGF-I plasma levels (21), an increase of IGF-I plasma levels in transgenic mice demonstrates a substantial increase of local IGF-I levels in the heart. Indeed, four- to eightfold increases of cardiac IGF-I levels, depending on the transgenic model, have been described (6, 31). The constitutively elevated IGF-I levels may lead to a chronic activation of IGF-IRs, which might exceed that of the activation achieved by endogenous IGF-I levels. It is also conceivable that the enhanced IGF-I levels may activate insulin/IGF-I hybrid receptors, which are activated by elevated IGF-I concentrations (33). Moreover, the local overexpression of IGF-I in the heart most likely does not affect only IGF-I signaling in cardiomyocytes. As we have shown in this study the cardiac endothelium represents another cell type expressing relevant levels of IGF-IR. Therefore, paracrine effects of IGF-I, which are not targeted in iCMIGF-IRKO mice, may contribute to the phenotype in IGF-I-overexpressing mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


