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Small heterodimer partner (SHP) contributes to insulin resistance in cardiomyocytes

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Abstract

Small heterodimer partner (SHP) is an atypical nuclear receptor expressed in heart that has been shown to inhibit the hypertrophic response. Here, we assessed the role of SHP in cardiac metabolism and inflammation. Mice fed a high-fat diet (HFD) displayed glucose intolerance accompanied by increased cardiac mRNA levels of Shp. In HL-1 cardiomyocytes, SHP overexpression inhibited both basal and insulin-stimulated glucose uptake and impaired the insulin signalling pathway (evidenced by reduced AKT and AS160 phosphorylation), similar to insulin resistant cells generated by high palmitate/high insulin treatment (HP/HI; 500 µM/100 nM). In addition, SHP overexpression increased Socs3 mRNA and reduced IRS-1 protein levels. SHP overexpression also induced Cd36 expression (-6.2 fold; p<0.001) linking to the observed intramyocellular lipid accumulation. SHP overexpressing cells further showed altered expression of genes involved in lipid metabolism, i.e., Acaca, Acadvl or Ucp3, augmented NF-κB DNA-binding activity and induced transcripts of inflammatory genes, i.e., Il6 and Tnf mRNA (-4-fold induction, p<0.01). Alterations in metabolism and inflammation found in SHP overexpressing cells were associated with changes in the mRNA levels of Ppara (79% reduction, p<0.001) and Pparg (-58-fold induction, p<0.001). Finally, co-immunoprecipitation studies showed that SHP overexpression strongly reduced the physical interaction between PPARα and the p65 subunit of NF-κB, suggesting that dissociation of these two proteins is one of the mechanisms by which SHP initiates the inflammatory response in cardiac cells. Overall, our results suggest that SHP upregulation upon high-fat feeding leads to lipid accumulation, insulin resistance and inflammation in cardiomyocytes.

Key words: Insulin resistance, diabetic cardiomyopathy, nuclear receptors, small heterodimer partner.
1. Introduction

Insulin resistance increases the risk for heart failure, the leading cause of death in subjects with type 2 diabetes (DM2) [1]. The underlying mechanisms involved in the pathogenesis of diabetic cardiomyopathy (DCM) include disturbances in myocardial energy metabolism and a chronic low-grade inflammatory process in cardiac tissue. Diabetic hearts are unresponsive to insulin-stimulation of glucose uptake, whereas (long-chain) fatty acid uptake and utilization are increased. This greater fatty acid influx exceeds the mitochondrial \( \beta \)-oxidative capacity [2], leading to a gradual intramyocellular build-up of inert triacylglycerol stores and bioactive lipid metabolites (diacylglycerols, ceramides). The accumulation of these lipid intermediates in cardiomyocytes elicits insulin resistance, impairing both insulin-stimulated glucose uptake [3] and oxidation [4]. Furthermore, elevated concentrations of these lipid intermediates have been linked to NF-\( \kappa \)B activation [5] and aberrant cytokine production, which may explain the putative link between the onset of chronic low-grade inflammation and metabolic disorders. Over time, both excessive lipid accumulation and progressive insulin resistance in combination with inflammatory processes will gradually initiate a remodelling process in the heart, which at first appears to be a compensatory adaptation, thereafter fostering the development of a functional impairment, i.e., heart failure [1, 6].

At the molecular level, nuclear receptors have emerged as relevant players in the regulation of gene expression profiles in metabolism and inflammation, not only acting as ligand-activated transcription factors, but also interfering with other gene expression regulators independent of their DNA-binding mechanisms. However, the role of nuclear receptors in the regulation of cardiac metabolism still remains partly uncharted. Small heterodimer partner (SHP) (NR0B2, according to the unified nomenclature system for the nuclear receptors [7]) is an atypical nuclear receptor characterized by the lack of a DNA binding domain (DBD) [7]. SHP predominantly acts by regulating the activity of other transcription factors through a number of different mechanisms [8]. Several studies have suggested the possible
involvement of SHP in peripheral insulin resistance (for review see [9]). In liver, SHP inhibits gluconeogenesis [10-15] and improves insulin sensitivity through the inhibition of the signal transducer and activator of transcription 3 (STAT-3), and the expression of suppressor of cytokine signalling 3 (SOCS-3) [16]. In the heart, SHP expression blocks cardiac hypertrophy by interfering with GATA binding protein 6 (GATA6) signalling [17]. Concerning the role of SHP in the regulation of the inflammatory response, conflicting results have been reported in different contexts, i.e., SHP exerts anti-inflammatory effects [18-20], and SHP takes part in NF-κB activation [21-24]. In particular, the role of this nuclear receptor in cardiac glucose/lipid metabolism and inflammation is understudied.

Here, we report that SHP mRNA levels in heart positively correlate with glucose intolerance in high-fat diet (HFD)-fed mice, and that SHP overexpression in HL-1 cardiomyocytes results in insulin resistance and upregulation of the inflammatory processes, which therefore mimics the cellular responses induced by high-palmitate/high-insulin (HP/HI) treatment.

2. Material and methods

2.1. Reagents

2-deoxy-D-[3H]-deoxyglucose was obtained from GE Healthcare. Palmitate, insulin and bovine serum albumin (BSA) were purchased from Sigma.

2.2. Animals

Animal experiments were performed in a well-characterized model [25-27]. Five-week-old CD-1 male mice were maintained on a standard light–dark cycle (12 h light/dark cycle) and temperature (21±1°C) conditions, with ad libitum access to food and water. Animals were randomly distributed in two experimental groups (n = 5 each) and fed with a standard chow diet (STD; Harlan Ibérica S.A.) or a high-fat diet (HFD; 35% fat by weight, 58% kcal from fat; Harlan Ibérica S.A.) for 3 weeks. Before the end the procedure, a glucose tolerance test was performed on mice fasted for 4 h. Briefly, animals received 2 g/kg body weight of glucose by
intraperitoneal injection, and blood was collected from the tail vein after 0, 15, 30, 60, and 120 min. Area Under the Curve (AUC) was determined as a measure of glucose intolerance. After 3 weeks, mice were sacrificed under isoflurane anaesthesia and the hearts were immediately frozen in liquid nitrogen and then stored at -80°C. These experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). All procedures were approved by the University of Barcelona Bioethics Committee, as stated in Law 5/21 July 1995 passed by the Generalitat de Catalunya (Autonomous Government of Catalonia).

2.3. Cell culture

HL-1 atrial cardiomyocytes were kindly provided by dr. W. Claycomb (Louisiana State University, New Orleans, LA, USA), and cultured in Claycomb medium (supplemented with 10% FCS, 0.1 mmol/l noradrenaline [norepinephrine], 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). Rat cardiomyocytes were isolated using a Langendorff perfusion system, as previously described [28] and seeded in laminin coated plates. After 90 min adhesion in modified Krebs–Ringer medium [28], cells were cultured according to the method described by Volz et al. [29] in modified serum-free medium M199 supplemented with 5 mM creatine monohydrate, 3.2 mM carnitine hydrochloride, 3.1 mM taurine, 100 units/ml penicillin and 10 mg/ml streptomycin. Both HL-1 cells and primary rat cardiomyocytes were maintained at 37°C and 5% CO₂. Cells were seeded in multi-well plates and transduced for 48 h with adenoviral particles at a multiplicity of infection (MOI) of 50, as previously described [14]. SHP over-expression was verified by real-time PCR. Alternatively, non-transduced cells were serum deprived in Dulbecco’s Modified Eagle Medium (DMEM; supplemented with 2 mM L-glutamine, 100 µM non-essential amino acids (NEAA), 100 U/ml penicillin and 100 µg/ml streptomycin) for 24 h, and then challenged with high palmitate (500 µM, palmitate:BSA 3:1)/high insulin (100 nM) (HP/HI) for 16 h.
HEK 293T (used to amplify and titrate adenovirus) were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).

2.4. Preparation of Recombinant Adenovirus

For ectopic expression of SHP, adenoviral delivery system was used. Adenoviruses encoding Enhanced Green Fluorescence Protein (Ad), or the full-length human SHP (Ad-SHP), were kindly provided by Dr. Hueng-Sik Choi (Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Gwangju) [10]. Large-scale amplification of adenovirus and viral titers were performed in HEK 293T cells. Briefly, cells were transduced with recombinant adenoviruses (Ad or Ad-SHP) and cells and supernatants were collected once cytopathic effect was observed. After three freeze and thaw cycles, cells debris were pelleted and supernatant containing adenoviral particles were used to determine the MOI by 10-fold serial dilutions.

2.5. Measurement of 2-deoxy-D-[^3H]-glucose uptake

2-deoxy-D-[^3H]-glucose uptake was measured as previously described [30, 31] in HL-1 cells transduced with adenoviral particles, or stimulated with HP/Hi. Briefly, cells were washed with uptake-buffer (117 mM NaCl, 2.6 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.2 mM MgSO\textsubscript{4}, 10 mM NaHCO\textsubscript{3}, 10 mM HEPES, 1 mM CaCl\textsubscript{2}) containing 4.6 mg/ml BSA, and challenged with 200 nM insulin for 30 min. Subsequently, deoxy-D-glucose was added to a final concentration of 4 µM with tracer amounts of 2-deoxy-D[^3H]-glucose (~2.17 µCi). After 10 min, uptake was stopped with ice-cold stop-solution (uptake-buffer containing 1 mg/ml BSA, 0.2 mM phloretin and 0.1% of DMSO). Then, cells were lysed with 1 M NaOH, and incorporated glucose was measured by scintillation counting of ^3H in a β-counter.

2.6. Immunoblotting
HL-1 cells transduced with adenoviral particles, or stimulated with HP/HI, were challenged with 200 nmol/l insulin for 30 min. To obtain whole cellular extracts RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Igepal, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) containing proteases and phosphatases inhibitors) was used. Protein concentration was measured by the BCA protein assay™ and equal amounts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes. Western blot analyses were performed using antibodies against phospho-Ser\textsuperscript{473}-AKT, AKT (Cell Signaling), phospho-Thr\textsuperscript{642}-AS160 (AKT substrate of 160 kDa), AS160 (Upstate), Insulin receptor substrate 1 (IRS-1) (Cell Signaling), succinate dehydrogenase complex iron sulfur subunit B Complex II (CII-SDHB) (Abcam) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling). Detection was performed using the appropriate horseradish peroxidase-labelled IgG and the Chemiluminescent Peroxidase Substrate-1 (Sigma). The size of detected proteins was estimated using protein molecular-mass standards (Thermo Scientific, Waltham, MA USA). Western blot images were analysed with a Molecular Imager (ChemiDoc XRS, BioRad) and quantified with Quantity One® (BioRad).

2.7. Oil-Red-O staining

Lipid content was measured in HL-1 transduced with adenoviral particles using the Oil-Red-O staining. Cells were fixed in ice-cold 4% paraformaldehyde for 15 min and stained with fresh Oil-Red-O (Sigma) solution for 30 min. Nuclei were counterstained with Haematoxylin and cells were mounted with Faramount mounting medium (Dako) after extensive washing. Pictures were taken at 40X magnification with a Nikon digital camera DMX1200 and ACT-1 v2.63 software from Nikon Corporation. The lipid content was quantified by Image J software from five random fields of three different experiments.

2.8. RNA preparation and quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) analysis
Levels of mRNA were assessed by the real time RT-PCR as previously described [31]. Total RNA was isolated using the TRI Reagent (Sigma, Saint Louis, USA) according to the manufacturer’s recommendations. RNA integrity was determined by electrophoresis in agarose gel. Total RNA (1 µg) was reverse-transcribed using the iScript™ cDNA Synthesis Kit (BioRad). Levels of mRNA were assessed by real-time PCR on an ABI PRISM 7900 sequence detector (Applied Biosystems). Primers for SYBR Green real-time PCR analysis are listed in Table 1. Cyclophilin A was used as endogenous control.

2.9. Mitochondrial inner membrane potential

The mitochondrial inner membrane potential were determined using the reagent MitoTracker Red CMXRos (Invitrogen), following the manufacturer’s instructions.

2.10. ATP measurement

Cellular ATP levels were determined using Cell-Titer-Glo® assay (Promega) according to manufacturer’s instructions.

2.11. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained as previously described [32] from HL-1 cells transduced with adenoviral particles. Protein concentration was measured by the BCA protein assay™. EMSA was performed using 5 µg of nuclear extracts and a double-stranded DNA probe containing the consensus binding site for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3'; Promega). DNA probe was labelled with [γ-32P]-ATP by using T4 polynucleotide kinase (Invitrogen) and purified on a Sephadex G-50 column (GE Healthcare). Competition assays were performed by adding an excess of unlabelled probe. Protein-DNA complexes were resolved by electrophoresis at 4ºC on 5% polyacrylamide gels. Gels were dried and subjected to autoradiography using a Storage Phosphor Screen (GE Healthcare). Shifted bands were detected using a Personal Molecular Imager™ System (Bio-Rad).
2.12. Co-immunoprecipitation

Cell nuclear extracts were brought to a final volume of 0.5 mL with IP buffer containing 20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% glycerol 1% Igepal for 6 h at 4 ºC and incubated with 4 µg of anti-p65 or an IgG of the same isotope (antibodies from Santa Cruz). Samples were incubated overnight at 4ºC with protein A-agarose, and agarose beads were collected by centrifugation. Then, pellets were washed with immunoprecipitation (IP) buffer containing protease inhibitors, resuspended in SDS–PAGE sample buffer and boiled for 5 min at 100 ºC. The resulting supernatant was then subjected to electrophoresis on 10% SDS-PAGE and immunoblot analysis.

2.13. Statistical Analyses

Results are expressed as mean ± standard deviation (SD). Significant differences were established by Student’s t-test. The correlation between two variables was evaluated using Spearman’s correlation coefficient. All data were analysed by using the GraphPad Instat programme (GraphPad Software V2.03). Differences were considered significant at p<0.05.

3. Results

3.1. Shp mRNA levels are induced in heart from mice fed with HFD

To explore whether glucose intolerance may be related to changes in cardiac Shp expression, we used hearts from animals in which glucose intolerance was induced by HFD [25]. Interestingly, Shp mRNA levels were up-regulated in hearts from HFD-fed mice compared to animals fed with a chow diet (Δ 1.4 fold, p<0.05) (Figure 1A), and positively correlated to the AUC values from the glucose tolerance test (r=0.6848, p<0.05) (Figure 1B). Therefore, the data suggest that HFD-induced SHP upregulation may either be an innocent secondary effect or directly contribute to the development of cardiac insulin resistance. To further evaluate key downstream factors involved in glucose metabolism, the expression of G6p, Pepck, Glut4 and Pdk4 were analysed, finding a slight but significant reduction (18% reduction, p<0.05) in the Glut4 mRNA levels in the hearts from HFD-fed mice (Figure 1C).
3.2. **SHP overexpression impairs glucose uptake and insulin signalling**

To explore the role of SHP in insulin responsiveness of cardiac myocytes, HL-1 cells were transduced with recombinant adenovirus in order to overexpress SHP. Alternatively, HL-1 cells were challenged with HP/HI to render the cells insulin resistant. Insulin stimulation induced glucose uptake in both non-transduced control (CT) and Ad-GFP (Ad) transduced cells (1.4 fold, p<0.001) (Figure 2A and 2C) showing that insulin responsiveness was not affected due to viral transduction. Interestingly, SHP overexpression caused a reduction in both basal and insulin-stimulated glucose uptake (Figure 2C) similar to the findings in HP/HI challenged cells (Figure 2A). To explore the role of SHP in the insulin signalling pathway, AKT and AS160 phosphorylation were analysed. Consistent with data from Figure 2A and 2C, HP/HI stimulation and SHP overexpression prevented both basal and insulin-stimulated AKT and AS160 (Figure 2B and 2D) phosphorylation, suggesting that impairment in the insulin signalling pathway could be related to the changes observed in cellular glucose uptake. Additionally, SHP overexpression also prevented the insulin-stimulated glucose uptake in primary cultures of rat cardiomyocytes (Figure 2E). To further characterize the role of SHP overexpression in the insulin resistance response in cardiomyocytes, the expression of Socs3, a protein involved in the inhibition of insulin signalling [33], was evaluated. In line with our expectations, SHP over-expression enhanced Socs3 mRNA levels (1.7-fold, p<0.05) (Figure 3A). SOCS3 promotes IRS-1 proteasomal degradation [34]. Accordingly, we found a reduction in the IRS-1 protein levels in SHP overexpressing cells (Figure 3B). Altogether, these results show that both SHP overexpression and HP/HI exposure each lead to insulin resistance in cardiac myocytes.

3.3. **SHP overexpression enhances intramyocellular lipid storage**

Because insulin resistance and decreases in insulin-stimulated glucose uptake are closely related to intramyocellular lipid accumulation [18], we explored whether SHP overexpression affects intramyocellular lipid content. Oil-Red-O staining revealed that SHP
overexpressing HL-1 cells exhibited a 3.7-fold (p<0.01) increase in intramyocellular lipid storage compared to cells transduced with the empty vector (Figure 4A and 4B). In agreement with the augmented lipid content, SHP overexpression raised mRNA levels of the membrane fatty acid transporter Cd36 (-6.2 fold; p<0.001) (Figure 4C), without changes in Acot1 expression encoding for acyl-CoA thioesterase 1 (data not shown), which hydrolyses fatty acyl-CoAs to free fatty acids and CoA. Lipid storage is often due to increased fatty acid uptake exceeding mitochondrial β-oxidation capacity [2]. Furthermore, SHP overexpression affected the expression of specific genes involved in fatty acid metabolism. Thus, no changes were found in the mRNA levels of Cpt1b (Figure 5A), which catalyses the entry of long-chain fatty acids into the mitochondrial matrix. Interestingly, Acaca was reduced in SHP overexpressing cells (42%, p<0.05) (Figure 5A). In addition, the Acaca protein product, ACC, showed enhanced phosphorylation in SHP overexpressing cells (Figure 5B). Once ACC is phosphorylated, the production of the allosteric CPT1B inhibitor malonyl-CoA is reduced, indicating an increased flux of fatty acids into mitochondria. Once inside mitochondria, fatty acyl-thioesters can undergo β-oxidation through enzymes including Acox1 or Acadvl. Therefore, we evaluated the expression of these genes in SHP overexpressing cells. Whereas SHP overexpression did not induce changes in the Acox1 mRNA levels, it significantly raised Acadvl expression (-1.8 fold; p<0.05) (Figure 5A).

3.4. SHP over-expression impairs mitochondrial function

Once long-chain and very-long-chain fatty acids are oxidized, they provide reducing equivalents (NADH) which are metabolized in the respiratory chain, resulting in the generation of an electrochemical gradient of protons, generally used to produce ATP via oxidative phosphorylation [35]. Nevertheless, under some circumstances, ATP synthesis can be impaired because of the proton electrochemical gradient dissipation through the UCPs, placed in the inner mitochondrial membrane [36]. To explore whether SHP overexpression may contribute to mitochondrial dysfunction, we determined the expression of Nrf1 and Nrf2, both involved in the control of DNA mitochondrial-encoded genes, such as the subunit 1 of
complex I (NADH dehydrogenase subunit 1, ND1), as well as Nd1 and Ucp3. Despite no changes were found in Nrf1 and Nd1 mRNA levels, the expression of Nrf2 was up-regulated in SHP over-expressing cells (Figure 6A). Accordingly, SHP over-expression induced the protein levels of the succinate dehydrogenase complex iron sulfur subunit B Complex II (CII-SDHB) of the mitochondrial respiratory chain (-4 fold, p<0.01) (Figure 6B), as well as the mitochondrial inner membrane potential (Figure 6C). Ucp3 mRNA levels were induced (-2.9 fold, p<0.05) (Figure 6A), whereas ATP levels were reduced (34% reduction, p<0.001) in SHP over-expressing cells.

3.5. SHP overexpression induced NF-κB activation in cardiomyocytes

Intramyocellular lipid storage may activate Ser/Thr-kinase cascades, activating the pro-inflammatory transcription factor NF-κB [5], and thereby linking the low-grade inflammatory process with metabolic disorders, such as insulin resistance. To explore whether the SHP-induced lipid accumulation was related to a rise in the inflammatory response, the expression of the pro-inflammatory genes Il6 and Tnf was evaluated. Along with increased lipid storage, SHP overexpression strongly augmented the mRNA levels of both messengers (-4-fold induction, p<0.01) (Figure 7A and 7B). Because these genes are under the transcriptional control of NF-κB, we tested the DNA-binding activity of this pro-inflammatory transcription factor in SHP overexpressing cells. EMSA studies showed the formation of a single specific complex, which disappeared in competition assays with an excess of unlabelled probe (Figure 7C, left). In agreement with the up-regulation of pro-inflammatory genes, SHP overexpression increased NF-κB DNA-binding activity (Figure 7C, right).

3.6. Decreased p65-PPARα interaction in SHP over-expressing cells

Metabolism and inflammation both are regulated by peroxisome proliferator-activated receptors (PPARs) in cardiomyocytes [26, 37-39]. Interestingly, alterations in the cardiac expression of PPARs are related to cardiac lipotoxicity, contributing to metabolic
disturbances related to insulin resistance and diabetic heart [40-45]. Therefore, we explored the PPAR gene expression profile in SHP overexpressing cells. As is shown in Figure 8A, SHP overexpression did not affect the mRNA levels of *Ppard*. However, SHP overexpression reduced the *Ppara* expression (79% reduction, p<0.001) and strongly induced the *Pparg* mRNA levels (-58-fold induction, p<0.001) (Figure 8A), together reflecting a similar profile to that found in the diabetic heart.

Finally, we further investigated the molecular mechanism by which SHP overexpression increases NF-κB activation in cardiac cells. PPAR activities are generally reduced by NF-κB activation [38, 44]. Vice versa, PPARα may inhibit NF-κB signalling through different mechanisms, including physical interaction between PPARα and the p65 subunit of NF-κB [46]. In order to evaluate whether the reduction in PPARα expression may affect its interaction with NF-κB in SHP overexpressing cells, we performed co-immunoprecipitation assays. Isolated nuclear extracts were immunoprecipitated using antibodies against the p65 subunit of NF-κB, subjected to SDS-PAGE, and immunoblotted with antibodies against PPARα. SHP overexpression strongly reduced the physical interaction between p65 and PPARα (Figure 7B), suggesting that dissociation of the PPARα-NF-κB protein complex may enable NF-κB transactivation and the development of the inflammatory response seen in SHP overexpressing cells.

4. Discussion

DM2 related alterations are especially relevant in the heart, because the myocardium needs to produce energy constantly, using both glucose and fatty acids, in order to maintain proper cardiac function. Despite advances in DM2 research, the prevalence of this disease is steadily rising worldwide [47]. The lack of an effective therapy for DM2 and its related disturbances such as DCM are alarming. Therefore, it is necessary to identify new emerging molecular targets for the potential treatment of the diabetic heart. In the present study, we
found that SHP, an atypical nuclear receptor involved in the regulation of peripheral insulin resistance [9], was induced in hearts from mice fed with a HFD and which displayed glucose intolerance compared with animals fed with a chow diet [25]. Hearts from these animals did not show changes in the expression of genes involved in gluconeogenesis, but yes a slight but significant reduction in Glut4 mRNA levels. Interestingly, cardiac SHP deficiency induces cardiac hypertrophy [17]. Given that during cardiac hypertrophy there is a shift in the substrate preference by heart, from fatty acids to glucose [48-50], these animals could show an increase in glucose utilization. However, the short period of feeding with HFD probably did not cause any structural changes or functional derangements in our animal model. These novel observations suggest that the induction of cardiac Shp expression after the HFD challenge could be related to the onset of the heart metabolic dysregulation mediated by the dietary fat. This hypothesis was further supported by subsequent data showing a positive correlation between the cardiac Shp mRNA levels and glucose intolerance, as measured by the AUC from the glucose tolerance test. Therefore, to explore the mode of action of SHP in cardiac metabolism we overexpressed this nuclear receptor in HL-1 cardiomyocytes.

At the cellular level, we demonstrate for the first time that SHP overexpression mimics the insulin resistance response elicited by a combination of HP/HI exposure in cardiomyocytes. It has been previously shown that in liver, SHP inhibits gluconeogenesis [10-15, 51] and improves insulin sensitivity [16] promoting the glucose delivery to mitochondria [13]. However, our data revealed that in HL-1 cardiomyocytes, SHP overexpression reduced both basal and insulin-stimulated glucose uptake, in similar degrees to those found in HP/HI challenged cells. In agreement with this, the insulin signalling pathway, analysed by AKT and AS160 phosphorylation, was also impaired in both models of cardiac insulin resistance. Insulin resistance was further analysed determining the expression of Socs3 [33]. Despite it has been previously shown that in hepatocytes SHP improves insulin sensitivity via inhibition of the STAT-3/SOCS-3 pathway [16], Socs3 mRNA levels were induced in SHP over-expressing cells. This data was in line with increased Socs1 and Socs3 expression found in
heart from HFD-fed mice [26]. Furthermore, SHP overexpression reduced the IRS-1 protein levels, suggesting that SHP may promote the SOCS-3-induced IRS-1 proteasomal degradation. Taken together, our data indicate that SHP may drive opposite responses in hepatocytes and cardiomyocytes. The discrepant role of SHP between these two tissues may be consequence of their different metabolic functions. While liver in the systemic context may be considered as an anabolic tissue, mainly involved in biosynthesis processes such as gluconeogenesis or triglyceride synthesis, heart is a catabolic organ that needs to produce energy constantly for maintaining its function. Thus, SHP could act as mediator differentially controlling physiological responses in various tissues.

Insulin resistance is commonly linked to intramyocellular lipid accumulation [52]. The increase in lipid mediators has been associated with cardiac dysfunction [53, 54], and vice versa cardiac function improved with their reduction [53]. According to the observed role of SHP as an originator of insulin resistance, we found increased lipid droplets in SHP over-expressing cells compared with those of controls. Consistent with this, the expression of the membrane fatty acid transporter Cd36 was augmented in SHP overexpressing cells, with no changes in Acot1 expression. These data suggested a positive flux of activated fatty acids into cardiomyocytes, as demonstrated with an increase in Oil-Red-O staining. Accumulated fatty acids can be used for lipid storage or channelled into mitochondria for oxidation. Reduction in the mRNA levels of Acaca and the up-regulation of Acadvl and Ucp3 in SHP over-expressing cells indicated a reprogramming in the expression of genes involved in fatty acid β-oxidation. Notably, increased Ucp3 activity in skeletal muscle has been associated with increased fatty acid oxidation rates [55]. However, because the heart needs to produce energy constantly, induction of Ucp3 expression has been previously reported in diabetic hearts as a hallmark of contractile dysfunction [56]. The induction of Ucp3 mRNA level was in parallel with decreased ATP levels in SHP over-expressing cells, which may reflect that increased fatty acid oxidation and mitochondrial respiration are less efficient for energy production upon Ucp3 induction [57].
Intramyocellular lipid accumulation is associated with the activation of the pro-inflammatory transcription factor NF-κB and abnormal cytokine production, thereby linking metabolic disorders with a low-grade inflammatory process [5]. Interestingly, recent work indicated a role of SHP in the inflammatory response, although results were somewhat conflicting. Indeed, while SHP was firstly described as transcriptional co-activator of NF-κB in several cell types [21-24], data from others support an anti-inflammatory role for this nuclear receptor [18-20]. Specifically, the molecular mechanisms by which SHP drives its anti-inflammatory effects involved dual regulatory functions in a canonical transcription factor NF-κB signalling pathway, acting as both a repressor of transactivation of the NF-κB subunit p65 and an inhibitor of polyubiquitination of the adaptor TRAF6 [20]. Thus, SHP may be considered as a modifier, able to differentially regulate the transcriptional activity of the same transcription factor, such as NF-κB, in function of the cell requirements. To clarify the role of SHP in cardiac inflammation we explored the degree of NF-κB activation. In accordance with a pro-inflammatory action, SHP over-expression induced the NF-κB DNA-binding activity, as well as the mRNA levels of Il6 and Tnf, two well-known NF-κB target genes involved in the heart failure progression [58]. Interestingly, a similar pro-inflammatory profile, characterized by increased Il6 and Tnf expression and an enhanced NF-κB DNA-binding activity, was found in the hearts from HFD-fed mice [26], where we observed increased Shp expression. As a result, our data indicates a contribution of SHP to NF-κB activation, and support a pro-inflammatory role of SHP, at least in cardiomyocytes.

PPARs have been proposed as potential modulators of both fatty acid metabolism [37, 38, 44] and inflammation [26, 37-39] in cardiomyocytes. Indeed, changes in the expression/activity of these transcription factors have been previously described in hearts with metabolic disturbances. Our data revealed that SHP overexpression strongly reduced Ppara mRNA levels. This data agrees with the Ppara reduction found in cardiomyocytes
chronically exposed to fatty acid excess [43] and in hearts from senescence-accelerated mice with enhanced ceramide levels [44]. Both \( Ppara \) and \( Ppard \) are expressed in comparable levels in heart and share similar functions in cardiomyocytes regarding fatty acid metabolism [59]. Despite the observed reduction of \( Ppara \) expression, no changes were observed in the expression of \( Ppara \) target-genes, such as \( Cpt1b \). However, it was previously reported that PPAR\( \delta \) can compensate for the lack of PPAR\( \alpha \) [60], which is consistent with our data showing unaltered \( Ppard \) expression in SHP overexpressing cells.

Unlike other PPARs, \( Pparg \) is barely detectable in heart, but it is up-regulated in hearts from rat models of DM2 [41, 42, 45], thereby contributing to the storage of intramyocellular lipid content [45]. In agreement with this, SHP overexpression strongly induced \( Pparg \), which is in line with the induction of its target gene \( Cd36 \), setting a potential bridge between changes in \( Pparg \) expression and the increased in lipid droplets observed in SHP overexpressing cells.

Reduced \( Pparg \) and \( Cd36 \) expression recently have been found in liver from \( Shp \)-deficient mice [61], thus suggesting a direct link between SHP and the regulation of the \( Pparg \) expression. The molecular mechanisms underlying such regulation may involve SHP-mediated FXR activation [62] and HNF4\( \alpha \) activation by RAR/Hes6 inhibition [63]. In addition, SHP may act as endogenous enhancer of PPAR\( \gamma \) transactivation through a mechanism involving a competition with the nuclear receptor corepressor (NCoR) for the direct binding to PPAR\( \gamma \) [64]. However, while SHP acts as endogenous enhancer of PPAR\( \gamma \) transactivation [64], it can activate or repress the PPAR\( \alpha \) transcriptional activity [65], suggesting the involvement of additional factors in the SHP-mediated transcriptional regulation of PPARs.

It has been previously reported that PPAR\( \alpha \) may act through DNA-binding independent mechanisms that involves a physical interaction of PPAR\( \alpha \) with NF-\( \kappa B \) [46]. This association prevents NF-\( \kappa B \) from binding to its response element, thereby inhibiting its ability to induce gene transcription [46]. Here, we demonstrate that SHP overexpression reduced protein-protein association between PPAR\( \alpha \) and p65, suggesting that dissociation between these
two proteins is one of the mechanisms by which SHP overexpression drives NF-κB activation and the inflammatory response in cardiomyocytes.

In summary, in the present study we show for the first time that ectopic expression of SHP in HL-1 cardiomyocytes induces the accumulation of intramyocellular lipid droplets and changes the expression of genes related to lipid metabolism and mitochondrial uncoupling. The SHP-induced lipid storage correlates to an inflammatory response involving the dissociation of PPARα from NF-κB and impairment of the insulin signalling pathway. Although further studies are necessary to explore the role of cardiac SHP in the context of normal physiological stimuli, our data show the relevance of this nuclear receptor in processes that have been linked to cardiac dysfunction, suggesting this nuclear receptor as a new potential therapeutic target for DCM.

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Abbreviations-Acaca: acetyl-CoA carboxylase; Acadvl: acyl-CoA dehydrogenase, very long chain; Acot1: acyl-CoA thioesterase 1; Acox1: acyl-CoA oxidase 1; AS160: AKT substrate of 160 kDa; BSA: bovine serum albumin; Cpt1b: carnitine palmitoyltransferase I; DBD: DNA binding domain; DCM: diabetic cardiomyopathy; DM2: type 2 diabetes mellitus; EMSA: electrophoretic mobility shift assay; GAPDH: glyceraldehyde 3-phosphate dehydrogenase;
References


metabolism precedes the onset of hyperglycemia and contractile dysfunction in two mouse models of insulin resistance and obesity, Endocrinology, 146 (2005) 5341-5349.


Fig. 1. HFD induces Shp mRNA levels in hearts from glucose intolerant mice. (A) Shp mRNA levels were analysed by real time RT-PCR in hearts from STD and HFD-fed mice. Data were normalized to Cyclophilin A mRNA levels. (B) Correlation between AUC and Shp mRNA levels data. (C) Analysis of the mRNA levels of Pepck, G6p, Glut4 and Pdk4 by real time RT-PCR in hearts from STD and HFD-fed mice. Data were normalized to Cyclophilin A mRNA levels. Data are expressed as mean ± SD of 5 mice per group. (*p<0.05 vs. STD-fed mice).

Fig. 2. SHP overexpression impairs glucose uptake and insulin signalling pathway. HL-1 cells were stimulated with HP/HI (500 µM/100 nM) for 16 h (A-B) or transduced with Ad or AdSHP (C-D), both in the absence (white bars) or presence (black bars) of insulin (200 nmol/L, 10 min). [3H]-deoxyglucose uptake was assessed in HP/HI-stimulated (A) and SHP over-expressing (C) cells. Protein levels of phospho-AKT (Ser473)/AKT and phospho-AS160 (Thr642)/AS160 (B, D) were analysed by western blot. Quantifications show the ratio between phosphorylated and total forms of each protein. (E) Glucose uptake in primary cultures of rat cardiomyocytes transduced with Ad or AdSHP. Data are expressed as mean ± SD of, at least 3 different experiments performed in duplicate. (*p<0.05; ***p<0.001 vs. control or Ad transduced cells without insulin stimulation; #p<0.05, ##p<0.01 and ###p<0.001 vs. control or Ad transduced cells stimulated with insulin).

Fig. 3. SHP overexpression induces Socs3 expression and reduces IRS-1 protein levels. (A) HL-1 cells were transduced with Ad or AdSHP and Socs3 mRNA levels were analysed by real time RT-PCR. Data were normalized to Cyclophilin A mRNA levels. (B) Western blot showing IRS-1 protein levels in HL-1 cells transduced with Ad or AdSHP. Protein quantification data were normalized to GAPDH and expressed as mean ± SD of at least 3 different experiments. (*p<0.05 vs. Ad transduced cells).
Fig. 4. SHP overexpression enhances intramyocellular lipid storage. Lipid content was analysed by Oil-Red-O staining in HL-1 transduced with Ad or AdSHP (A and B). (A) Representative microphotography showing lipid droplets in cells counterstained with Haematoxylin (bar 20 µm). Squares indicate the areas shown at high magnification. (B) Quantification of stained areas relative to cell surface. Data are expressed as mean ± SD of 5 different pictures from 3 independent experiments (**p<0.01 vs. Ad transduced cells). (C) Cd36 mRNA levels were analysed by real time RT-PCR in HL-1 cells transduced with Ad or AdSHP. Data were normalized to Cyclophilin A mRNA levels and expressed as mean ± SD of 4 different experiments. (**p<0.001 vs. Ad transduced cells).

Fig. 5. SHP overexpression regulates the expression of genes involved in fatty acid metabolism. (A) Analysis of the mRNA levels of Cpt1b, Acaca, Acox1 and Acadvl by real time RT-PCR in HL-1 cells transduced with Ad or AdSHP. Data were normalized to Cyclophilin A mRNA levels. (B) Western blot showing protein levels of phospho-ACC and total ACC in HL-1 cells transduced with Ad or AdSHP. Quantifications show the ratio between phosphorylated and total form of ACC. Data are expressed as mean ± SD of at least 3 different experiments. (*p<0.05 vs. Ad transduced cells).

Fig. 6. SHP over-expression impairs mitochondrial function. (A) Analysis of the mRNA levels of Nrf1, Nrf2, Nd1 and Ucp3 by real time RT-PCR in HL-1 cells transduced with Ad or AdSHP. Data were normalized to Cyclophilin A mRNA levels. (B) Western blot showing protein levels of CII-SDHB in HL-1 cells transduced with Ad or AdSHP. Protein quantification data were normalized to GAPDH. (C) Mitochondrial inner membrane potential and (D) ATP levels in HL-1 cells transduced with Ad or AdSHP. Data are expressed as mean ± SD of at least 3 different experiments. (*p<0.05; **p<0.01 and ***p<0.001 vs. Ad transduced cells).

Fig. 7. SHP overexpression induces inflammatory response in HL-1 cardiac cells. Analysis of the mRNA levels of Il6 (A) and Tnf (B) by real time RT-PCR in HL-1 cells transduced with
Ad or AdSHP. Data were normalized to *Cyclophilin A* mRNA levels and expressed as mean ± SD of 4 different experiments. (**)p<0.01 and (***)p<0.001 vs. Ad transduced cells. (C) Autoradiograph of EMSA performed with a $^{32}$P-labeled NF-κB binding DNA fragment and crude nuclear protein extract (NE) from HL-1 transduced with Ad or AdSHP. The unlabelled DNA probe is used as competitor. The specific NF-κB/DNA complex is indicated by arrows.

**Fig. 8.** SHP overexpression modulates the PPAR gene expression profile and promotes dissociation between PPARα and the p65 subunit of NF-κB. (A) Analysis of mRNA levels of *Ppara, Ppard* and *Pparg* by real time RT-PCR in HL-1 cells transduced with Ad or AdSHP. Data were normalized to *Cyclophilin A* mRNA levels and expressed as mean ± SD of 4 different experiments. (***)p<0.001 vs. Ad transduced cells. (B) Nuclear extracts from HL-1 transduced with Ad or AdSHP were subjected to immunoprecipitation using anti-p65 antibody coupled to protein A-agarose beads. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-PPARα antibodies. Displayed data are representative of three separate experiments.
Figure 1

A

B

C

Figure 1
Figure 2

A

B

C

D

E

Figure 2
Figure 3
Figure 4

A

B

Ad
AdSHP

Ad
AdSHP

C

Ad
AdSHP

Figure 4
Figure 5

A

B

Figure 5
Figure 6

A

B

C

D

Figure 6
Figure 7

(A) 

(B) 

(C) 

NF-κB free probe

NF-κB free probe

NE - + +
COMPETITOR - +

NE - Ad AdSHP

Figure 7
Figure 8

A

![Graph showing mRNA levels](image)

B

- **IP:** anti-p65
- **Blot:** anti-PPARα

![Western blot image](image)
Graphical abstract
Table 1. Primers for SYBR Green real-time PCR

<table>
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### Highlights.

- Glucose intolerance and cardiac *Shp* expression linearly correlate in HFD-fed mice.
- SHP overexpression impairs glucose uptake and insulin signaling in cardiomyocytes.
- Intramyocellular lipid accumulation is increased in SHP overexpressing cardiomyocytes.
- SHP overexpression impairs mitochondrial function in cardiac cells.
- Overexpression of SHP in cardiomyocytes is pro-inflammatory.
- Decreased p65-PPARα interaction may underlie the SHP observed effects.

This box summarizes the key points of the article.