Transient postnatal over nutrition induces long-term alterations in cardiac NLRP3-inflammasome pathway

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TRANSIENT POSTNATAL OVER NUTRITION INDUCES LONG TERM ALTERATIONS IN CARDIAC NLRP3-INFLAMMASOME PATHWAY

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ABSTRACT

Aims

The prevalence of obesity is increasing worldwide at an alarming rate. Altered early nutrition, in particular neonatal overfeeding, is a risk factor for impaired cardiac function in adulthood. In the understanding of the initiation or progression of heart diseases, NLRP3 inflammasome and non-coding RNAs have been proposed as key players. In this context, the aim of this study is to decipher the role of NLRP3 inflammasome and its post transcriptional control by micro-RNAs in the regulation of cardio-metabolic function induced by postnatal overfeeding (PNOF) in mice.

Method and results:

Based on a model of mice exposed to PNOF through litter size reduction, we observed increased cardiac gene and protein expression levels of NLRP3 and ETS-1 associated with alterations in insulin signaling. *Additionally*, miR-193b levels were down-regulated in the adult hearts of overfed animals. In a cardiomyocyte cell line, transfection with miR-193b induced down-regulation of ETS-1 and NLRP3 and improved insulin signaling.

Conclusions:

These findings suggest that the miR-193b could be involved in cardiac phenotypic changes observed in adulthood induced by PNOF likely through the regulation of ETS-1 and NLRP3 expression, and through this of insulin signaling.

INTRODUCTION

A worldwide upward trend in the burden of non-communicable chronic diseases (NCDs), such as obesity, diabetes, hypertension, cancers, infertility, immune, mental and cardiovascular diseases, is currently observed[1]. Epidemiological and experimental approaches indicate that environmental exposures during early life, such as parental lifestyle, smoking, exposure to toxicants, diabetes, and obesity, increase the susceptibility of offspring to develop these chronic diseases. Gravely, the prevalence of obesity has been recently increasing at an alarming rate, given that approximately 40% of the adults in United States are obese and that approximately 20% of children and adolescents are overweight[2]. Maternal over-nutrition during lactation and postnatal overfeeding (PNOF) have been shown to program post-weaning obesity and cardio-vascular complications in rat pups[3,4]. Given that turnover of human cardiomyocytes is limited over a lifetime, the heart may be particularly sensitive to early events, such as exposure to nutritional excess. Indeed, neonatal overfeeding represents a risk factor for impaired cardiac function in adulthood by inducing excessive lipid accumulation in myocardial cells[5], defects in cardiac insulin signaling[6], increased heart rate and left ventricular wall thickness, hypertrophy and fibrosis[6]. Strong efforts have been implemented in approaches that could reverse the dysfunctions induced by early life overfeeding. Recently, the increased risk of cardio-metabolic disorders induced by perinatal overfeeding was reversed by a short-term nutritional adjustment at a time when the phenotype seems to be definitively acquired[7]. Despite its clear benefits, caloric restriction remains difficult to implement as a long-term therapy in obese patients. Thus, a major challenge in developing targeted therapeutics for this type of heart disease is to understand how the molecular and genetic changes induced by neonatal overfeeding lead to altered cardiac functions[8]. Inflammasomes are attractive candidates to understand these mechanisms, given that they are strongly implicated in the initiation or progression of chronic diseases, including heart disease. The inflammasomes are key signaling platforms that detect sterile stressors as well as transduce signals triggering an inflammatory cascade reaction. The specific sensor, nucleoside-triphosphatase domain (NACHT), leucine-rich repeat (LRR), and pyrin domain (PYD) domains-containing protein 3 (NLRP3), has been widely studied due to its ability to sense a large number of endogenous activators,

including metabolic substrates, such as glucose, ATP and fatty acids. NLRP3 connects to CASPASE-1 via the adapter Apoptosis-associated speck-like protein containing a CARD domain (ASC) and its activation leads to CASPASE-1 self-cleavage and activation. Active CASPASE-1 proteolytically activates proinflammatory cytokines, including pro-Interleukin-1β (pro-IL-1b). In addition to its role in inflammation, the inflammasome regulates metabolism[9], lipid deposition[10], and insulin resistance[11].

In the early origins of chronic diseases, epigenetic mechanisms are likely key players. These mechanisms regulate different steps of heart development[12], and regulate lipid metabolism, glucose homeostasis and insulin signaling[13]. The major epigenetic features include DNA methylation, histone modifications and non-coding RNA. Importantly, approximately 60% of the human genes are under the control of micro-RNAs. The dysregulation of their balance can lead to the development of cardio-metabolic disorders[14-16]. In this context, the inflammasome and its regulation through micro-RNAs represent a potential candidate in the understanding of the long-term effects induced by postnatal overfeeding on cardiac function. The aim of the present study was to delineate the role of the NLRP3 inflammasome in a model of cardio-vascular alteration induced by PNOF, and to determine the role of upstream micro-RNAs regulators.

METHODS

Materials

H9C2 cardiomyoblast cell line, cell culture medium, retinoic acid, Tween 20, sodium oleate and other reagents were purchased from Sigma Aldrich. Antibodies raised against phospho-AKT pSer473, Phospho-IRS-1 pSer307, NF-kB2, and IL-1β; DMEM/Glutamax medium; BCA Protein Assay Kit; RIPA buffer; phosphatase inhibitor cocktail; Trizol reagent; High Capacity Reverse Transcriptions Kit, TaqMan Advanced miRNA cDNA Synthesis Kit, miRNAs Advanced Taqman assays, lipofectamine, and RNAimax were purchased from Thermo Fisher Scientific, Inc. Antibodies raised against IRS-1, AKT, and β-Actin anti-rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Technology, Inc.

Heat-inactivated fetal bovine serum was purchased from PAN Biotech. I-Taq Universal SybrGreen Master Mix, protease inhibitor cocktail (BioRad), rno-pre-miR-193b and control pre-miR were purchased from Exiquon. PVDF membranes were obtained from Amersham. Immobilon Chemiluminescent Western HRP Substrate was obtained from Millipore. High Pure miRNA Isolation Kit was purchased from Roche Diagnostics, and Magnetic Luminex Screening Assay was purchased from R&D Systems. Caspase-1 antibody was obtained from Santa Cruz Biotechnology. ETS-1 antibody was obtained from Bethyl laboratories. NLRP3 antibody was obtained from Novus Biologicals. TXNIP antibody was obtained from Abgent.

***In vivo* experimental studies**

All animals received humane care, and the study protocol complied with the institution's guidelines. The investigation was performed in accordance with Directive 2010/63/EU of the European Parliament, the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the local ethics committee (Comité d'Ethique de l'Expérimentation Animale Université-Bourgogne-Franche-Comté, Dijon, France, protocol agreement number: 00412.03), which specifically approved this study. Adult female C57BL/6 mice (Charles River, L'Arbresle, France) were mated with male mice, housed in individual cages, and given water and a standard pellet diet (A03, SAFE Diets Augy, France) *ad libitum* during pregnancy and lactation. On the third day of life, male pups were randomly distributed among mothers to achieve cross fostering. The litter size was adjusted to 9 male pups (normal fed, NF group) or reduced to 3 male pups to induce postnatal overfeeding (overfed, OF group). Each litter included pups from one to six different dams, which increased genetic variability within the litter. Excess pups were rapidly sacrificed by decapitation after brief isoflurane anesthesia. After weaning (day 24), mice of both groups had free access to a standard diet (A04, SAFE Diets Augy, France: 19.3% proteins, 8.4% lipids, 72.4% carbohydrates) and water.

Plasma and Heart Sampling

In 7-month-old mice, intraperitoneal injection of sodium pentobarbital (80 mg/kg) mixed with heparin (500 IU/kg) was performed. Blood was collected from the heart, and plasma was rapidly separated by

centrifugation (3,000 × g, 4 °C for 10 min) and stored at -80°C until assayed. The heart was quickly harvested; the ventricles were separated from the atria and immediately frozen in liquid nitrogen.

Cell cultures and transfection

H9C2 cardiomyoblast cell line was maintained in DMEM/Glutamax medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified, CO_2-controlled (5%) incubator. H9C2 cells grown on 6-well plates were differentiated into cardiomyocytes by reducing FBS to 1% and with daily treatments of 10 nM retinoic acid for 6 days. Then, cells were transfected with 50 pmol rno-pre-miR-193b or a scramble pre-miR-control using Lipofectamine RNAimax Transfection reagent, according to the manufacturer's protocol. Forty-eight hours following transfection, cells were washed with ice-cold phosphate-buffered saline and harvested for protein extraction.

Protein extraction

Frozen cardiac tissue was ground in liquid nitrogen to obtain tissue powder. Aliquots (~20 mg) of powder and H9C2 cells were homogenized in RIPA buffer (supplemented with 1% proteases and phosphatases inhibitor cocktail). Protein concentration was determined with the bicinchoninic acid assay. In total, 10 to 30 µg were used for Western blot analysis.

Western blotting analysis

Lysates were separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 1% BSA in phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 and were incubated with primary antibodies and horseradish peroxidase-conjugated secondary anti-rabbit antibodies. The proteins of interest were visualized using Immobilon chemiluminescence reagent. Membranes were scanned with a luminescent image analyzer camera G:Box (Syngene) and quantified with Gene Tools software (Syngene).

RNA isolation

Total RNA was isolated from frozen heart powder using 1 ml TRIzol reagent and 200 µl chloroform. The aqueous phase was precipitated with 1.5 vol of ethanol 100% at 4°C overnight followed by 75% ethanol. Total RNAs were further purified on a column. The quantities of total RNAs and microRNAs were evaluated with a fluorescent-based method on a Qubit apparatus (Life technologies, Inc).

Real-time quantitative PCR for mRNA expression

In total, 200 ng of RNAs were reverse transcribed using High Capacity Reverse Transcription Kit according to the manufacturer's protocols in a SimplyAmp Thermal Cycler (Life Technologies, Inc.). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with 2 µl of diluted cDNA (1/20) using the I-Taq Universal SybrGreen Master Mix and both sense and antisense primers (5 mM) in a final volume of 10 µL in a Quantstudio 3 apparatus (Life Technologies, Inc.). The primers that were used are listed in supplemental table S1. Data were normalized to β-Actin using the Ct method.

MicroRNAs expression quantification by RT-qPCR

In total, 10 ng of RNAs were reverse transcribed with Advanced miRNA RT kit on a SimplyAmp Thermal Cycler (Life technologies, Inc.). RT product was diluted by 10-fold, and 2.5 µl were used for qPCR analyses. qPCR was performed with TaqMan Advanced MasterMix according to the manufacturer's protocol on a Quantstudio 3 apparatus (Life technologies, Inc.). The relative expression levels of miRNAs were calculated using the comparative ΔΔCt method by normalizing to miR-16 levels.

Circulating cytokines

IL-1β, IL-6, CRP, and TNFα plasma levels were measured using a magnetic Luminex screening assay according to the manufacturer's instructions on a Bio-Rad Bio-plex 200 System (Luminex xMAP Technology).

Data analysis

The data from the different experiments were analyzed with GraphPad Prism software version 6.05 (GraphPad Software, Inc.). The values were expressed as the mean ± SEM to account for sample and animal variation within a dataset. The Student's *t* test was performed to determine whether there were differences between all groups ($P < 0.05$).

RESULTS

Global inflammation status (figure 1)

The heart dysfunction and the metabolic phenotype induced by PNOF have been previously reported by Li et al[7]. In the same set of overfed animals, in order to investigate the expression pattern of pro-inflammatory cytokines, and inflammatory biomarkers, we measured the systemic concentration of C-reactive protein (CRP), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). The pro-inflammatory factor IL-6 significantly increased in overfed animals. These data indicate a global low inflammatory status in overfed animals.

PNOF induces dual effects on NLRP3 inflammasome (figure 2)

To delineate the role of the NLRP3 inflammasome in adult cardiac dysfunctions induced by PNOF, we compared the expression levels of the inflammasome components in cardiac tissues from adult mice (7 months) postnatally exposed to overfeeding (PNOF) through litter size reduction with normal fed mice (NF) raised in litters of normal sizes. In adult PNOF mice, we observed a significant increase (37%) in cardiac NLRP3, protein levels **(figure 2A)**. This protein up-regulation was associated with the other members of the inflammasome, cleaved CASP1 and IL-1β **(figure 2A).**

The NLRP3 inflammasome can be activated by reactive oxygen species (ROS) production through the Thioredoxin-interacting protein (TXNIP)[17]. Here, TXNIP mRNA levels were upregulated by PNOF **(figure 2B)** which indicates that oxidative stress-induced inflammasome activation is increased by overfeeding.

The alterations in NLRP3, CASP1, IL-1b and TXNIP appeared only in adulthood and were not detected at PND21 at the end of PNOF period at the protein and mRNA levels **(supplemental figure S1)**.

Cardiac levels of inflammation-priming factors are regulated by PNOF (figure 3)

We next examined the role of proteins involved in the priming of the inflammasome including nuclear factor-κB (NF-κB) that regulates NLRP3, Pro-IL-1β, and V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog 1 (ETS-1), which targets Caspase-1. When compared with normal fed mice, PNOF mice exhibited elevated NF-kB1, NF-κB2 **(figure 3A)**, and ETS-1 protein levels **(figure 3B)**.

MiR-193b regulates inflammasome in cardiomyocyte cell line (figure 4)

The aforementioned data demonstrated that PNOF alters NLRP3 inflammasome components levels in cardiac tissue. Using in silico analysis with MiRTarBase (version 6.0, http://mirtarbase.mbc.nctu.edu.tw/), we identified miR-193b as a regulator of genes involved in the inflammasome, such as NLRP3 and ETS-1 **(figure 4A).**

The organization of the miR-193b- 365 cluster is highly conserved among vertebrates[18]. Here, we analyzed its possible involvement in ETS-1 and NLRP3 regulation in cardiomyocytes. The interaction of miR-193b with ETS-1 3'UTR was previously well documented[19]. The region of human, mouse and rat NLRP3 3'UTR sequences that may interact with miR-193b was aligned with Clustal W (version 1.81), and the homologies were highlighted **(figure 4A).**

The regulation of the NLRP3 inflammasome by miR-193b was then tested in the H9C2 cardiomyocyte cell line transfected with pre-miR-193b (miR-193b) or a control (scramble nucleotide) pre-miRNA (miR-CTRL). As described in other cell types, the transfection with pre-miR-193b decreased the levels of ETS-1, indicating that miR-193b regulates ETS-1 levels in rat cardiomyocyte. Furthermore, NLRP3 and the target of ETS-1, the Caspase 1 showed decreased levels with premiR-193b transfection **(Figure 4B)**. No modification was detected neither in NF-κB2, TXNIP nor IL-1β protein levels **(Figure 4B)**.

In vivo, PNOF induced a decrease in cardiac miR-193b levels in adult mice **(Figure 4C)**. The alterations in cardiac miR-193b levels were detected only in adulthood, not at PND21, **(supplemental figure S1)**, which was consistent with the appearance of NLRP3 and ETS-1 alterations.

Cardiac metabolic disruptions induced by PNOF (figure 5)

In addition to its role in inflammation, the inflammasome has been described as a regulator of insulin signaling and metabolism, especially through IL1-β, NLRP3, ETS-1, and CASPASE-1. To assess whether miR-193b-inflammasome alterations induced by PNOF were associated with cardiac insulin resistance, we evaluated insulin signaling. Increased levels of phosphorylated AKT pser473 and IRS-1 pser307 were observed in mice exposed to postnatal overfeeding compared with normally fed mice **(figure 5A)**.

The role of miR-193b in insulin signaling was then assessed *in vitro*. In H9C2 cells, miR-193b over-expression induced IRS1 and AKT decreased phosphorylation levels indicating that miR-193b regulates insulin signaling possibly through inflammasome regulation **(figure 5B)**. NLRP3 inflammasome activation induces lipid accumulation through the regulation of lipid metabolism-related genes in the liver[20], macrophages[10], and kidneys[21]. In H9C2 cells, we visualized lipid droplet accumulation with BODIPY staining procedures and found that oleate treatment (100 μM) increased fat stores in H9C2 cells compared with untreated cells **(Figure 5C).** Interestingly, transfection with pre-miR-193b (miR-193b) decreased the amount of lipid droplets in oleate-treated cells compared with those transfected with pre-miR-CTRL **(Figure 5C),** indicating a role for miR-193b in the regulation of fat stores in cardiomyocytes.

DISCUSSION

Heart and vessel diseases are closely linked to modern lifestyle and diet. Particularly, child obesity has been implicated in the origins of cardiovascular diseases later in life. The deterioration in cardiovascular function in adulthood could be a direct effect of early altered programming by postnatal overnutrition or explained by the side-effects of adult-onset overweight and modifications in the metabolic environment in adulthood or both. Thus, the understanding of heart diseases induced by early life diet and the possibility of their reversal represent a major challenge. Interestingly, Li et al[7] demonstrated that short-term (one month) 20% food restriction in adulthood could ameliorate metabolic profile and reverse the cardiac dysfunction induced by early overnutrition in mice.

However, caloric restriction remains difficult to implement as a long-term therapy. In this context, our aim was to understand the molecular bases of cardiovascular dysfunctions induced by postnatal nutritional programming. Sustained activation of NF-κB appears to be detrimental and promotes heart failure by triggering chronic inflammation through enhanced induction of TNFα, IL-1, and IL-6, leading to endoplasmic reticulum stress responses and cell death. Consistent with these findings, our study revealed NF-κB increase induced by PNOF. Here, in addition to the alterations in the inflammatory signaling, our findings highlighted a mechanism that could explain cardiac metabolic regulations through nutritional approaches represented in the **figure 6**. Indeed, neonatal programming of heart dysfunctions could be related to metabolic regulations through the regulation of NLRP3 inflammasome by miR-193b.

NLRP3 represents a central platform in the sensing of endogenous stress and the regulation of metabolism[22]. Ablation of NLRP3 attenuates type 2 diabetes[23] and age-related functional decline[24]. Thus, identification of mechanisms that control NLRP3 inflammasome components may provide insights into the control of these chronic diseases. Here, we described the regulation of ETS-1 by mir-193b in cardiomyocyte, and for the first time, NLRP3 regulation by miR-193b. Further studies should determine whether this regulation occurs through direct interaction between miR-193b and the 3'UTR end of NLRP3. In the heart, miR-193 expression is localized to cardiomyocytes and fibroblasts but is not detected in telocytes[25] suggesting that miR-193 may play an important role in cardiomyocytes' functions. In addition, through the targeting of ETS-1 and MAX, miR-193b participates in the negative regulation of the local inflammatory web and contributes to limiting the strength of inflammation[26]. In this study, we reported a novel role for miR-193b in cardiomyocyte functions through the regulation of NLRP3. Furthermore, miR-193b transcription was reported to be regulated by NF-κB[27] which is consistent with our observation showing increased NF-κB cardiac levels in animals exposed to postnatal overfeeding, and no effect of miR-193b transfection *in vitro* on NF-κB levels in cardiomyocytes. Various NF-κB inhibitors have been tested and their effects were described as highly pleiotropic, and giving undesired side effects. Therefore, the targeting of miR-193b appears as an alternative pathway to treat the metabolic dysfunctions driven by NF-κB. Overfeeding related to

small-litter rearing during lactation contributes to NAFLD phenotype when combined with a high-fat diet, possibly through up-regulated hepatic lipogenesis[28]. Evidence from human and animal studies suggests that the amount of lipids in the myocardium contributes to the development of cardiac dysfunction through lipotoxic effects. Recently, miR-193b has been reported as a circulating micro-RNA associated with glycemic impairment. Indeed, miR-193b interacts with ETS-1 mRNA, which is a transcription factor that plays an important role in the pathogenesis of insulin resistance in different tissues[29]. Here, we found that alterations in miR-193b may be a possible cause for cardiac lipid accumulation and disrupted insulin signaling in cardiomyocytes.

Thus, miR-193b and its targets in NLRP3 inflammasome appear as an interesting target for the reversal of cardiac inflammation and metabolic dysfunctions induced by postnatal overnutrition. To date, pharmacological approaches using Metformin[30] and Resveratrol[31] up-regulate miR-193b levels, possibly through epigenetic mechanisms. Indeed, the miR-193b promoter can be methylated[32] which opens the possibility of its regulation through the targeting of DNA methylation processes. In addition, the recent remarkable progress in innovation in the areas of miRNA inhibitors and mimics provides exciting perspectives for *in vivo* delivery of this miRNA therapeutics[33]. The in vivo use of miR-193b mimics to reverse the cardio-metabolic dysfunctions induced by neonatal overfeeding remains to be tested.

In our animal model, of note the changes in miR-193b levels are not observed immediately after the overfeeding (PND21) but later in adulthood (7 months), which is concomitant to the appearance of inflammasome alterations. The delayed effects on the miR-193b and inflammasome in the adult heart after neonatal exposure to overfeeding is an observation that is reminiscent of the Barker hypothesis related to the concept of the developmental origins of health and disease. In this context, it is noteworthy that altered miRNA patterns may be associated with disturbed developmental processes during fetal or neonatal life[34]. Also, miRNAs are strikingly stable and can be detected in body fluids, including blood. This observation has raised the possibility that miRNAs may be probed in the circulation and can serve as novel diagnostic markers. Consistent with our study, the analysis of circulating microRNAs in heart failure revealed that miR-193b levels are decreased in patient groups

with reduced and preserved left ventricular ejection fraction[35]. These data suggest that miR-193b is a sensitive and early marker of heart failure and needs to be further explored. MiR-193b could provide a novel strategy for diagnosis and therapy in chronic cardiac dysfunctions programmed by environmental stressors early in life.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

SUPPLEMENTARY INFORMATION

Supplemental figure S1**:** Gene and miR-193b expression analysis at PND21 in normal fed and overfed mice hearts

Supplemental table 1: List of primers

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FIGURE LEGENDS

Figure 1: Effect of neonatal overfeeding on adult circulating cytokines

Luminex analysis of proinflammatory cytokines in the blood of 7-month-old mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). Levels of plasma cytokines/chemokines and growth factors, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6), were measured. Data are expressed as the mean ± SEM; 9<n<12 per group. ∗ indicates statistical significance *($p < 0.05$).

Figure 2: Cardiac levels of NLRP3 inflammasome members are regulated by PNOF

Protein levels of NLRP3, CASP-1 and IL-1β **(A)** and mRNA levels of TXNIP **(B)** were analyzed in the hearts from adult (7 month) mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). Data are expressed as the mean ± SEM; 9<n<12 per group. ∗ indicates statistical significance ($p < 0.05$).

Figure 3: Cardiac levels of inflammation priming factors are regulated by PNOF

(A) NF-kB1 and NF-kB2 mRNA levels were analyzed in the hearts of adult (7 month) mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). **(B)** Cardiac protein and mRNA levels of ETS-1 were measured in adult mice. Data are expressed as the mean ± SEM; 9<n<12 per group. ∗ indicates statistical significance ($p < 0.05$).

Figure 4: miR-193b expression regulates NLRP3 inflammasome

(A) Human, mouse and rat NLRP3 3'UTR region were aligned. Conserved nucleotides are indicated in blue. In silico prediction of the interaction between human NLRP3 and hsa-miR-193b-3p sequence is represented. **(B)** Differentiated H9C2 cells plated in 6-well plates were transfected with 50 pmol pre-miRNA control or pre-miR-193b for 48 hours. The results are representative of two independent experiments. NLRP3, ETS-1, Pro CASPASE-1, cleaved CASP-1, IL-1β, NF-κB2, TXNIP and β-ACTIN were assessed by Western blot analysis. **(C)** MiR-193b levels were analyzed by RT-qPCR in the hearts from adult (7 month) mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). Data are expressed as the mean ± SEM; 9<n<12 per group. ∗ indicates statistical significance ($p < 0.05$).

Figure 5: PNOF induces cardiac metabolic disruptions (figure 5)

(A) Expression levels of cardiac phospho IRS-1 ser307 (protein), IRS-1 (mRNA) phospho AKT ser473 (protein), total AKT (protein), and β–ACTIN (protein) were measured in the hearts of adult (7 month) mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). **(B)** and **(C)** Differentiated H9C2 cells plated in 6-well plates, were transfected with 50 pmol pre-miRNA control or pre-miR-193b for 48 hours. The results are representative of two independent experiments. **(B)** Phospho AKT ser473, total AKT, phospho IRS-1 ser307, IRS-1, and β-ACTIN were assessed by Western blot analysis in H9C2 cells transfected with pre-miRNA control or premiR-193b. **(C)** Forty-eight hours after transfection, cells were incubated in the presence or absence of sodium oleate (at 100 μM) for 12 hours. At the end of the treatment, a neutral lipid droplet staining was performed with Bodipy. Oleate-treated cells exhibited intensive green staining with Bodipy compared with untreated cells. Data are expressed as the mean ± SEM; 9<n<12 per group in adult animals and 6<n<7 per group in PND21 animals. ∗ indicates statistical significance ($p < 0.05$).

Figure 6: Proposed mechanism of cardiac inflammation and metabolic dysfunction induced by postnatal **overfeeding (PNOF)**

PNOF up-regulates NF-κB levels, which increases NLRP3 and pro-IL-1β levels. In parallel, PNOF decreases miR-193b levels, which in turn leads to ETS-1, CASPASE-1 and NLRP-3 up-regulation. Increased levels of TXNIP activates the inflamasomme, leading to IL-1β release which finally induces inflammation state, altered insulin signaling and increased fat storage in cardiomyocytes.

Supplemental figure S1: Gene and miR-193b expression analysis at PND21 in NF and PNOF mice heart

Protein levels **(A)** of pro-Caspase-1, cleaved Caspase-1, IL-1β, TXNIP, ETS-1 and NLRP3 were analyzed at PND21 in the hearts of mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). mRNA levels **(B)** of NLRP3, Caspase-1, IL-1β, ETS-1, NF-κB1, and NF-κB2 were analyzed at PND21 in the hearts of mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). **(C)** Mir-193b levels were measured in the hearts of mice with postnatal normal feeding (NF) or postnatal overfeeding (PNOF). Data are expressed as the mean ± SEM; $6<n<7$ per group. ∗ indicates statistical significance ($p < 0.05$).

Supplemental table 1: Primer list used for RT-qPCR analyses

List of the forward and reverse mouse primers used for gene expression analyses by RT-qPCR.

FIGURE 1

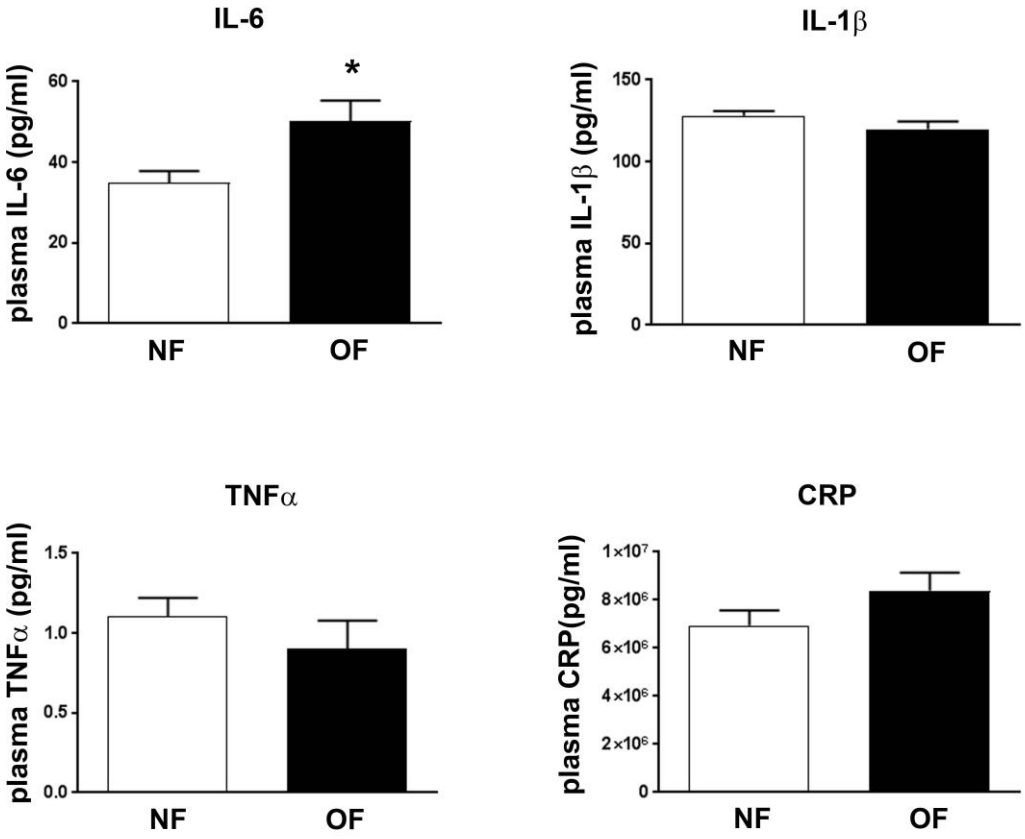

IL-6
plasma IL-6 (pg/ml)
60
40
20
0
*
NF
OF
IL-1β
plasma IL-1β (pg/ml)
150
100
50
0
NF
OF
TNFα
plasma TNFα (pg/ml)
1.5
1.0
0.5
0.0
NF
OF
CRP
plasma CRP(pg/ml)
1×10⁷
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
NF
OF

FIGURE 2

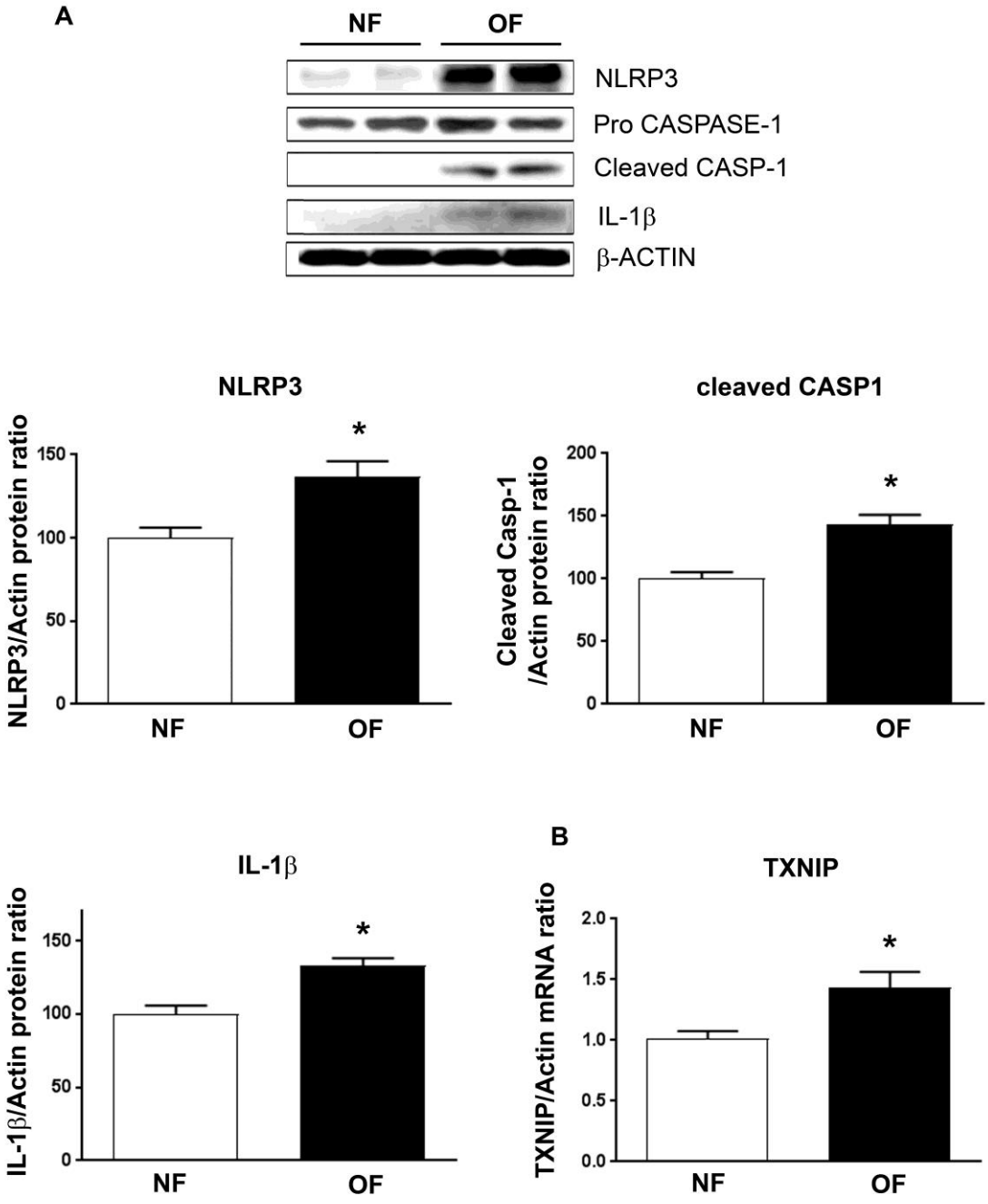

A
NF
OF
NLRP3
Pro CASPASE-1
Cleaved CASP-1
IL-1β
β-ACTIN
NLRP3
NLRP3/Actin protein ratio
150
100
50
0
*
NF
OF
cleaved CASP1
Cleaved Casp-1 /Actin protein ratio
200
150
100
50
0
*
NF
OF
IL-1β
IL-1β/Actin protein ratio
150
100
50
0
*
NF
OF
B
TXNIP
TXNIP/Actin mRNA ratio
2.0
1.5
1.0
0.5
0.0
*
NF
OF

FIGURE 3

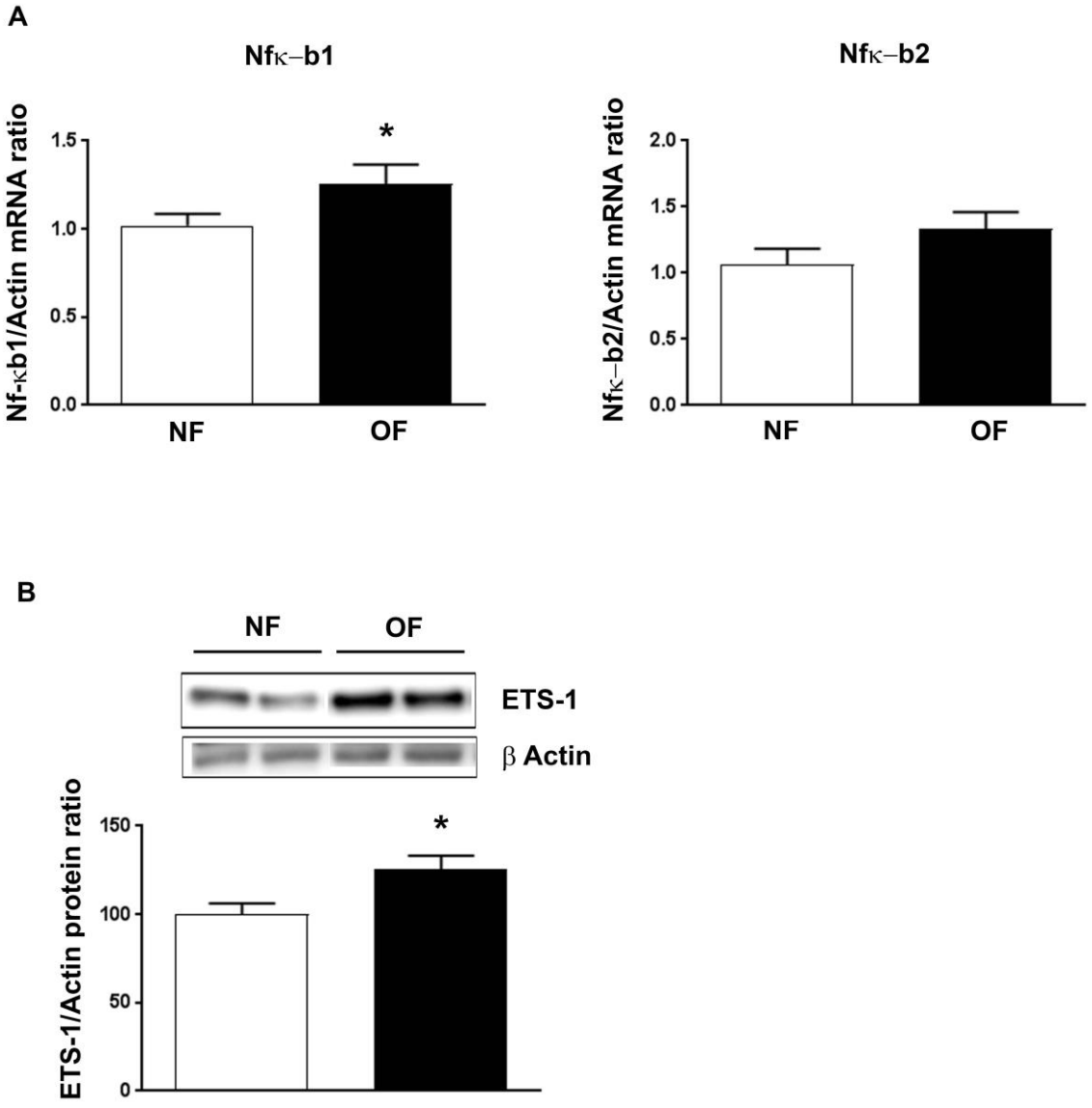

A
Nfκ–b1
Nf-κb1/Actin mRNA ratio
1.5
1.0
0.5
0.0
*
NF
OF
Nfκ–b2
Nfκ–b2/Actin mRNA ratio
2.0
1.5
1.0
0.5
0.0
NF
OF
B
NF
OF
ETS-1
β Actin
ETS-1/Actin protein ratio
150
100
50
0
*

FIGURE 4

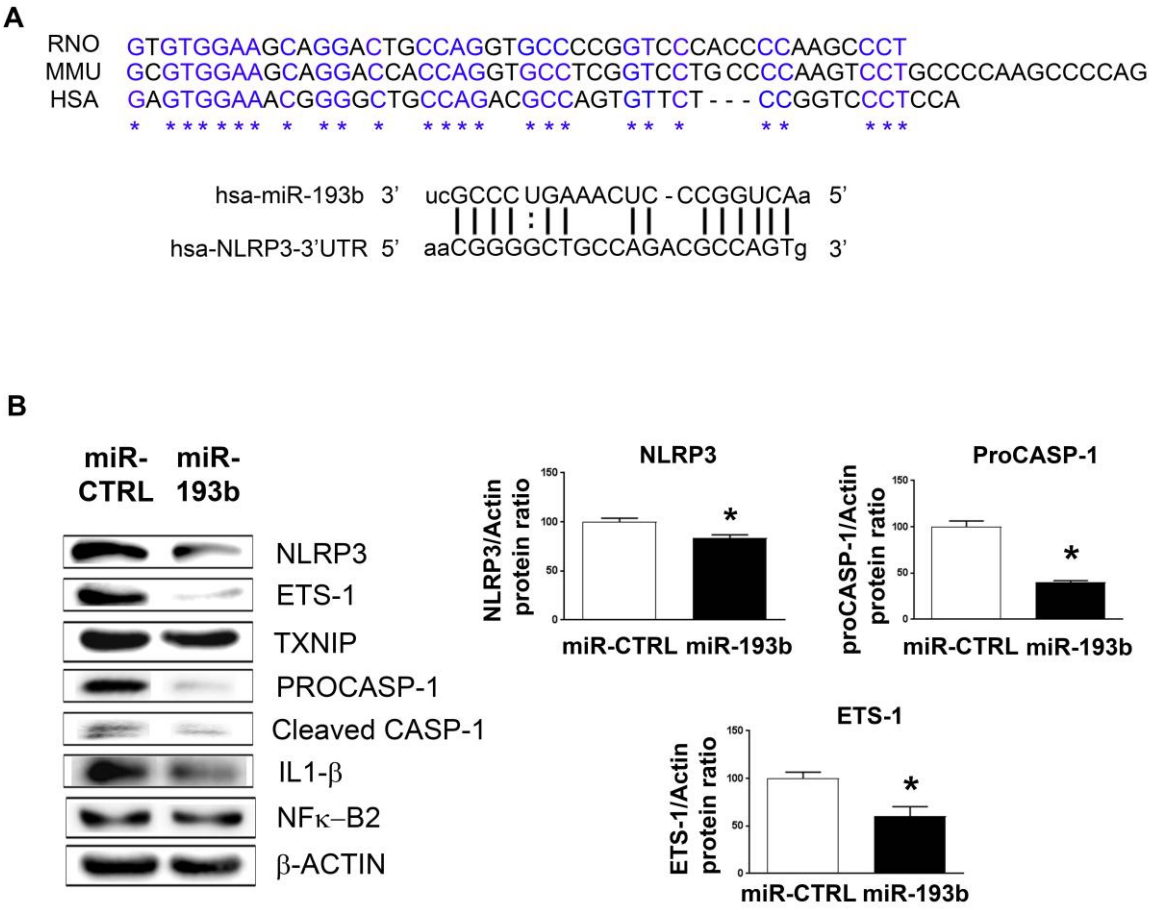

A
RNO GTGTGGAAGCAGGACTGCCAGGTGCCCCGGTCCCACCCCAAGCCCT
MMU GCGTGGAAGCAGGACCACCAGGTGCCTCGGTCCTG CCCCAAGTCCTGCCCCAAGCCCCAG
HSA GAGTGGAAACGGGGCTGCCAGACGCCAGTGTTCT - - - CCGGTCCCTCCA
hsa-miR-193b 3' ucGCCC UGAAACUC - CCGGUCAa 5'
hsa-NLRP3-3'UTR 5' aaCGGGGCTGCCAGACGCCAGTg 3'
B
miR-CTRL
miR-193b
NLRP3
ETS-1
TXNIP
PROCASP-1
Cleaved CASP-1
IL1-β
NFκ–B2
β-ACTIN
NLRP3
NLRP3/Actin protein ratio
miR-CTRL miR-193b
ProCASP-1
proCASP-1/Actin protein ratio
miR-CTRL miR-193b
ETS-1
ETS-1/Actin protein ratio
miR-CTRL miR-193b
C
miR-193b
miR-193b/miR-16 ratio
NF
OF

FIGURE 5

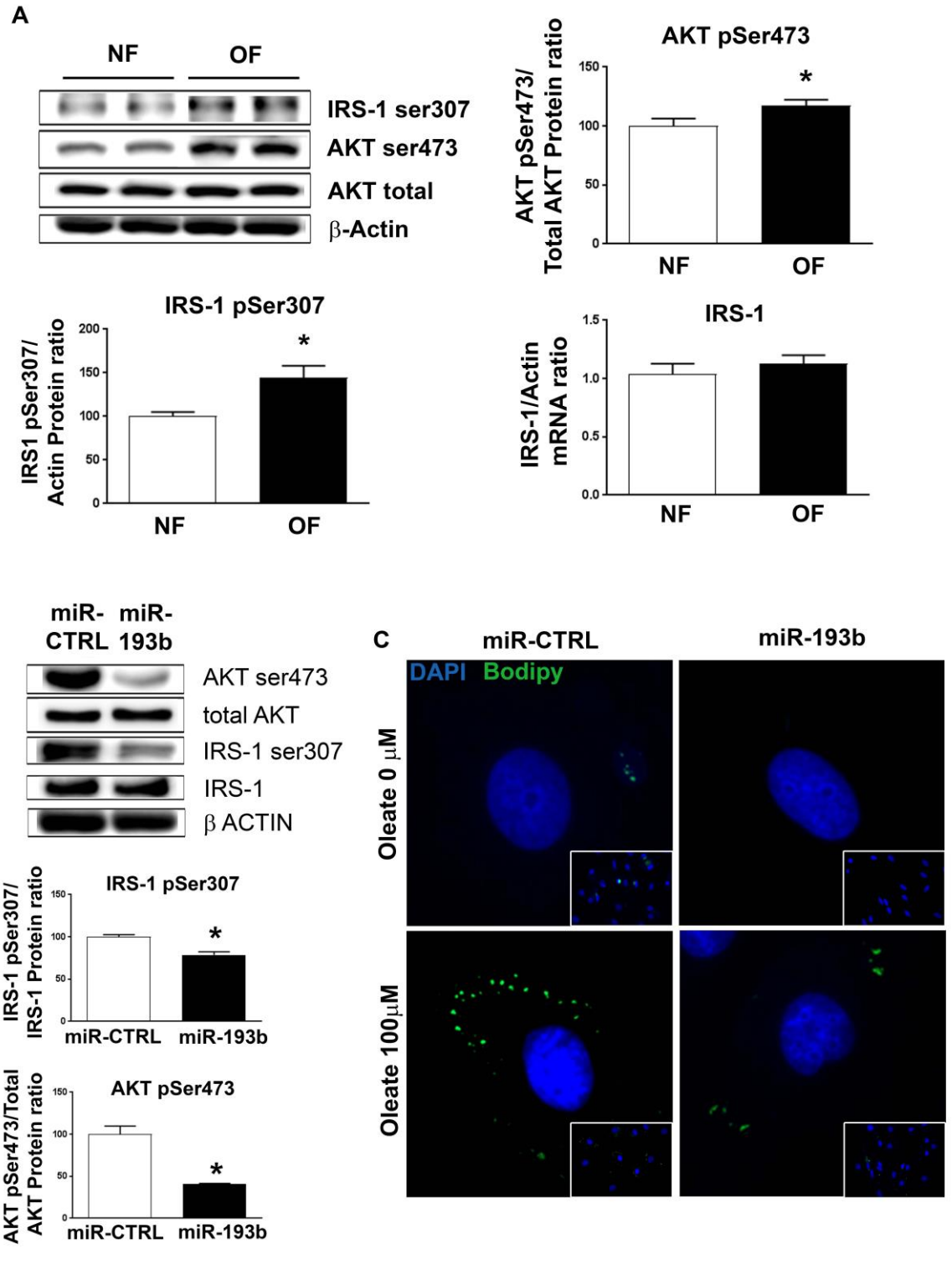
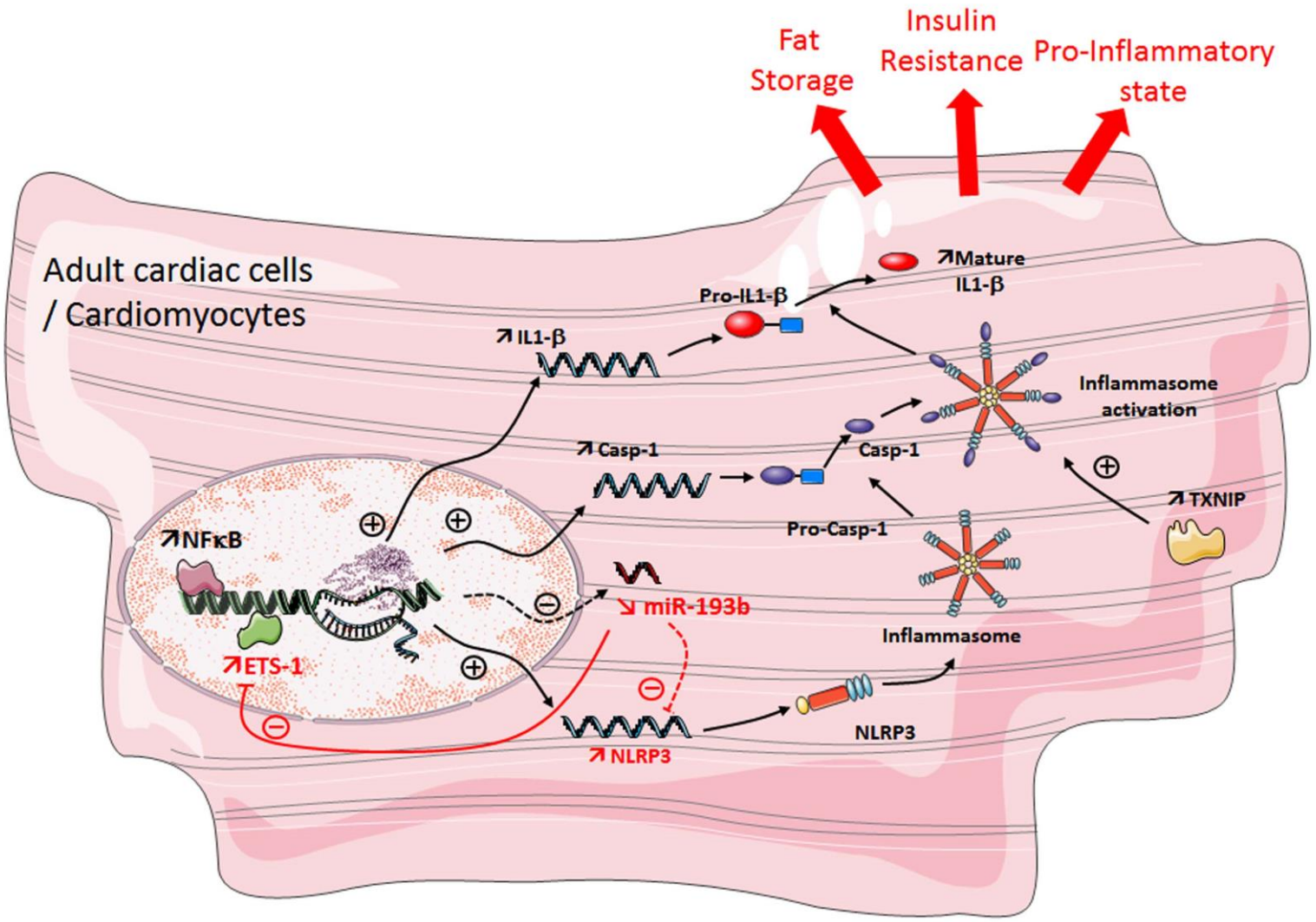
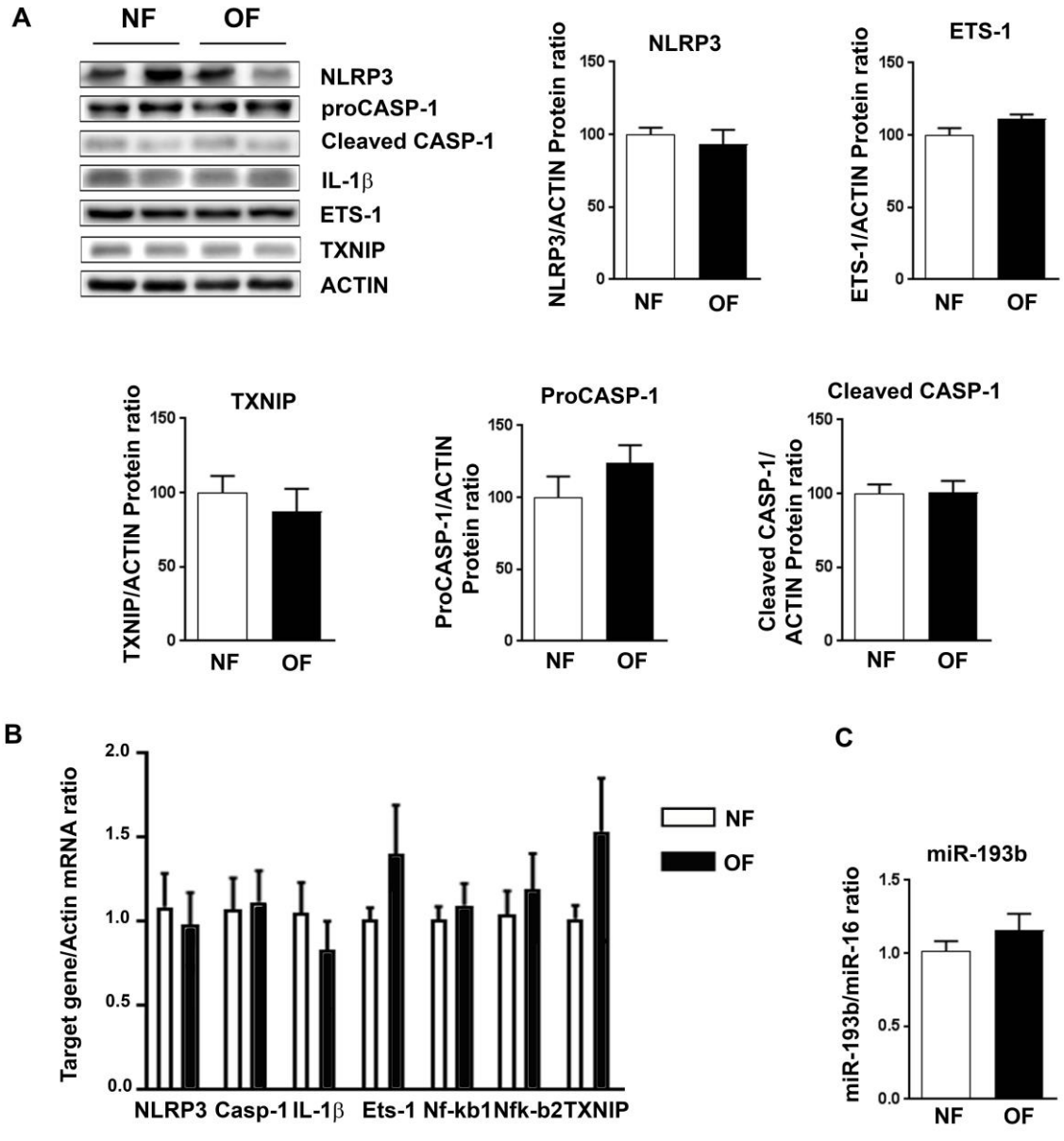

A
NF
OF
IRS-1 ser307
AKT ser473
AKT total
β-Actin
AKT pSer473
AKT pSer473/ Total AKT Protein ratio
150
100
50
0
*
NF
OF
IRS-1 pSer307
IRS1 pSer307/ Actin Protein ratio
200
150
100
50
0
*
NF
OF
IRS-1
IRS-1/Actin mRNA ratio
1.5
1.0
0.5
0.0
NF
OF
B
miR- CTRL
miR- 193b
AKT ser473
total AKT
IRS-1 ser307
IRS-1
β ACTIN
IRS-1 pSer307
IRS-1 pSer307/ IRS-1 Protein ratio
150
100
50
0
*
miR-CTRL
miR-193b
AKT pSer473
AKT pSer473/Total AKT Protein ratio
150
100
50
0
*
miR-CTRL
miR-193b
C
miR-CTRL
miR-193b
DAPI Bodipy
Oleate 0 μM
Oleate 100μM

FIGURE 6


Fat Storage
Insulin Resistance
Pro-Inflammatory state
Adult cardiac cells / Cardiomyocytes
↗IL1-β
Pro-IL1-β
↗Mature IL1-β
Inflammasome activation
↗Casp-1
Casp-1
Pro-Casp-1
↗TXNIP
↗NFκB
↘ miR-193b
↗ETS-1
Inflammasome
NLRP3
↗ NLRP3

SUPPLEMENTAL FIGURE S1



SUPPLEMENTAL TABLE 1

Gene	Forward primer	Reverse primer
Caspase-1	AGATGGCACATTTCCAGGAC	GATCCTCCAGCAGCAACTTC
Ets-1	ACAGCTTTGTTGTCCATCTG	AGATCTGTCCATCCTTCCTG
Il-1β	CACAGCAGCACATCAACAAG	GTGCTCATGTCCTCATCCTG
IRS-1	TCCTATCCCGAAGAGGGTCT	TGGGCATATAGCCATCATCA
Nf-kb1	GAAATTCCTGATCCAGACAAAAAC	ATCACTTCAATGGCCTCTGTGTAG
Nf-kb2	CTGGTGGACACATACAGGAAGAC	ATAGGCACTGTCTTCTTTCACCTC
NLRP3	AGCCTTCCAGGATCCTCTTC	CTTGGGCAGCAGTTTCTTTC
TXNIP	ATCCCAGATACCCCAGAAGC	TGAGAGTCGTCCACATCGTC
β-actin	TCCCTGGAGAAGAGCTATGA	CGATAAAGGAAGGCTGGAA