Insulin stimulates mitochondrial fusion and function in cardiomyocytes via the Akt-mTOR-NFκB-Opa-1 signaling pathway

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Running title: Insulin controls cardiomyocyte mitochondrial fusion

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ABSTRACT

Insulin regulates heart metabolism through the regulation of insulin-stimulated glucose uptake. Recent studies indicate that it can also regulate mitochondrial function. Relevant to this, mitochondrial function is impaired in diabetic individuals. Furthermore, the expression of Opa-1 and mitofusins, two proteins of the mitochondrial fusion machinery are dramatically altered in obese and insulin resistant patients. Given the role of insulin in the control of cardiac energetics, our goal was to investigate whether insulin impacts mitochondrial dynamics in cardiomyocytes. Confocal microscopy and the mitochondrial dye Mitotracker green were used to obtain three-dimensional images of the mitochondrial network in cardiomyocytes and L6 skeletal muscle cells in culture. Three hours of insulin treatment increased Opa-1 protein levels, promoted mitochondrial fusion, increased mitochondrial membrane potential, and elevated both intracellular ATP levels and oxygen consumption in cardiomyocytes in vitro and in vivo. Consequently, the silencing of Opa-1 or Mfn2 prevented all the metabolic effects triggered by insulin. Finally, we also provide evidence indicating that insulin increases mitochondrial function in cardiomyocytes through the Akt-mTOR-NFκB signaling pathway. These data indicate for the first time that insulin acutely regulates mitochondrial metabolism in cardiomyocytes through a mechanism that is dependent upon increased mitochondrial fusion, Opa-1 and the Akt-mTOR-NFκB pathway.

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INTRODUCTION

Heart requires a constant supply of metabolic substrates and oxygen. Although in the adult heart nearly 70% of energy requirements are met by free fatty acid oxidation, cardiomyocytes have a high metabolic flexibility evidenced by their ability to use different molecules as energetic substrates (1). Although dependence on alternative substrates is well documented under pathological conditions such as cardiac hypertrophy and heart failure (2), there is accumulating evidence that glycolysis-derived ATP is also an important energy source that contributes to the excitation-contraction coupling in healthy cardiomyocytes (3).

In the heart, insulin regulates glucose transport, glycolytic rate, glycogen synthesis, growth, cardiomyocyte contractility and survival (4) acting mainly through the insulin receptor substrate family proteins (IRS-1/2), phosphoinositide 3-kinase (PI3K), Akt (5) and the metabolic signaling hub mammalian target of rapamycin (mTOR) (6). Epidemiological evidence has associated diabetes with a higher risk of cardiovascular disease (7), that is largely attributable to insulin resistance, hyperlipidemia and hyperglycemia (8). Although insulin-mediated glucose uptake is impaired in the heart in obesity and diabetes, changes in insulin signaling to mTOR are less well understood (9). Interestingly, mitochondrial function is impaired in diabetic individuals (10); their skeletal muscle contains fewer mitochondria than age-matched healthy individuals (11) and the expression of optic atrophy protein-1 (Opa-1) and dynamin-related GTPases mitofusin-2 (Mfn2), two proteins of the mitochondrial fusion machinery are dramatically altered in obese and insulin resistant patients (12). Moreover, it has been suggested that mitochondrial dynamics are associated with distinct states of mitochondrial function (13). Thus, we hypothesized that insulin and its downstream signaling pathways regulate cardiomyocyte metabolism by controlling the morphology of the mitochondrial network. Our study
demonstrates that insulin stimulates cardiomyocyte mitochondrial metabolism by promoting mitochondrial fusion via a mechanism involving Akt/mTOR/NFκB, and Opa-1.

RESEARCH DESIGN AND METHODS

Reagents. Insulin was obtained from Novo Nordisk Pharma. Antibodies against Opa-1 (polyclonal and monoclonal), Mfn1, Mfn2, PGC1α and OXPHOS subunits were purchased from Abcam. Phospho-mTOR, mTOR, phospho-Akt, Akt and IκBα antibody were from Cell Signaling and mtHsp70 antibody was from Affinity BioReagents. Tetramethylrhodamine methyl ester (TMRM), Mitotracker green FM and FBS were from Invitrogen. Anti-Drp-1 and Cyt-c antibodies were from Becton-Dickinson, whereas anti-Fis1 antibodies was from ENZO Life Sciences. Anti-β-tubulin antibody, carbonyl cyanide m-chlorophenylhydrazone (CCCP), DMEM, M199 medium, Bay 11-7085 and other reagents were from Sigma-Aldrich Corp. All the inhibitors (Rapamycin, Akti VIII, genistein, Y-294002, cycloheximide and actinomycin D) were from Calbiochem. Protein assay reagents were from Bio-Rad. AsMfn2 and microOpa-1 (Ad5 serotype, both) generation and use was previously described (14). Cardiomyocytes were transduced with adenviral vectors at a multiplicity of infection (MOI) of 1000, except for AdIκBα (MOI 300), 48 h before insulin treatment. Empty adenovirus (mock), AdLacZ or a scrambled microRNA were used as controls.

Culture of cardiomyocytes. Cardiomyocytes were isolated from hearts of neonatal Sprague–Dawley rats as described previously (15). Rats were bred in the Animal Breeding Facility of the University of Chile. All studies conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by our Institutional Ethics Review Committee. Primary cell cultures were
incubated with or without insulin (10 nmol/L) for 0–24 h in DMEM/M199 (4:1) medium containing 10% FBS, in the presence or absence of the different inhibitors.

**Cardiomyocyte transfection.** Small interfering RNAs (siRNA) for Oma-1 I and II and negative control (Mission, Sigma-Aldrich Corp.) were used following the manufacturer's instructions. The siRNAs used for knockdown experiments were as follows: a) Negative control, catalog number SIC001; Oma-1 I, sense (5"-GACCUAACACACUGUCA -3"), antisense (5"-UGACAGUGAUGUGUAGUGUC -3"), Oma-1 II, sense (5"-GCCAUAGAGAGGUCCGGA -3"), antisense (5"-UCCGGACCUCUCUAUGGC -3"). Both siRNA’s were tested, obtaining the best results with the siRNA that we called II, which was the one used for all the subsequent experiments (Supplementary Fig. 4).

**Cell lines culture.** L6 rat muscle cells were maintained in myoblast monolayer culture as previously described (16). For all experiments, cells were seeded in α-MEM 10% FBS for two days. After this period, α-MEM was changed to a 2% FBS for the next two days when experiments were executed. Parallel, HL-1 human cardiomyocytes, were seeded in Claycomb media supplemented with 10% FBS for two days (17), then media was replaced and the experiments performed.

**Mitochondrial dynamics analysis.** Cells were incubated for 30 min with Mitotracker green FM (400 nmol/L) and maintained in Krebs solution. Confocal image stacks were captured with a Zeiss LSM-5, Pascal 5 Axiovert 200 microscope, using LSM 5 3.2 image capture and analysis software and a Plan-Apochromat 63x/1.4 Oil DIC objective, as previously described (15). Images were deconvolved with ImageJ and then, Z-stacks of thresholded images were volume-reconstituted. The number and individual volume of each object (mitochondria) were quantified using the ImageJ-3D Object counter plug-in. Each experiment was done at least four times and
16–25 cells per condition were quantified. An increase in mitochondrial volume and a decrease in the number of mitochondria were considered as a fusion criteria (15,18). Percentage of cells with a fusion pattern was also determined (18).

**Immunofluorescence studies, colocalization analysis and cytochrome-c release.** Cells were fixed, permeabilized, blocked and incubated with primary antibodies (anti-mtHsp70, Drp-1, Fis1 or cytochrome-c). Secondary antibodies were anti-mouse Alexa 456 for mtHsp70 and Drp-1, anti-mouse 488 for cytochrome-c and anti-rabbit IgG 488 for Fis1. For the colocalization analysis only one focal plane was analyzed. Images obtained were deconvolved and background was subtracted using the ImageJ software. Colocalization between proteins was quantified using the Mander’s algorithm, as previously described (15,19). Cyt-c immunofluorescence and redistribution was evaluated by counting the cells with a diffuse pattern (15,20).

**Fluorescence recovery after photobleaching (FRAP).** For FRAP measurements, cells were loaded with 200 nmol/L TMRM for 30 min at 37°C in Krebs solution. TMRM was excited at 561 nm, and fluorescence emission was detected with a 650/710-emission filter. Bleaching of TMRM fluorescence was applied in an approximately 25 µm² square at randomly chosen regions and fluorescence intensity was normalized to the intensity levels before and after bleaching. The fluorescent images were collected every 0.4–2.0 s and analyzed frame by frame with ImageJ software (NIH).

**ATP measurements.** ATP content in cells was determined using a luciferin/luciferase based assay (Cell-Titer Glo Kit, Promega).

**Flow cytometry analysis of Ψmt and mitochondrial mass.** Ψmt and mitochondrial mass were measured after loading cardiomyocytes with TMRM (200 nmol/L, 30 min) or Mitotracker green
FM (400 nmol/L, 30 min). Afterwards, cells were trypsinized and fluorescence was assessed by flow cytometry (excitation/emission = 543/560 and 488/530 for TMRM and Mitotracker green FM, respectively) using a FACScan system (Becton-Dickinson). CCCP 50 µM and oligomycin 10 µM for 30 min were used as positive and negative controls for the mitochondrial membrane potential measurements.

**Mitochondrial DNA quantification.** Mitochondrial DNA (mtDNA) content was determined by quantitative real-time polymerase chain reaction (qPCR), as previously described (21). β-actin and mitochondrial cytochrome b were used as nuclear and mtDNA markers, respectively.

**Quantitative RT-PCR for Opa-1 and Mfn2.** Real-time PCR was performed using SYBR green (Applied biosystems), as previously described (21). Data for each transcript were normalized to both GAPDH and 18S rRNA as internal controls using the 2-ΔΔCt method. Primers used are: Opa-1 Rat Forward 5’ TGACAAACTTAAGGAGGCTGTG 3’; Opa-1 Rat Reverse 5’ CATTGTGCTGAATAACCCTCAA 3’; Mfn2 Rat Forward 5’ CAGCGTCCTCTCCCTCTGAC 3’; Mfn2 Rat Reverse 5’ GGTCCAGGTCAGTCGCTCAT 3’.

**Live cells oxygen consumption determination.** Oxygen consumption determinations in cells were assayed polarographically, (22), using a Clark electrode 5331 (Yellow Springs Instruments).

**Transmission electron microscopy.** Cells were fixed in 2.5% glutaraldehyde in sodium cacodylate buffer, embedded in 2% agarose, post-fixed in buffered 1% osmium tetroxide and stained in 2% uranyl acetate, dehydrated with an ethanol graded series and embedded in EMbed-812 resin. Thin sections were cut on an ultramicrotome and stained with 2% uranyl acetate and lead citrate. Images were acquired on a FEI Tecnai G2 Spirit electron microscope equipped with
a LaB6 source and operating at 120 kV. Measurements of mitochondrial area, circularity and mitochondrial cristae integrated density were made using the Multi measure ROI tool of ImageJ (National Institutes of Health) software.

**Western blot analysis.** Cells and tissues total protein extracts were analyzed as described previously (15). Protein contents were normalized by β-tubulin or β-actin.

**Hyperinsulinemic-euglycemic clamps procedures in mice.** Wild type C57BL6 mice were catheterized under avertin anesthesia, and after a 48 h recovery period, they were fasted overnight and infused with insulin at a constant flow rate and 50% dextrose at a variable rate to maintain glucose at 100–150 mg/dL as described by Huang et al. Glucose was measured at 5 min intervals and glycemia was considered at steady state after three successive equal glucometer readings (23). Mice were sacrificed and their hearts harvested for fiber isolation and Western blot analysis.

**Mitochondrial respiration in permeabilized cardiac fibers.** Mitochondrial oxygen consumption and ATP production were measured in permeabilized cardiac fibers obtained from 5-7 week old mice infused with saline (sham or control) or following euglycemic hyperinsulinemic clamps, using previously described protocols (24).

**Transgenic animals, rapamycin administration and diet.** MyrAkt1 mice were previously generated by K. Walsh (25,26). MyrAkt1 mice have two transgenes: tetracycline transactivator (tTA) driven by α-myosin heavy chain promoter and a myrAkt1 transgene under the 'tet'O promoter. MyrAkt1 mice were fed with a doxycycline (DOX) chow (1 g/kg) until the predetermined time (8 wk old) for myrAkt1 transgene induction. myrAkt1 mice are on a mixed background. Rapamycin (2 mg/kg/day) was administered by i.p. injection 1 d before withdrawal.
of DOX chow and continued until 1 d before tissue harvest. For high-fat diet studies, mice were given a diet containing 36% fat (60% calories from fat) (F3282, Bio Serv., NJ, USA) starting at weaning (27). Body weight was measured weekly.

**Statistical analysis.** Data shown are mean ± SEM of the number of independent experiments indicated ($n$) and represent experiments performed on at least three separate occasions with similar outcomes. Data were analysed by one-way ANOVA and comparisons between groups were performed using a protected Tukey’s test. Statistical significance was defined as $P < 0.05$.

**RESULTS**

**Insulin promotes mitochondrial fusion in cardiomyocytes.** Treatment of rat cardiomyocytes with insulin (10 nmol/L for 3 h) induced the appearance of large interconnected mitochondria, mainly in the perinuclear area of cells (Fig. 1A) and significantly increased the percentage of cells that displayed fused mitochondria from 19±10 to 85±7% (Figure 1B, top panel). Mitochondrial network integrity was assessed by measuring the total number of mitochondria per cell and the volume of individual mitochondrion through 3D reconstitution of confocal stacks (15). Insulin gradually increased mean volume per individual mitochondrion (or detected element), showing a 32% and 129% increase after 0.5 and 3 h of incubation, respectively (Fig. 1B, middle panel), an effect that was reversed after 6 h. Conversely, the number of mitochondria per cell decreased significantly from 150±38 (control) to 61±8 at 3 h of insulin treatment (Fig. 1B, lower panel). To further validate our assay of mitochondrion volume, we also quantified the volume of individual mitochondrion along the different stacks of one micrograph (Fig. 1C, left panel). If the elements were connected during the stack, the software automatically assigned the same colour pattern to the same mitochondrion. With this approach we were able to calculate the
volume for each mitochondrion and build histograms with this information (Fig. 1C, right panel). The treatment of cardiomyocytes with insulin (3 h) changed the distribution pattern of mitochondrion size, decreasing the abundance of smaller mitochondria and increasing the number of mitochondria with intermediate and higher values. With this approach we corroborated the results obtained with the quantification of the mean mitochondrion volume and total number per cell. Thus, in all subsequent experiments we used these values as an index of mitochondrial fusion. A 30-min insulin exposure was both required and sufficient to achieve the subsequent fusion 3 h later (Supplementary Fig. 1A and B), but this effect dissipated by 6 h of insulin treatment (Supplementary Fig. 1C and D). To further support our findings with Mitotracker green fluorescence, we performed the same analysis using indirect immunocytochemistry (IIMH) for mtHsp70 to detect mitochondria (Supplementary Fig. 1E and F). We found that insulin increased mitochondrial mean volume (21%) and decreased the number of mitochondria per cell (from 140±16 to 98±8). Using this technique, the decreases in mitochondrial number where similar to those seen with Mitotracker green, however, the increase in volume was less. We postulate that the difference may be due to morphological changes during the fixation and permeabilization steps required for this method. Despite these limitations, IIMH corroborated the trends documented in live cells with Mitotracker green.

To examine detailed changes in mitochondrion morphology in response to insulin stimulation, we performed electron microscopy analysis. The data showed a significant insulin-dependent increase in the number of large mitochondria and organelle redistribution towards the perinuclear zone (Fig. 1D). In addition, insulin increased mitochondrion size (+107%), decreased circularity index (from 0.78±0.02 to 0.66±0.04) and mitochondrial density (36%). Furthermore,
insulin increased the percentage of cells displaying a dense *cristae* structure (64%), which was quantified using the parameter of integrated density for this organelle (Fig. 1E and F).

In order to evaluate the functional connectivity of the mitochondrial network, we carried out FRAP experiments on cardiomyocytes labeled with TMRM. Figure 1G shows that after randomly bleaching chosen regions in control or insulin treated mitochondrial networks, the rate and magnitude of fluorescence recovery was greater in the insulin treated mitochondria (slope for the 10 first sec after the bleach, control: 0.030±0.007 and insulin: 1.647±1.250, *P* < 0.001) indicating an increased connectivity of the mitochondrial network.

**Insulin increases the levels of the mitochondrial protein Opa-1 and increases mitochondrial function.** In mammalian cells, Mfn and Opa-1 are the main regulators of mitochondrial fusion (28). The two isoforms of Mfn (Mfn1 and Mfn2) are localized to the outer mitochondrial membrane (OMM) (29), whereas Opa-1 is localized to the inner mitochondrial membrane (IMM), where they are required for membrane tethering and fusion. Opa-1, also participates in *cristae* remodelling, an important determinant of mitochondrial metabolism. At least 8 isoforms of Opa-1 exist, grouped as long (L) and short (S), whose distribution depends on the energetic state of the cell. All isoforms are capable of participating in normal IMM fusion (30,31). In the whole heart, 5 Opa-1 isoforms have been detected, although whether there is cell type specificity is not known (32).

To elucidate the mechanism of insulin-dependent mitochondrial fusion, changes in Opa-1 and Mfn2 levels were assessed, as well as the levels and localization of the fission protein Drp-1. Western blot (WB) analysis showed that Drp1, Mfn1 and Mfn2 levels did not change after insulin treatment, whereas the total content of Opa-1 increased (Fig 2A). Two different
antibodies were used to probe for Opa-K1. One, a polyclonal antibody, recognized at least three Opa-K1 isoforms in cardiomyocytes (fold increase with insulin 2.0±0.2) and the other, a monoclonal recognized two bands corresponding to the long (L) and short (S) isoforms of the protein (fold increase with insulin 2.5±0.4) (Fig. 2A and B). Interestingly, mitochondrial localization of the fission protein Drp-1 also decreased in our experimental condition, suggesting that insulin may decrease the rate of mitochondrial fission in addition to stimulating mitochondrial fusion (Supplementary Fig. 2A). Since mitochondrial uncoupling induces Opa-K1 proteolysis (33), cells treated with either insulin or CCCP were analyzed using the polyclonal antibody. Whereas insulin promoted an increase in both L and S isoforms, CCCP decreases total Opa-1. This antibody also detected a nonspecific cross-reacting band that did not change with CCCP (Supplementary Fig. 2B). Because the monoclonal Opa-1 antibody was most effective at detecting early changes in Opa-1 protein levels, this antibody was used in all the subsequent in vitro experiments in cardiomyocytes (except those where the information of different isoforms was important), as well as all the in vivo studies. The polyclonal Opa-1 antibody proved preferable for work with other cell types.

Gene expression analysis using qPCR indicated an increase in the Opa1 mRNA transcripts following insulin treatment (Supplementary Fig. 2C). Accordingly, preincubation of cardiomyocytes with either actinomycin D or cycloheximide promoted mitochondrial fission and abrogated all the insulin-dependent effects on mitochondrial morphology (Supplementary Fig. 2D). Cycloheximide also prevented the insulin-dependent increase in Opa-1 protein, supporting a model in which insulin acts by increasing Opa-1 synthesis rather than inhibiting its degradation (Supplementary Fig. 2E).
To determine whether the changes observed in OpaK1 occurred in conjunction with an increase in mitochondrial biogenesis, we evaluated changes in the total mitochondrial mass by quantifying different constitutive mitochondrial proteins, as mtHsp70 (Fig. 2A) and three core proteins of the oxidative phosphorylation (OXPHOS) machinery. Insulin did not change the levels of any of these mitochondrial components, nor was there a change in the level of PGC1α, a central regulator of mitochondrial biogenesis (Fig. 2C). These results were further confirmed using flow cytometry in cells stained with Mitotracker green and by determining mtDNA abundance (Fig. 2D). These results suggest that insulin increases Opa-1 levels and mitochondrial fusion in cardiomyocytes without altering the overall mitochondrial mass.

Mitochondrial fusion has been associated with increases in mitochondrial OXPHOS (34,35). Therefore, we evaluated Ψmt and cellular ATP content as a measure of mitochondrial function after insulin treatment (Fig. 2E and F). Both Ψmt and ATP levels increased as early as 0.5 h after insulin addition and remained elevated until 3 h (21% and 19%, respectively). However, prolonged stimulation (6 h) resulted in a significant decrease in both parameters. To directly assess changes in mitochondrial OXPHOS we measured cardiomyocytes oxygen consumption rates at baseline and under maximal uncoupling conditions. Insulin promoted a rise in both baseline and CCCP-uncoupled cellular oxygen consumption, increasing the respiratory control ratio from 1.7 to 2.2 after 3 h of insulin (Fig. 2F).

**Insulin stimulates mitochondrial fusion and function in the human cardiomyocyte cell line HL-1 and in rat skeletal muscle myoblasts.** We next proceed to further evaluate the mitochondrial morphology and oxygen consumption rates in human cardiomyocyte HL-1 and rat skeletal muscle L6 cell lines. Under similar experimental conditions, 3 h of insulin treatment (100 nmol/L) we replicated the response of cardiomyocytes in both cell lines (Supplementary
Collectively, these results indicate that insulin controls mitochondrial function and morphology. Studies were then performed to determine if these phenomena were mechanistically linked.

**Knockdown of Opa-1 and Mfn2 suppresses the effects of insulin on cardiomyocyte mitochondrial morphology and function.** To determine if mitochondrial fusion changes are directly related to the effects of insulin on mitochondrial function, we used a microRNA directed against Opa-1 (Supplementary Fig. 4A). Knock-down of Opa-1 in cardiomyocytes increased the percentage of cells with fragmented mitochondria (from 25%±8 to 80%±5), decreased mitochondrion mean volume (from 1,067±66 to 802±91 voxels) and markedly increased the number of mitochondria per cell (from 150±10 to 183±13) relative to cells treated with a scrambled microRNA (Fig. 3A and B). Opa-1 knockdown prevented all the insulin-induced changes in mitochondrial function (Fig. 3C), suggesting that Opa-1 is necessary for the enhancement in mitochondrial function observed after insulin treatment.

Since Opa-1 also controls mitochondrial cristae remodelling (31), which might have a direct effect on mitochondrial metabolism, independent of its role in fusion, we also increased mitochondrial fission using an antisense adenovirus against Mfn2 (AsMfn2) (Supplementary Fig. 4B) (15). AsMfn2 induced mitochondrial fragmentation, decreased the mitochondrial mean volume by 25% and also prevented the increase in mitochondrial metabolism in response to insulin (Fig. 3D, E and F), supporting our hypothesis that mitochondrial fusion is a critical element in the metabolic effect of insulin in cardiomyocytes.

In order to establish a direct relationship between the rise in the Opa-1 protein levels and increased mitochondrial fusion and function, we down-regulated the mitochondrial protease
Oma-1 in cardiomyocytes. We hypothesize that the down-regulation of this protease, known as a regulator of the Opa-1 levels in mitochondria (33,36), could transiently increase Opa-1 levels, enhancing mitochondrial fusion and function. We used two different siRNAs, obtaining the best results with the second one in terms of Oma-1 knock down, however, knockdown of Oma-1 with either lead to an increase in Opa-1 (Supplementary Fig. 4C). Oma-1 knock down itself stimulated mitochondrial fusion, measured as an increase in mitochondrial mean volume and a decrease in the number of mitochondria per cell (Supplementary Fig. 4D and E), as well as boosting mitochondrial respiration (Supplementary Fig. 4F). Pre-treatment with rapamycin did not alter the outcome of Oma-1 knockdown.

Interestingly, Opa-1 can also help preserve cardiomyocyte survival, as si-RNA against Opa-1, decreases cellular survival in cells treated with C2-ceramides, an apoptotic factor known to cause cellular death through mitochondrial fragmentation (15,20). Insulin treatment protected cardiomyocytes from C2-ceramides, whereas insulin protection was lost with knock down of Opa-1 (Supplementary Fig. 4G).

**Insulin increases Opa-1 and mitochondrial function in vivo.** To evaluate the effects of insulin in vivo, C57BL6 mice were subjected to hyperinsulinemic-euglycemic clamps (23). After 2 h of treatment, hearts were harvested and used for protein analysis and oxygen consumption determination in permeabilized cardiac fibers. Akt Ser473 phosphorylation in hearts was significantly increased relative to sham controls, showing that insulin signaling was activated during the glucose clamp (Supplementary Fig. 5A). Using the monoclonal Opa-1 antibody, the euglycemic-hyperinsulinemic clamp increased the total levels of Opa-1 protein by 1.8±0.1 fold without changing Mfn2 levels (Fig. 4A and B). Similar changes were observed when analyzing the mRNA levels for Opa-1 and Mfn2 (Fig. 4C). As shown in Fig. 4D, permeabilized fibers
obtained from insulin-perfused mice also exhibited enhanced ADP-stimulated (state 3) mitochondrial respiration rate (38%), ATP synthesis (53%) and ATP/O ratio (33%) following incubation with palmitoyl-carnitine. Furthermore, hearts from mice on a high fat diet for 16 weeks showed decreased Opa-1 protein levels compared with regular diet controls (Supplementary Fig. 5B and C). These results provide evidence that insulin stimulation of mitochondrial function is correlated with an increase in Opa-1 protein levels in vivo, and that loss of insulin sensitivity may lead to a decrease in Opa-1.

**Insulin controls mitochondrial morphology and function through the Akt-mTor-NFkB signaling pathway.** Insulin controls metabolic homeostasis via the IRS–PI3K–Akt signaling cascade, acting in part through FOXO1 and mTOR (37). Whether this pathway plays a role in the regulation of mitochondrial morphology and function remains unknown. We used a transgenic model where inducible Akt activation in the heart was achieved in mice expressing a myrAkt1 transgene (25,26). Akt activation, following DOX withdrawal triggered an increase in Opa-1 protein levels by 1.40±0.02 fold relative to WT littermates. This change was inhibited by treating the mice with rapamycin (Fig. 4E and 4F). Moreover, rapamycin inhibited the phosphorylation of S6K (a downstream effector of mTOR), but had no effect on Akt phosphorylation (Fig. 4E, G and H).

Similar to the in vivo findings, insulin treatment of cultured cardiomyocytes induced a significant increase in both Akt and mTOR phosphorylation at 0.5 and 3 h (Fig. 5A and B). Pre-incubation with rapamycin (100 nmol/L) inhibited insulin-induced mTOR phosphorylation (Fig. 5B). After 6 h of insulin treatment, phosphorylation of both proteins decreased in agreement with prior evidence suggesting that prolonged insulin stimulation leads to Akt desensitization (38,39). Consistent with this, a second insulin pulse (insulin 10 nmol/L for 15
min) after a prolonged insulin exposure (6–24 h) showed a lower Akt phosphorylation in comparison to cells not previously treated with the hormone (Fig. 5C). To correlate the results obtained in mice with those obtained in cells, we used pharmacological inhibitors to determine if Akt and mTOR signaling were involved in the increase in mitochondrial fusion following insulin. Both Akti (a specific inhibitor of Akt1/2) and rapamycin fully suppressed the insulin-dependent increase in mitochondrion mean volume as well as the decrease in the number of mitochondria per cell (Fig. 5D). The general inhibition of the insulin receptor and PI3K, using genistein and LY-294002, respectively, showed this same inhibitory effect (Supplementary Fig. 6A). These results confirm the involvement of the IRS-PI3K-Akt-mTOR signaling pathway in regulating mitochondrial morphology. Combined use of Akti and rapamycin had no synergistic effect (Supplementary Fig. 6B), consistent with mTOR acting downstream of Akt in cardiomyocytes (40,41). Preincubation of cardiomyocytes with cytochalasin B (a Glut transporter inhibitor) diminished mitochondrial fusion response to insulin (Supplementary Fig. 6C). This result supports previous studies, demonstrating the need for glucose uptake for full mTOR activation (41). Inhibition of Akt and mTOR with Akti or rapamycin completely abolished the insulin-dependent Opa-1 increase (Fig. 5E).

Because mTOR appeared to be required for insulin to influence mitochondrial morphology and metabolism, we assayed the effect of rapamycin treatment on Ψmt, intracellular ATP levels, and oxygen consumption. Fig. 5F shows that the ability of insulin to increase mitochondrial function was completely abrogated after rapamycin preincubation. Collectively, these data strongly suggest that insulin stimulation enhances mitochondrial function in cardiomyocytes via a mechanism that is mediated in part by an mTOR dependent increase in Opa-1 regulated mitochondrial fusion.
Currently, transcriptional factors involved in the regulation of Opa-1 expression remain unknown. In light of previous reports describing Akt-mTOR-dependent regulation of the transcription factor NFκB (42) and its demonstrated ability to influence insulin responses and mitochondrial function (43), we investigated the relationship between NFκB and mitochondrial morphology. As shown in the Supplementary Figs. 2D and 7A-B, treatment with actinomycin D to inhibit transcription inhibited the insulin-dependent increase in Opa-1 protein levels and mitochondrial fusion. In silico analysis indicated the presence of an evolutionarily conserved NFκB binding site in the Opa-1 promoter (Supplementary Fig. 7C). Moreover, insulin decreased the protein levels of the NFκB repressor IκBα, consistent with an increase in NFκB activity (Supplementary Fig. 7D). Both mitochondrial fusion (Fig. 5G) and the increase in Opa-1 protein, induced by insulin (Fig. 5H) were abolished by the NFκB inhibitor, Bay 11-7085. Expression of a NFκB super-repressor also cancelled the Opa-1 increase induced by insulin (Fig. 5I). Together these data suggest that insulin-mediated changes in Opa-1 protein levels and mitochondrial fusion are influenced by NFκB, acting downstream of the Akt-mTOR pathway.

DISCUSSION

In cardiomyocytes, mitochondrial dynamics are emerging as fundamental biological processes, important for controlling not only mitochondrial shape but also its function, which can have a key role in determining cell survival. A full understanding of the contribution of mitochondrial dynamics to cardiac physiology and function is still lacking. Our findings are the first to show that insulin controls mitochondrial dynamics in cultured cardiomyocytes by stimulation of mitochondrial fusion without changing mitochondrial biogenesis. This process is
controlled by an increase in Opa-1 protein leading to an enhanced mitochondrial function. These changes are, at least in part, dependent on the Akt-mTOR-NFκB pathway.

One of the most interesting findings of our study is the increase in the levels of the Opa-1 protein in response to insulin. Mammalian Opa-1 has been studied in in vitro cell culture systems and beyond its role in mitochondrial fusion, it has been suggested to play roles in preventing apoptosis and maintaining mtDNA (44). Opa-1 also controls cristae structure of the IMM and thereby the release of cytochrome c during apoptosis (44). However, studies focusing on the role of Opa-1 in mammalian tissues have been limited, as homozygous mutations of Opa-1 lead to embryonic lethality in mice. Mice with heterozygous mutations of Opa-1 show normal phenotypes except for age-dependent degradation of optic nerves (45,46). Recent studies showed that dysregulation of Opa-1 in Oma1 knockout mice induced insulin resistance and impaired glucose homeostasis and thermogenesis. Importantly, Oma1−/− mice develop metabolic defects similar to those seen with high fat feeding, illustrating the importance of a proper balance between long and short forms of Opa-1 for maintaining mitochondrial function (47). In Drosophila, an Opa-1 heterozygous mutation is associated with decreased heart rate and cardiac arrhythmia (48). In adult mice with decreased Opa-1 expression (Opa-1−/−), oxidative and respiratory capacity changes only after an important hemodynamic stress. These changes likely result from incompletely understood compensatory adaptations, that help to maintain a fused mitochondrial morphology prior to stress (49). The results presented in our work, establish for the first time, a role for the Opa-1 protein in insulin-dependent control of muscle cell metabolism. These data provide novel insights that may link the changes in Opa-1 levels observed in obesity and diabetes with the pathogenesis and progression of insulin resistance. Our results complement the studies of Keller et al., who showed that islet Opa-1 levels de cease
before the onset of diabetes in ob/ob mice (50), and with a more recent that used the Cre-loxP system to delete mouse Opa-1 in pancreatic beta cells. In this model, Zhang et al., showed that beta cells lacking Opa-1 maintained normal mtDNA copy numbers; however, the amount and activity of electron transport chain complex IV were significantly decreased, leading to impaired glucose-stimulated ATP production and insulin secretion (51). These findings in beta cells are consistent with the data from cardiomyocytes presented in our study.

Nowadays, there is strong evidence implicating mitochondrial dysfunction in diseases such as diabetes mellitus and insulin resistance, however, the relationship between mitochondrial dynamics, mitochondrial biogenesis, and mitophagy in the pathophysiology of these disorders is incompletely understood. In cardiomyocytes, these important aspects of mitochondrial biology have only recently begun to be addressed. Progress may have been limited by the general perception that the highly structured organization of adult ventricular cardiomyocytes prevents mitochondrial dynamics from playing a relevant role in cellular physiology (52). However, new studies are rapidly changing this assumption. Cardiac tissues contain higher levels of proteins involved in mitochondrial dynamics than other tissues (14,15,53) and in vitro data suggest that hyperglycemia induces mitochondrial fragmentation in cardiac cells, resulting in cell death (18). Moreover, Makino et al. demonstrated that coronary endothelial cells from murine diabetic hearts displayed mitochondrial fragmentation associated with reduced Opa-1 protein levels (54). However, a full understanding of the contribution of mitochondrial dynamics to cardiac physiology and function is still incomplete.

Thus the present study is significant in that we define an important mechanism linking mitochondrial dynamics in cardiomyocytes with the regulation of mitochondrial energy metabolism by insulin. We propose a novel regulatory pathway involved in the control of Opa-1
protein levels that is mediated by Akt, mTOR and NFκB. This signaling module has been implicated in the onset of a variety of metabolic diseases. Our data suggest an important relationship not only between Opa-1, mitochondrial morphology and metabolism, but also with insulin signaling, and potentially with the onset of insulin resistance by revealing the existence of a novel link between mitochondrial morphology and insulin signalling in cardiac and sekeletal muscle cells.

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No potential conflicts of interest relevant to this article were reported.
V.P. carried out project design and all experiments, except those stated separately, and wrote the manuscript; H.V. helped with project design and manuscript writing; M.I. made the qPCR and mtDNA analysis; A. de C. contributed with the L6 experiments; R.T. carried out the mTOR WB; D.L.J. and Y.Z. helped with the in vivo glucose clamps studies in mice and help in the processing of the cardiac samples from those experiments; J.K. helped with Akt Western blots, high-fat diet studies and data discussion; C.P. and C.L. carried out the adenovirus purification and helped with their standardization; F.J. and J.F. helped with the normalization of oxygen consumption measures and data discussion; E.N. built the Opa-1 micro RNA adenovirus; M.C. guided the adenovirus purification and helped with figures preparation; D.A,B contributed with the high-fat diet studies; A.K. contributed with data discussion and manuscript edition; J.A.H. and B.A.R. helped with electron microscopy studies, FRAP experiments and data discussion; E.D.A. conceived the design and discussion of the in vivo experiments with transgenic animals and rapamycin administration; A.Z. provided all the adenovirus, discussed the data and helped in the conceiving of the project and S.L. conceived and supervised the whole project, discussed the data and edited the final manuscript. S.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

FIG 1. Insulin increases mitochondrial fusion in cultured cardiomyocytes. A: Time course of insulin on mitochondrial morphology. Cells were incubated with insulin (Ins, 10 nmol/L) at indicated times and then loaded with Mitotracker green. Multi-slice imaging reconstitution was obtained by confocal microscopy to show mitochondrial morphology. The scale bar is 10 µm. B: Percentage of cells with fused mitochondria; the individual mitochondrial volume and the number of mitochondria per cell were determined. Values are the mean ± SEM, n=4, *P < 0.05, **P < 0.01 and ***P < 0.001 vs. 0 h, ##P < 0.01 and ###P < 0.001 vs. 3 h. C: Representative Z-stack reconstruction of individual mitochondrial structures and volume histograms of the representative images, n=4. D: Representative transmission electron microscopy images from control or treated cells. Three different magnifications of the same cell are shown. Red arrows indicate fused mitochondria. E: Mitochondrial area, circularity index, the number of mitochondria per mm² and, F: the percentage of mitochondria with dense cristae and the cristae integrated density were quantified from the images in D. Data were obtained from 100 mitochondria from each control and insulin treated cells examined from three separate experiments. Values are the
mean ± SEM, n=3, *P<0.05 and **P<0.01 vs. Ctrl. G: Fluorescence recovery after photobleaching (FRAP) analysis of the mitochondrial network (upper panel). Bleaching of TMRM fluorescence was applied in an approximately 25 µm² square at randomly chosen regions where indicated (bleach), and fluorescence intensity was normalized to the intensity levels before and after bleaching. Data were obtained from 15 cells examined from three separate experiments.

FIG. 2. Insulin increases the levels of the mitochondrial Opa-1 protein without changing mitochondrial total mass. A: Total protein extracts were prepared from cells incubated with insulin (10 nmol/L) for the indicated times. Opa-1, Drp-1, mtHsp70, Mfn1, Mfn2 and β-tubulin levels were determined by Western blot. For the determination of Opa-1 protein levels two antibodies were used, a polyclonal (first panel) and a monoclonal (fourth panel). Representative Western blots are shown (n=4). Protein content was normalized using anti-β-tubulin. B: Densitometric analysis of normalized Opa-1 levels from total extracts is shown. Data are the mean ± SEM, n=4, *P < 0.05 and **P < 0.01 vs. 0 h. C: Mitochondrial OXPHOS and PGC1α protein levels were analyzed from total protein extracts from cells incubated with insulin (10 nmol/L) for the indicated times. Representative Western blots of fourth with a similar outcome (n=4). D: Mitochondrial mass analysis using the dye Mitotracker green and flow cytometry and real-time PCR for mtDNA. Data are the mean ± SEM, n=5. H: Quantification of ∆Ψm in cells treated with insulin (10 nmol/L) for the times indicated. CCCP 50 µM and oligomycin 10 µM were used as negative and positive controls, respectively. Values are the mean ± SEM, n=5, *P < 0.05, ***P < 0.001 vs. 0 h and #P < 0.05 vs. 3 h. F: Intracellular ATP levels and oxygen consumption determined in control or treated cells with 10 nmol/L insulin for 3 h. For the oxygen consumption assays respiration
was assayed under basal and uncoupled conditions (maximal respiration) using 200 nmol/L CCCP, $n=7$ and $n=4$ for ATP and oxygen measurements, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. 0 h (basal) and #$P < 0.05$ vs. basal insulin respiration 3 h.

FIG. 3. Down-regulation of mitochondrial fusion proteins Opa-1 and Mfn2 alters mitochondrial morphology and the metabolic response to insulin in cardiomyocytes. A: Cells were transduced for 48 h with an adenovirus encoding a scrambled microRNA (miCtrl) or a microRNA against Opa-1 (miOpa-1). Representative confocal images of cells loaded with mitotracker green are shown. Cells were transduced with miCtrl (MOI=1,000) or with the miOpa-1 (MOI=1,000) for 48 h before imaging. Scale bar represents 10 µm.

B: Percentage of cells with fragmented mitochondria (upper panel); individual mitochondrial volume (middle panel) and number of mitochondria per cell (lower panel) were determined for the miControl (gray bars) and miOpa-1 (black bars) treated cells. Values are the mean ± SEM ($n=4$), *$P < 0.05$, **$P < 0.01$ vs. miCtrl. C: After 48 h of transduction with respective adenoviruses, the cells were exposed to insulin (10 nmol/L) for the times indicated and mitochondrial membrane potential (upper panel); intracellular ATP content (lower panel) and oxygen consumption (right panel) were quantified. Values are the mean ± SEM ($n=4$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. miCtrl 0 h, #$P < 0.05$ vs. miCtrl insulin 3 h, δ$P < 0.05$ and δδ$P < 0.01$ vs. respective time with microCtrl. D-F: Cells were transduced for 48 h with an adenovirus coding for an antisense against Mfn2 (AsMfn2) or an empty adenovirus (Mock) and subjected to the same analysis as miOpa-1 treated cells. Analysis of mitochondrial morphology (E) and metabolism (F) was performed as described previously. Cells were transduced with the AsMfn2 (MOI=1,000, gray bars) or
Mock (MOI=1,000, black bars) for 48 h before insulin treatment. Values are shown as mean ± SEM (n=4), *P < 0.05, vs. mock 0 h, #P < 0.05 vs. mock insulin 3 h.

FIG. 4. Insulin regulates Opa-1 protein levels and mitochondrial function in vivo. A: Wild type mice were subjected to hyperinsulinemic-euglycemic clamps for 2 h. Hearts of sham and operated animals were collected and the proteins obtained used for western blot detection of Opa-1, Mfn2 and β-tubulin. B: Quantitative analysis of protein expression is shown. Data are the mean ± SEM, n=6 for each group, *P < 0.05 vs. sham group. C: Opa-1 and Mfn2 gene expression in hearts isolated after 90-min of hyperinsulinemic euglycemic clamp. mRNA was amplified by real-time PCR and normalized to β-actin. Data are the mean ± SEM, n=8, *P < 0.05 vs. control 0 h. D: Cardiac fibers obtained from sham and (2 h)-clamped animals were saponin-permeabilized, incubated with palmitoyl-carnitine and used for the determination of state 3 mitochondrial respiratory parameters, ATP synthesis rate, and ATP/O ratios. Open bars represent fibers obtained from sham hearts (n=6) and black bars fibers from clamped hearts (n=6). State 3 oxygen consumption refers to ADP-stimulated respiration (ADP 1 µmoles/L). Values are the mean ± SEM, *P < 0.05 vs. sham group. E: Opa-1, pAkt, Akt, pS6K and S6K protein levels in myrAkt1 transgenic mice, treated with DOX and Rapamycin (Rapa, 2 mg/kg/day)). Hearts of sham and treated animals were collected and the proteins obtained used for Western blot detection of Opa-1 and β-tubulin. F-H: Quantitative analysis of protein expression is shown. Data are the mean ± SEM, n=4 for each group, *P < 0.05, **P < 0.01 and ***P < 0.001 vs. WT vehicle; #P < 0.01 and ###P < 0.001 vs. myrAkt1 vehicle and &&&P < 0.001 vs. WT Rapa.
FIG. 5. Signaling through the IR-PI3K-Akt-mTOR-NFκB pathway mediates the changes observed in mitochondrial morphology, Opa-1 protein levels and cardiomyocyte mitochondrial metabolism following insulin stimulation. A-C: Time course of insulin-mediated Akt and mTOR phosphorylation. Cardiomyocytes were preincubated with insulin (Ins, 10 nmol/L) and Rapamycin (Rapa, 100 nmol/L) for the times indicated. p-Akt (ser 473), total Akt, p-mTOR (ser 2448) and total mTOR levels were determined by Western blot. Densitometric analysis for normalized p-Akt (A, C) and p-mTOR (B) levels are shown. Data are the mean ± SEM, n=3, *P < 0.05 and **P < 0.01 vs. 0 h. D-E: Effect of pre-incubation with Akti VIII (Akti, 10 µmol/L) or Rapa (100 nmol/L) on the modulation of mitochondrial fusion (D) or Opa-1 levels induced by insulin (E), n=4 and n=3, respectively, *P < 0.05, **P < 0.01 vs. Ctrl and #P < 0.05 and ##P < 0.01 vs. insulin 3 h. F: mTOR inhibition abolishes the metabolic boost induced by insulin treatment. Ψmt (upper panel), intracellular ATP levels (middle panel) and oxygen consumption rate (lower panel) were determined in cells treated with insulin, with (black bars) or without (white bars) Rapa pre-incubation, n=4, *P < 0.05, ***P < 0.001 vs. 0 h and #P < 0.05, ###P < 0.001 vs. insulin 3 h. G-H: Effect of pre-incubation with Bay 11-7085 (10 µmol/L) on the modulation of mitochondrial fusion (G) and Opa-1 levels induced by insulin (H), n=4 and n=3, respectively, **P < 0.01, ***P < 0.001 vs. Ctrl and #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. insulin 3 h. I: Blunted insulin-mediated Opa-1 protein induction by insulin in cardiomyocytes transduced with an AdIκBα adenovirus. Representative Western blots are shown (n=3). Protein content was normalized using anti-β-tubulin. Data are the mean ± SEM, **P < 0.01 vs. AdLacZ without insulin treatment.
Insulin increases mitochondrial fusion in cultured cardiomyocytes.
Insulin increases the levels of the mitochondrial Opa-1 protein without changing mitochondrial total mass.
Down-regulation of mitochondrial fusion proteins Opa-1 and Mfn2 alters mitochondrial morphology and the metabolic response to insulin in cardiomyocytes.
Insulin regulates Opa-1 protein levels and mitochondrial function in vivo.
Signaling through the IR-PI3K-Akt-mTOR-NFkB pathway mediates the changes observed in mitochondrial morphology, Opa-1 protein levels and cardiomyocyte mitochondrial metabolism following insulin stimulation.

204x151mm (300 x 300 DPI)
ONLINE SUPPLEMENTAL FIGURE LEGENDS

FIG. 1. Minimum time and reversibility of insulin-induced mitochondrial fusion in cardiomyocytes. A-B: Cardiomyocytes were treated with insulin (Ins, 10 nmol/L) at indicated times and then visualized after a period of 3 h once they were loaded with mitotracker green. C-D: Cardiomyocytes were treated with a pulse of insulin (Ins, 10 nmol/L) for 30 min and then visualized at the indicated times. For both experiments, multi-slice imaging reconstitutions were obtained by confocal microscopy to quantify mitochondrial morphology. The individual mitochondrial volume and the number of mitochondria per cell were determined. Values are the mean ± SEM, n=4, *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control (Ctrl). E-F: Effect of insulin on mitochondrial morphology assessed by indirect immunofluorescence against mtHsp70. Representative confocal images of mitochondrial morphology in cardiomyocytes stained for mtHsp70 (red) and incubated with insulin (Ins 10 nmol/L, 3 h). The individual mitochondrial volume and the number of mitochondria per cell were evaluated. Scale bar is 10 µm. Values are given as mean ± SEM, n = 4, *P < 0.05 vs. Ctrl and #P < 0.05 vs. Ins 3 h.

FIG. 2. Insulin decreases Drp-1 translocation to the mitochondria and increases Opa-1 protein levels and mRNA expression. A: Control cells or cells incubated with insulin (Ins, 10 nmol/L) for 3 h were stained for Drp-12 (red) or Fis1 (green) to determine Pearson and Mander’s coefficients. The scale bar is 20 µm. Mean values ± SEM, n = 5 (20 cells each one), *P < 0.05; **P < 0.01 vs. 0 h and #P < 0.05 vs. Ins 3 h. B: Total protein
extracts were prepared from cells incubated with insulin (Ins 10 nmol/L, 3 h) or CCCP (50 µmol/L, 0.5 h). Opa-1 and β-tubulin levels were determined by Western blot. The protein contents were normalized against β-tubulin. Data are the mean ± SEM, n=3, **P < 0.01 vs. Control. C: Opa-1 expression in cardiomyocytes after insulin treatment. mRNA was amplified by real-time PCR and normalized to β-actin. Data are mean ± SEM, n=3, *P<0.05 vs. control 0 h, #P<0.05 vs. 3 h. D: Representative confocal images of mitochondrial morphology in cardiomyocytes incubated with insulin (Ins 10 nmol/L, 3 h) with or without a 30 min pre treatment with actinomycin D (Act, 2.5 mg/mL) or cycloheximide (Cyclo, 10 mg/mL). Individual mitochondrial volume and number of mitochondria per cell were evaluated. Scale bar is 10 µm. Values are the mean ± SEM, n=4, *P < 0.05, **P<0.01 vs. Ctrl; ##P< 0.01 and ###P < 0.001 vs. Ins 3 h. E: Effect of Cyclo pre-incubation on the insulin-induced increase in Opa-1 levels (Ins 10 nmol/L, 3 h). Total protein extracts were subjected to Western blot analysis of Opa-1 and normalized to β-tubulin. A representative blot and densitometric analysis are shown. Data are the mean ± SEM, n=3, **P < 0.01 vs. Ctrl without insulin and ##P < 0.01 vs. Ins 3 h.

FIG. 3. Insulin increases mitochondrial fusion and function in HL-1 human cardiomyocytes and L6 rat skeletal muscle cells. A: HL-1 cells were incubated with insulin (Ins, 100 nmol/L) at indicated times and then loaded with mitotracker green. Multi-slice imaging reconstitution was obtained by confocal microscopy to show mitochondrial morphology. The scale bar is 10 µm. B: The individual mitochondrial volume and the number of mitochondria per cell were determined. Values are mean ±
SEM, n=4, *P < 0.05 and **P < 0.01 vs. 0 h. C: Total protein extracts were prepared from cells incubated with insulin (Ins 10 nmol/L, 3 h) with or without a 30 min pre-treatment of the inhibitors Akti VIII (Akti, 10 µmol/L) or rapamycin (Rapa, 100 nmol/L). Opa-1 and β-tubulin levels were determined by Western blot. Opa-1 protein levels in the HL-1 cells were determined using the polyclonal antibody. Representative Western blots are shown (n=4). Protein content was normalized using anti-β-tubulin. Densitometric analysis of normalized Opa-1 levels from total extracts is shown below the Western blot. Data are mean ± SEM, n=4, *P < 0.05 vs. 0 h; ##P < 0.01 and ###P < 0.001 vs. Ins 3 h.

D: Oxygen consumption was determined in control or treated cells with 100 nmol/L insulin for 3 h under basal and uncoupled conditions (maximal respiration) using 200 nmol/L CCCP, n=4, *P < 0.05, **P < 0.01 vs. 0 h (basal) and #P < 0.05 vs. basal insulin respiration 3 h. E-H: L6 skeletal muscle cells were incubated with insulin (Ins 10 nmol/L, 0-6 h) and mitochondrial morphology (n=4), Opa-1 protein levels (n=3) and oxygen consumption (n=3) was determined as described above. *P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 h; #P < 0.05 and ##P < 0.01 vs. Ins 3 h.

FIG. 4. Down-regulation of Opa-1 or Mfn2 protein levels. A-B: Cells were transduced for 48 h with an adenovirus encoding a scrambled microRNA (miCtrl), a microRNA against Opa-1 (miOpa-1), an adenovirus coding for an antisense against Mfn2 (AsMfn2) or an empty adenovirus (Mock). Total extracts (A) or mitochondrial fractions (B) were obtained and Opa-1, β-tubulin, Mfn2 and mtHsp70 protein levels were determined by Western blot (upper panel). The lower panel shows the quantification by densitometric
analysis. Values are the mean ± SEM, n=3. *P < 0.05 vs. miCtrl or mock, respectively.  
C: Cells were transfected for 48 h with two different siRNA’s against Oma-1 (siOma-1 I/II) or a control siRNA (Ctrl). Total extracts were obtained and Opa-1, Oma-1 and β-tubulin were determined by Western blot (left panel). The right panel shows the quantitation by densitometric analysis. Values are mean ± SEM, n=4, *P < 0.05, **P < 0.01 and ***P < 0.001 vs. siRNA Ctrl respectively.  
D-F: Oma-1 siRNA transfected cardiomyocytes incubated with insulin (Ins 10 nmol/L, 3 h) or with a 3 h pretreatment with rapamycin (Rapa, 100 nmol/L) were used for mitochondrial morphology analysis (n=4) and oxygen consumption measures (n=3). *P < 0.05, **P < 0.01, vs. siRNA Ctrl; ##P < 0.01 and ###P < 0.001 vs. siRNA Ctrl Rapa.  
G: Effect of Oma-1 knock down and insulin treatment (Ins 10 nmol/L, 3 h) on the redistribution of cytochrome-c in cells treated with C2-ceramide (C2, 20 µmol/L, 6 h). Scale bar = 20 µm, n=5, **P < 0.05, ***P < 0.01 vs. miCtrl, #P < 0.05 vs. miCtrl C2 6 h and &P <.0.05 vs. miCtrl C2 + Ins.  

FIG. 5. Insulin induces phosphorylation of Akt in the heart in vivo.  
A: Wild type mice were subjected to hyperinsulinemic-euglycemic clamps for 2 h. Hearts of sham and operated animals were collected and the proteins obtained, used for Western blot detection of phospho-Akt (serine 473) and Akt. Representative Western blots are shown (n=3).  
B-C: Wild type mice were subjected to a regular (RD) or high fat diet (HFD) for 16 weeks. Hearts of RD and HFD mice were collected, and protein extracts were obtained and used for Western blot detection of Opa-1 and β-tubulin. The lower panel
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shows the quantification by densitometric analysis. Values are mean ± SEM, \( n=3 \) and \( n=4 \) for RD and HFD, respectively, *\( P < 0.05 \) vs. RD.

**FIG. 6.** Effects of various inhibitors on the insulin-mediated mitochondrial fusion. *A-C:* Representative confocal images of mitochondrial morphology in mitotracker green-loaded cardiomyocytes incubated with insulin (Ins 10 nmol/L, 3 h) with or without a 30 min pre treatment with genistein (Gen, 50 µmoles/L), LY-294002 (LY, 50 µmol/L), Akt-VIII (Akti, 10 µmol/L) and rapamycin (Rapa, 100 nmol/L) or cytochalasin B (Cyto, 10 µmol/L). The individual mitochondrial volume and the number of mitochondria per cell were evaluated for all the treatments. Scale bar is 10 µm. Values are the mean ± SEM, \( n=4 \), *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) vs. Ctrl; *\( P < 0.05 \), **\( P < 0.01 \) and ***\( P < 0.001 \) vs. Ins 3 h.

**FIG. 7.** Transcriptional regulation of Opa-1 expression in response to insulin. *A:* Effect of actinomycin D (Actino, 2.5 mg/mL) pre-incubation on the insulin-dependent increase in Opa-1 levels (Ins 10 nmol/L, 3 h). Total protein extracts were subjected to Western blot analysis of Opa-1 and normalized to β-tubulin. A representative blot and densitometric analysis are shown. Data are the mean ± SEM, \( n=3 \), ***\( P < 0.001 \) vs. Ctrl without insulin and **\( P < 0.01 \) vs. Ins 3 h. *B:* Opa-1 expression in cardiomyocytes after actinomycin and insulin treatment. mRNA was amplified by real-time PCR and normalized to β-actin. Data are the mean ± SEM, \( n=3 \), **\( P < 0.01 \) vs. Ctrl without
insulin and $^P < 0.05$ vs. Ins 3 h. C: NFκB binding site and its conservation in the Opa-1 promoter sequence. D: Effect of insulin on IkBα protein levels. Cardiomyocytes were preincubated with insulin (Ins, 10 nmol/L) and IkBα and β-tubulin levels were determined by Western blot. Protein contents were normalized against β-tubulin. Values are the mean ± SEM, $n=5$, *$P < 0.05$ vs. 0 h; **$P < 0.01$ and ###$P < 0.001$ vs. Ins 0.5 h.
Supplementary Figure 1

A

Mitochondrial volume (voxels x 10^-6)

B

Number of mitochondria per cell

C

Mitochondrial volume (voxels x 10^-6)

D

Number of mitochondria per cell

E

F

Mitochondrial volume (voxels x 10^-9)

Number of mitochondria per cell

With text and annotations as per the figure.
Supplementary Figure 4

A

B

C

D

E

F

G

Supplementary Figure 4
Supplementary Figure 5

A

|        | Sham | Clamp |
|--------|------|-------|
| pAkt (Ser 473) |      |       |
| Akt    |      |       |

B

Body weight (g)

|        | RD    | HFD   |
|--------|-------|-------|
| ***    |       |       |

C

|        | RD    | HFD   |
|--------|-------|-------|
| Opa-1  |       |       |
| β-tubulin |      |       |

Opa-1 level (fold)

|        | RD    | HFD   |
|--------|-------|-------|
| *      |       |       |
Supplementary Figure 6

A

| Ctrl | Ins | Gen + Ins | LY + Ins |
|------|-----|-----------|----------|

B

| Ctrl | Akti-Rapa | Ins | Akti-Rapa + Ins |
|------|-----------|-----|-----------------|

C

| Ctrl | Cyto | Ins | Cyto + Ins |
|------|------|-----|-----------|

Graphs showing mitochondrial volume (μm² x 10⁴) and number of mitochondria per field (μm² x 10⁴).
Supplementary Figure 7

A

B

C

Rattus norvegicus
Mus musculus
Homo sapiens

-298 TGCTGCCGACGTAATATTTACCTGGGGATTTCCCAGCCAGCCAGCCAGCAGCTC -240
-191 GGCTGCCGACTAAATATTAACATTGCCGATGAAATTCTGCCACCCAGCCCAGCCAGTTTCAT -133
-453 TTGGCACTCGAGTTCAGATCCCTGACTCTGATTTCAGGACTTTAGGCTTCTCCATGACTCTTCGCTCG -395

NPxB binding site

D

Time (h)

0 0.5 3 6

0 0.5 1.0 1.5

0 0.5 3 6