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Reduced Neuronal Nitric Oxide Synthase Expression Contributes to Cardiac Oxidative Stress and Nitroso-Redox Imbalance in *ob/ob* Mice

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Abstract

Disruption of leptin signaling in the heart may contribute to obesity-related cardiac disease, as leptin deficient (*ob/ob*) mice display cardiac hypertrophy, increased cardiac apoptosis and reduced survival. Since leptin maintains a tonic level of neuronal nitric oxide synthase (NOS1) expression in the brain, we hypothesized that leptin deficiency would decrease NOS1 cardiac expression, in turn activating xanthine oxidoreductase (XOR) and creating nitroso-redox imbalance. We studied 2–6 month old *ob/ob* (n=26) and C57Bl/6 controls (n=27). Cardiac NOS1 protein abundance ($P<0.01$) and mRNA expression ($P=0.03$) were reduced in *ob/ob* (n=10 and 6, respectively), while NOS3 protein abundance and mRNA expression were unaltered. Importantly, cardiac NOS1 protein abundance was restored towards normal in *ob/ob* mice after leptin treatment (n=3; $P<0.05$ vs leptin untreated *ob/ob* mice). NO metabolite (nitrite and nitrate) production within the myocardium was also reduced in *ob/ob* mice (n=5; $P=0.02$). Furthermore, oxidative stress was increased in *ob/ob* mice as GSH/GSSG ratio was decreased (n=4; $P=0.02$). Whereas XOR activity measured by Amplex Red fluorescence assay kit was increased (n=8; $P=0.04$), XOR and NADPH oxidase subunits protein abundance were not changed in *ob/ob* mice (n=6). Leptin deficiency did not disrupt NOS1 subcellular localization, as NOS1 co-localized with ryanodine receptor but not with caveolin-3. In conclusion, leptin deficiency is linked to decreased cardiac expression of NOS1 and NO production, with a concomitant increase in XOR activity and oxidative stress, resulting in nitroso-redox imbalance. These data offer novel insights into potential mechanisms of myocardial dysfunction in obesity.

Keywords

obesity; heart; free radicals; leptin; nitric oxide synthase; xanthine oxidoreductase

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Introduction

Obesity, an epidemic of growing proportions in the US, has substantial health implications, as it is associated with the development of diabetes [1], and cardiovascular diseases, such as heart failure (HF) [2;3], and sudden death [2]. Currently, biochemical mechanisms linking obesity to HF remain incompletely understood. Leptin, the product of the *ob* gene [4], is a hormone produced by adipose tissue that regulates food intake and body weight [5]. In addition, leptin has widespread biologic activity and its receptors are also expressed at the heart [6–8]. In this regard, there is growing interest in the cardiovascular activity of the leptin signaling pathway [6;9–11] as dysregulation of its pathway is present in hypertension and HF [12–15], and leptin plasma levels is a predictor for cardiovascular events [16;17]. Leptin deficient (*ob/ob*) mice express a myriad of cardiovascular alterations including development of cardiac hypertrophy [6], impaired β -adrenergic signaling [18], increased cardiac apoptosis and reduced survival [19].

The leptin signaling pathway is linked with nitric oxide (NO) pathway, as leptin stimulates production of NO by cardiomyocytes [10]. In the heart, NO synthase (NOS) isoforms are compartmentalized to specific subcellular microdomains and are involved in many aspects of cardiac excitation-contraction coupling and energetics [20–22]. The balance between the production of reactive oxygen species (ROS) and nitrogen species (RNS) – nitroso-redox balance – is a proposed mechanism for the regulation of multiple functions inside the cell in normal and pathological conditions [23;24], and both endothelial NOS (NOS3) [25–28] and neuronal NOS (NOS1) [29;30] isoforms are protective in cardiac disease. NOS1 participates in several critical physiologic cardiac processes. This isoform facilitates the SR Ca^{2+} cycle that is central to excitation-contraction coupling [31;32], and constrains the activity of the xanthine oxidoreductase (XOR) [33;34], a major source of $\text{O}_2^{\bullet -}$ in the heart, thus contributing to the maintenance of normal nitroso-redox balance inside the cell. Furthermore, NOS1 deficient mice develop cardiac changes that are also present in *ob/ob* mice, as cardiac hypertrophy, and reduced response to β -adrenergic stimulus [31]. Interestingly, leptin regulates NOS1 expression in the brain contributing to keep a ‘tonic’ level of its expression [35]. Accordingly, we tested the hypothesis that *ob/ob* mice display reduced NOS1 cardiac expression with consequent increase in XOR activity and development of nitroso-redox imbalance, which can contribute to the alterations described in the cardiac phenotype of *ob/ob* mice.

Materials and Methods

Animals

We studied 2–6 month old *ob/ob* (n=26) and age-matched C57BL6 wild type (WT) controls (n=27). All animals were purchased from Jackson Laboratories, Bar Harbor, ME. Mice were housed in an animal facility with a 12-hour light-dark cycle and allowed water and food *ad libitum*. The Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all protocols and experimental procedures.

We induced weight loss in a group of *ob/ob* mice for 4 weeks by leptin infusion. Mice were anesthetized with 2% isoflurane for the procedure. Osmotic minipumps (Alzet, Cupertino, CA) 100 μL were filled with recombinant mouse leptin (R&D Systems, Minneapolis, MN, 0.3 mg/kg per day) or PBS and implanted subcutaneously in the interscapular area. Pumps were replaced every 14 days. The leptin group (n=7) received continuous leptin infusion and food *ad libitum*. Controls (n=7) received PBS infusion and food *ad libitum*. A group of WT controls were also treated with leptin (n=7) or PBS (n=7) infusion.

Western-Blotting

Whole heart proteins were prepared and western blots analysis was performed as described [32;33]. The blots were incubated with primary polyclonal anti-NOS1 antibody (1:1000, BD Transduction Laboratories, San Diego, CA), anti-xanthine dehydrogenase/XOR antibody (1:1000, NeoMarkers Inc., Fremont, CA), anti-p47^{phox} antibody (1:500, Upstate, Lake Placid, NY), anti-p22^{phox} antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-NOS3 antibody (1:1000, BD Transduction Laboratories), anti-p67^{phox} antibody, or anti-gp91^{phox} antibody (1:500, BD Transduction Laboratories). A monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (1:100000, Research Diagnostic Inc., Flanders, NJ) was used separately as a normalizer. Later on the membranes were incubated for 1 hour with peroxidase-conjugated chicken anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology) in 1:5000 dilutions. Bands were visualized by chemiluminescence (SuperSignal Substrate kit, Pierce, Rockford, IL) and quantified using the NIH Image software.

Quantification of mRNA

Fluorescence based real-time qPCR was used to determine relative expression of cardiac mRNA. Total RNA was isolated, cDNA was synthesized and each sample was run in duplicate on a GeneAmp 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) and was analyzed using SDS 2.0 software (Applied Biosystems) as we have described [33]. All samples were run in triplicate and repeated with GAPDH used as normalizer. A subset (n=3) of the NADPH subunits data of the WT animals has been previously reported [29]. The primer sequences are described in Table 1.

Immunofluorescence

Hearts from WT and *ob/ob* mice were obtained, perfused with PBS to wash out blood, fixed in 4% paraformaldehyde overnight at 4°C and re-hydrated in 25% sucrose at 4°C. The tissue samples were then embedded in O.C.T. and stored at -80°. All immunohistochemical studies were carried out on 6-µm-thick frozen sections. After blocking (10% normal donkey serum) and permeabilization, the tissue sections were incubated with anti-goat caveolin-3 (1:100, Santa Cruz Biotechnology), anti-goat ryanodine receptor (RYR; 1:50, Santa Cruz Biotechnology) and/or anti-rabbit NOS1 (1:40, BD Transduction Laboratories) antibodies for 1 hour at 4°C. After washing, the tissue sections were incubated with Alexa Fluor 594-labeled donkey anti-goat (1:250, Molecular Probes, Eugene, OR) and Alexa Fluor 488-labeled donkey anti-rabbit (1:250, Molecular Probes) antibodies for one hour at 4°C. The slides were viewed on a Zeiss Axiovert 200 confocal microscope with 510-Meta confocal laser scanning module and digital images were obtained. The excitation/emission wavelengths for the donkey anti-goat antibody were 543/560 and for the donkey anti-rabbit antibody were 488/505.

NO Metabolites

NO metabolites in heart homogenates were determined by the Griess reaction (nitrate/nitrite colorimetric assay kit, Cayman chemical, Ann Arbor, MI), which measures the combined oxidation products for NO (nitrites and nitrates) after reduction with nitrate reductase. Griess reagent was formed of 2% sulfanilamide in 1M H₃PO₄ and 0.2% sulfanilamide in water. Nitrite was quantified colorimetrically at 540 nm. Standards were made by serial dilutions of sodium nitrite.

GSH/GSSG Ratio—Determination of the reduced glutathione/glutathione disulfide (GSH/GSSG) ratio was performed by using the glutathione assay kit (Cayman chemical). Briefly, animals were sacrificed, the hearts harvested and perfused with cold PBS and snap frozen. The tissue was homogenized and the supernatant obtained after centrifugation (10,000 g, 15 min) was deproteinated following the instructions of the manufacturer. The deproteinated sample

was divided into two aliquots: one for measurement of total GSH and the other for measurement of GSSG. The samples were assayed spectrophotometrically at 405 nm using a microplate spectrophotometer.

XOR Activity

XOR activity was measured using the horseradish peroxidase-linked Amplex Red fluorescence assay (Molecular Probes), as previously described [36]. Heart homogenates were passed through Sephadex G-25 columns (GE Healthcare, Piscataway, NJ). The processed effluent was added to working solution containing Amplex Red reagent (50 μ M), xanthine (0.1 mM) and horseradish peroxidase type II (0.1 U/ml), and the H₂O₂ production was measured in the absence and presence of allopurinol (300 μ M), in order to subtract the background. The reactions were incubated at 37 °C for 30 minutes. Fluorescence readings were made in duplicate in a 96-well plate at Ex/Em = 544/590 nm using 100- μ l total volume per each well. The obtained values were plotted against a standard curve with known concentrations of XOR, in order to determine the XOR activity in mU/mg protein in the samples tested.

Statistics

Data were expressed as mean $\pm$ standard error of the mean (SEM). Data between groups were compared using Student's t-test when comparing two groups or one-way ANOVA, followed by Student-Newman-Keuls post-hoc analysis, when comparing four groups. The null hypothesis was rejected at $P < 0.05$.

Results

NOS1 and NOS3 Abundance/Expression

We evaluated cardiac NOS1 and NOS3 protein abundance and mRNA expression in *ob/ob* mice and controls. There was no significant difference in NOS3 protein abundance between leptin treated (n=3) and untreated (n=6) controls, and leptin treated (n=3) and untreated (n=7) *ob/ob* mice. In contrast, NOS1 protein abundance was decreased by 52% in *ob/ob* mice (n=10) compared to WT (n=9; $P < 0.01$; Figure 1A–C). Whereas leptin treatment did not alter NOS1 protein abundance in controls (n=3), it restored cardiac NOS1 protein abundance towards normal in *ob/ob* mice (n=3; $P < 0.05$ vs leptin untreated *ob/ob* mice). Cardiac NOS1 mRNA expression was also decreased by 47% in *ob/ob* mice (n=6; $P = 0.03$) in relation to WT (n=6), while NOS3 mRNA expression was unchanged (WT n=3; *ob/ob* n=5; Figure 1D).

NOS1 Subcellular Localization

We evaluated whether NOS1 spatial localization was altered within the cardiomyocyte from *ob/ob* mice using dual-photon immunofluorescence. NOS1 immunostaining (in green) localized to the sarcoplasmic reticulum (SR) in both strains and not to the plasma membrane (Figure 2), as seen by superposition of NOS1 and RYR immunostaining (Figure 3), but no superposition of NOS1 and caveolin-3 (Figure 2).

Nitroso-Redox Balance

In order to identify the consequences of NOS1 downregulation on nitroso-redox balance of *ob/ob* mice, we analyzed both NO production and oxidative stress. NO production, evaluated by total nitrate/nitrite concentration in heart homogenates, was 47% lower ($P = 0.02$) in *ob/ob* mice (n=5) than in controls (n=5; Figure 4A). Conversely, oxidative stress was higher in *ob/ob* mice (n=4) than in controls (n=4) as demonstrated by decreased GSH/GSSG ratio ($P = 0.02$; Figure 4B). Taken together, these results demonstrate that in *ob/ob* mice there is concomitant increase in O₂^{•−} and reduction in NO production, the hallmarks of nitroso-redox imbalance.

XOR Activity

We examined the source for the increased $O^{\bullet}_2$ production in *ob/ob* mice by analyzing the activity of XOR. XOR activity was 68% higher ($P = 0.04$) in *ob/ob* (n=8) than in WT mice (n=8; Figure 4C).

Expression/Abundance of XOR and NADPH oxidase

We investigated if increased XOR activity in *ob/ob* mice could be attributed to differences in XOR expression. Cardiac XOR protein abundance (Figure 5A) and mRNA expression (Figure 5B) were similar in *ob/ob* (n= 6 for each experiment) and WT (n=6 and 3, respectively) mice. We also investigated if nicotinamide adenine dinucleotide 3-phosphate (NADPH) oxidase, another $O^{\bullet}_2$ source in the heart, could have its expression changed in *ob/ob* mice. NADPH oxidase subunits (Gp91^{phox}, P22^{phox}, P47^{phox} and P67^{phox}) protein abundance (Figure 6) and mRNA expression were similar in *ob/ob* (n=6) and WT (n=6) mice (Figure 7). Leptin repletion did not further affect protein abundance of either XOR (Figure 5) or NADPH oxidase subunits (Figure 6) in *ob/ob* (n=6) or WT (n= 6) mice.

Discussion

The major new finding of this study is that leptin deficiency is associated with cardiac NOS1 downregulation, but not translocation, and that this leads to nitroso-redox imbalance in a murine model of obesity associated with the development of phenotypes associated with heart failure. These findings suggest that NOS1 related nitroso-redox imbalance is a general mechanism for cardiac dysfunction and is not limited to overt heart failure [29]. Nitroso-redox imbalance is proposed to participate in deregulated signaling throughout biological systems, as it impairs physiological S-nitrosylation of key proteins involved in excitation-contraction coupling [23].

Under physiologic conditions, NOS1 localizes to the cardiac SR where it likely participates in S-nitrosylation of RYR thereby facilitating Ca^{2+} -induced Ca^{2+} release [37]. XOR is also present in the SR in association with NOS1 and RYR suggesting that it also participates in this process, through mechanisms that are presently unclear [33;34]. Importantly, XOR activity is upregulated with NOS1 deficiency representing a potential source of oxidative stress. From a functional standpoint, NOS1 deficiency blunts the response of myocardial contractility to both catecholamines [31] and increased heart rate stimuli [32;33], and increased XOR activity is implicated in depressed myofilament responses to activator calcium [33].

Several phenotypic features of the *ob/ob* mouse are reminiscent of the phenotype attributed to nitroso-redox imbalance. These mice exhibit attenuated β -adrenergic inotropic responses [18], develop cardiac hypertrophy [6] and increased myocyte apoptosis [19]. Here we directly addressed the hypothesis that these changes may be associated with nitroso-redox imbalance and demonstrated downregulation of NOS1 as well as increased XOR-driven ROS production. Importantly, when *ob/ob* mice were treated by exogenous leptin repletion the cardiac NOS1 protein abundance restored towards normal. This confirms that leptin deficiency in *ob/ob* mice was responsible for downregulation of cardiac NOS1 expression.

Nitroso-redox imbalance develops in *ob/ob* mice as a consequence of increased XOR-driven ROS production associated to reduced NO production. The lower NO generation in *ob/ob* mice is probably due solely to NOS1 downregulation, as NOS3 is not downregulated in *ob/ob* mice. Importantly, the increased level of XOR activity in *ob/ob* mice is independent of XOR abundance, since proteins levels are similar in WT and *ob/ob* mice. This phenomenon is in accordance with previous findings from our group [33] and others [34] that have shown higher XOR activity in NOS1^{-/-} mice resulting in higher ROS production despite unchanged levels

of XOR protein abundance. The increase in oxidative stress in *ob/ob* mice was not related to NADPH oxidase, which is another important source of ROS in the heart [23], as the expression of its subunits was similar between *ob/ob* mice and controls. Besides that, NADPH oxidase is reported to be linked to NOS3[25] and both protein abundance and mRNA expression of NOS3 were unaltered by leptin deficiency. Another important aspect in NOS1 cardiobiology in disease states is its translocation from SR to sarcolemmal membranes [38–40]. We were unable to demonstrate such translocation in our conditions, but we must stress that the conditions where NOS1 translocation was described were associated with increased NOS1 expression [38–40], whereas in our condition we observed NOS1 down-regulation.

Interestingly, ROS have been implicated as one of the second messengers of the leptin pathway in endothelial cells [41;42] and ROS in hyperleptinemia states is implicated in atherogenesis [41]. Nitroso-redox imbalance has been implicated in a series of adverse phenomena and can contribute to depressed cardiac performance in several ways [23;24]. Therefore, nitroso-redox imbalance can potentially contribute to the previous description of cardiac hypertrophy [6], impaired β -adrenergic signaling [18], increased cardiac apoptosis and reduced survival in *ob/ob* mice [19]. Additionally, NO is reported to be anti-hypertrophic [43] and NOS1 deficient mice develop cardiac hypertrophy [31;44]. The impaired β -adrenergic signaling previously observed in *ob/ob* mice [18] can be related not only to the decreased protein kinase A (PKA) activity [18] but also to our observation of reduced NOS1 expression and consequent increased XOR activity. Isolated myocytes from NOS1 deficient animals present impaired β -adrenergic response [32], while XOR inhibition restores it [33].

Limitations

Several potential limitations warrant mention. First, we noted biologic variation in the level of some proteins in the different groups studied and addressed this by assaying large enough group sizes so as to achieve statistical significance in our measurements. In terms of addressing the adequacy of our leptin infusion technique, we have previously validated that this method achieves physiologic plasma leptin levels [6] accordingly, we did not repeat those measurements in this study. Finally, in terms of the Griess reaction employed to evaluate NO metabolites concentrations in biological specimens, we report results from a widely used method that involves nitrate reduction to nitrite (so-called nitrate recovery) [45]. We did not measure nitrate recovery from our samples, but feel confident that our technique is accurate given reports of 90–99% nitrate recovery from serum samples, kidney homogenates [46], and pleural exudates [47] using the same technique.

Conclusion

From a clinical standpoint, diabetes and obesity are currently considered to be a pre HF condition. Here, we unravel a novel mechanism in obesity - nitroso-redox imbalance- whose putative consequences over the cardiovascular system can contribute to the development of obesity-related heart disease. Nitroso-redox imbalance described in this murine obesity model warrants further studies to evaluate the intervention over these pathways as a preventive and/or treatment strategies for cardiac disease development in obesity and diabetes.

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References

1. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. *JAMA* 1999;282:1523–1529. [PubMed: 10546691]
2. Contaldo F, Pasanisi F, Finelli C, de SG. Obesity, heart failure and sudden death. *Nutr Metab Cardiovasc Dis* 2002;12:190–197. [PubMed: 12514939]
3. Kenchaiah S, Gaziano JM, Vasan RS. Impact of obesity on the risk of heart failure and survival after the onset of heart failure. *Med Clin North Am* 2004;88:1273–1294. [PubMed: 15331317]
4. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994;372:425–432. [PubMed: 7984236]
5. Friedman JM. Leptin, leptin receptors and the control of body weight. *Eur J Med Res* 1997;2:7–13. [PubMed: 9049588]
6. Barouch LA, Berkowitz DE, Harrison RW, O'donnell CP, Hare JM. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. *Circulation* 2003;108:754–759. [PubMed: 12885755]
7. Wold LE, Relling DP, Duan J, Norby FL, Ren J. Abrogated leptin-induced cardiac contractile response in ventricular myocytes under spontaneous hypertension: role of Jak/STAT pathway. *Hypertension* 2002;39:69–74. [PubMed: 11799081]
8. Breslow MJ, Min-Lee K, Brown DR, Chacko VP, Palmer D, Berkowitz DE. Effect of leptin deficiency on metabolic rate in ob/ob mice. *Am J Physiol* 1999;276:E443–E449. [PubMed: 10070008]
9. Illiano G, Naviglio S, Pagano M, Spina A, Chiosi E, Barbieri M, Paolisso G. Leptin affects adenylate cyclase activity in H9c2 cardiac cell line: effects of short- and long-term exposure. *Am J Hypertens* 2002;15:638–643. [PubMed: 12118913]
10. Nickola MW, Wold LE, Colligan PB, Wang GJ, Samson WK, Ren J. Leptin attenuates cardiac contraction in rat ventricular myocytes. Role of NO. *Hypertension* 2000;36:501–505. [PubMed: 11040226]
11. Rajapurohitam V, Gan XT, Kirshenbaum LA, Karmazyn M. The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. *Circ Res* 2003;93:277–279. [PubMed: 12893740]
12. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996;334:292–295. [PubMed: 8532024]
13. Bray GA, York DA. Clinical review 90: Leptin and clinical medicine: a new piece in the puzzle of obesity. *J Clin Endocrinol Metab* 1997;82:2771–2776. [PubMed: 9284693]
14. Pladevall M, Williams K, Guyer H, Sadurni J, Falces C, Ribes A, Pare C, Brotons C, Gabriel R, Serrano-Rios M, Haffner S. The association between leptin and left ventricular hypertrophy: a population-based cross-sectional study. *J Hypertens* 2003;21:1467–1473. [PubMed: 12872039]
15. Sader S, Nian M, Liu P. Leptin: a novel link between obesity, diabetes, cardiovascular risk, and ventricular hypertrophy. *Circulation* 2003;108:644–646. [PubMed: 12912793]
16. Wolk R, Berger P, Lennon RJ, Brilakis ES, Johnson BD, Somers VK. Plasma leptin and prognosis in patients with established coronary atherosclerosis. *J Am Coll Cardiol* 2004;44:1819–1824. [PubMed: 15519013]
17. Soderberg S, Ahren B, Jansson JH, Johnson O, Hallmans G, Asplund K, Olsson T. Leptin is associated with increased risk of myocardial infarction. *J Intern Med* 1999;246:409–418. [PubMed: 10583712]
18. Minhas KM, Khan SA, Raju SV, Phan AC, Gonzalez DR, Skaf MW, Lee K, Tejani AD, Saliaris AP, Barouch LA, O'donnell CP, Emala CW, Berkowitz DE, Hare JM. Leptin repletion restores depressed {beta}-adrenergic contractility in ob/ob mice independently of cardiac hypertrophy. *J Physiol* 2005;565:463–474. [PubMed: 15760936]
19. Barouch LA, Gao D, Chen L, Miller KL, Xu W, Phan AC, Kittleson MM, Minhas KM, Berkowitz DE, Wei C, Hare JM. Cardiac myocyte apoptosis is associated with increased DNA damage and decreased survival in murine models of obesity. *Circ Res* 2006;98:119–124. [PubMed: 16339484]
20. Hare JM. Spatial confinement of isoforms of cardiac nitric-oxide synthase: unravelling the complexities of nitric oxide's cardiobiology. *Lancet* 2004;363:1338–1339. [PubMed: 15110487]

21. Hare JM. Nitric oxide and excitation-contraction coupling. *J Mol Cell Cardiol* 2003;35:719–729. [PubMed: 12818561]
22. Belge C, Massion PB, Pelat M, Balligand JL. Nitric oxide and the heart: update on new paradigms. *Ann N Y Acad Sci* 2005;1047:173–182. [PubMed: 16093495]
23. Hare JM, Stamler JS. NO/redox disequilibrium in the failing heart and cardiovascular system. *J Clin Invest* 2005;115:509–517. [PubMed: 15765132]
24. Hare JM. Nitroso-redox balance in the cardiovascular system. *N Engl J Med* 2004;351:2112–2114. [PubMed: 15533853]
25. Smith RS Jr, Agata J, Xia CF, Chao L, Chao J. Human endothelial nitric oxide synthase gene delivery protects against cardiac remodeling and reduces oxidative stress after myocardial infarction. *Life Sci* 2005;76:2457–2471. [PubMed: 15763077]
26. Scherrer-Crosbie M, Ullrich R, Bloch KD, Nakajima H, Nasseri B, Aretz HT, Lindsey ML, Vancon AC, Huang PL, Lee RT, Zapol WM, Picard MH. Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice. *Circulation* 2001;104:1286–1291. [PubMed: 11551881]
27. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. *Proc Natl Acad Sci U S A* 2003;100:4891–4896. [PubMed: 12676984]
28. Janssens S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M, Jans P, Scherrer-Crosbie M, Picard MH, Szelid Z, Gillijns H, Van de WF, Collen D, Bloch KD. Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. *Circ Res* 2004;94:1256–1262. [PubMed: 15044322]
29. Saraiva RM, Minhas KM, Raju SV, Barouch LA, Pitz E, Schuleri KH, Vandegaer K, Li D, Hare JM. Deficiency of neuronal nitric oxide synthase increases mortality and cardiac remodeling after myocardial infarction: role of nitroso-redox equilibrium. *Circulation* 2005;112:3415–3422. [PubMed: 16301341]
30. Dawson D, Lygate CA, Zhang MH, Hulbert K, Neubauer S, Casadei B. nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. *Circulation* 2005;112:3729–3737. [PubMed: 16344403]
31. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, Hobai IA, Lemmon CA, Burnett AL, O'Rourke B, Rodriguez ER, Huang PL, Lima JA, Berkowitz DE, Hare JM. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* 2002;416:337–339. [PubMed: 11907582]
32. Khan SA, Skaf MW, Harrison RW, Lee K, Minhas KM, Kumar A, Fradley M, Shoukas AA, Berkowitz DE, Hare JM. Nitric oxide regulation of myocardial contractility and calcium cycling: independent impact of neuronal and endothelial nitric oxide synthases. *Circ Res* 2003;92:1322–1329. [PubMed: 12764022]
33. Khan SA, Lee K, Minhas KM, Gonzalez DR, Raju SV, Tejani AD, Li D, Berkowitz DE, Hare JM. Neuronal nitric oxide synthase negatively regulates xanthine oxidoreductase inhibition of cardiac excitation-contraction coupling. *Proc Natl Acad Sci U S A* 2004;101:15944–15948. [PubMed: 15486091]
34. Kinugawa S, Huang H, Wang Z, Kaminski PM, Wolin MS, Hintze TH. A defect of neuronal nitric oxide synthase increases xanthine oxidase-derived superoxide anion and attenuates the control of myocardial oxygen consumption by nitric oxide derived from endothelial nitric oxide synthase. *Circ Res* 2005;96:355–362. [PubMed: 15637297]
35. Isse T, Ueta Y, Serino R, Noguchi J, Yamamoto Y, Nomura M, Shibuya I, Lightman SL, Yamashita H. Effects of leptin on fasting-induced inhibition of neuronal nitric oxide synthase mRNA in the paraventricular and supraoptic nuclei of rats. *Brain Res* 1999;846:229–235. [PubMed: 10556640]
36. Minhas KM, Saraiva RM, Schuleri KH, Lehrke S, Zheng M, Saliaris AP, Berry CE, Vandegaer KM, Li D, Hare JM. Xanthine oxidoreductase inhibition causes reverse remodeling in rats with dilated cardiomyopathy. *Circ Res* 2006;98:271–279. [PubMed: 16357304]
37. Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science* 1998;279:234–237. [PubMed: 9422697]

38. Bendall JK, Damy T, Ratajczak P, Loyer X, Monceau V, Marty I, Milliez P, Robidel E, Marotte F, Samuel JL, Heymes C. Role of myocardial neuronal nitric oxide synthase-derived nitric oxide in beta-adrenergic hyporesponsiveness after myocardial infarction-induced heart failure in rat. *Circulation* 2004;110:2368–2375. [PubMed: 15466641]
39. Damy T, Ratajczak P, Shah AM, Camors E, Marty I, Hasenfuss G, Marotte F, Samuel JL, Heymes C. Increased neuronal nitric oxide synthase-derived NO production in the failing human heart. *Lancet* 2004;363:1365–1367. [PubMed: 15110495]
40. Sun J, Picht E, Ginsburg KS, Bers DM, Steenbergen C, Murphy E. Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca²⁺ channel alpha1 subunit and reduced ischemia/reperfusion injury. *Circ Res* 2006;98:403–411. [PubMed: 16397145]
41. Bouloumie A, Marumo T, Lafontan M, Busse R. Leptin induces oxidative stress in human endothelial cells. *FASEB J* 1999;13:1231–1238. [PubMed: 10385613]
42. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* 2001;276:25096–25100. [PubMed: 11342529]
43. Calderone A, Thaik CM, Takahashi N, Chang DL, Colucci WS. Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. *J Clin Invest* 1998;101:812–818. [PubMed: 9466976]
44. Barouch LA, Cappola TP, Harrison RW, Crone JK, Rodriguez ER, Burnett AL, Hare JM. Combined loss of neuronal and endothelial nitric oxide synthase causes premature mortality and age-related hypertrophic cardiac remodeling in mice. *J Mol Cell Cardiol* 2003;35:637–644. [PubMed: 12788381]
45. Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the L-arginine/nitric oxide area of research. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2006in press
46. Tugcu V, Ozbek E, Tasci AI, Kemahli E, Somay A, Bas M, Karaca C, Altug T, Cekmen MB, Ozdogan HK. Selective nuclear factor kappa-B inhibitors, pyrolidinium dithiocarbamate and sulfasalazine, prevent the nephrotoxicity induced by gentamicin. *BJU Int* 2006;98:680–686. [PubMed: 16925772]
47. Tracey WR, Nakane M, Kuk J, Budzik G, Klinghofer V, Harris R, Carter G. The nitric oxide synthase inhibitor, L-NG-monomethylarginine, reduces carrageenan-induced pleurisy in the rat. *J Pharmacol Exp Ther* 1995;273:1295–1299. [PubMed: 7540689]

Figure 1.

(A) Representative Western blots of myocardial tissue extracts from leptin treated and untreated WT and *ob/ob* mice probed with polyclonal anti-NOS1 antibody, monoclonal anti-NOS3 antibody, and monoclonal anti-GAPDH antibody. GAPDH is used to normalize any potential differences across samples. (B) Densitometric analysis depicting average NOS1 protein abundance in leptin treated (n=3) and untreated (n=9) WT and leptin treated (n=3) and untreated (n=10) *ob/ob* mice. (C) Densitometric analysis depicting average NOS3 protein abundance in leptin treated (n=3) and untreated (n=6) WT and leptin treated (n=3) and untreated (n=7) *ob/ob* mice. (D) Graph bar representation of cardiac NOS1 and NOS3 mRNA expression measured by qPCR in myocardial tissue extracts from WT (n=6 and 3, respectively) and *ob/ob* (n=6 and 5, respectively) mice. (n) *P < 0.01 vs WT and P < 0.05 vs *ob/ob* plus leptin; †P = 0.03.

Figure 2. Dual photon immunofluorescence for caveolin-3 (CAV-3; red) and NOS1 (green) in heart sections from WT and *ob/ob* mice.

Figure 3.

(A) Dual photon immunofluorescence for ryanodine receptor (RYR; red) and NOS1 (green) in heart sections from WT and *ob/ob* mice.. (B) Detail of images overlays of cardiomyocytes from *ob/ob* mice in higher augmentation.

Figure 4. Nitroso-redox balance and XOR activity in *ob/ob* mice. (A) Graph bar representation of nitrate/nitrite levels in heart homogenates from *ob/ob* (n=5) and WT (n=5) mice. (B) Graph bar representation of GSH/GSSG ratio in cardiac tissue from *ob/ob* (n=4) and WT (n=4) mice. (C) Graph bar representation of XOR activity in heart homogenates from *ob/ob* (n=8) and WT (n=8) mice. * $P < 0.05$.

Figure 5.

Expression and abundance of XOR. (A) Representative Western blots of myocardial tissue extracts from leptin treated and untreated WT and *ob/ob* mice probed with anti-XOR antibody. Group data depicting average cardiac XOR protein abundance in leptin treated and untreated WT and *ob/ob* mice (n=6 for each group). (B) Graph bar representation of cardiac XOR mRNA expression measured by qPCR in myocardial tissue extracts from WT (n=3) and *ob/ob* mice (n=6).

Figure 6. NADPH oxidase protein abundance. (A) Representative Western blots of cardiac tissue extracts from leptin treated and untreated WT and *ob/ob* mice probed with antibodies against NADPH oxidase subunits (Gp91^{phox}, P22^{phox}, P47^{phox}, P67^{phox}). GAPDH is used to normalize any potential differences across samples. (B) Group data depicting average cardiac NADPH oxidase subunits protein abundance in leptin treated and untreated WT and *ob/ob* mice (n=6 for each group).

Figure 7. NADPH oxidase mRNA expression. Graph bar representation of cardiac NADPH oxidase subunits (Gp91^{phox}, P22^{phox}, P47^{phox}, P67^{phox}) mRNA expression measured by qPCR in myocardial tissue extracts from WT (n=6) and *ob/ob* (n=6) mice.

Table 1
Oligonucleotide primers for mRNA quantitative assays

Gene	Acession No	Primer	
XDH/XO	NM_011723	F(1693-1712)	TCCAGCTAACGTCCAGCTTT
		R(1910-1889)	TGGCTTCTGAAGTGTCGATG
Gp91 ^{phox}	U_43384	F (951-970)	CAAGATGGAGGTGGGACAGT
		R (1120-1101)	GCTTATCACAGCCACAAGCA
P22 ^{phox}	NM_007806	F (344-363)	TTCCTGTTGTCGGTGCCTGC
		R (558-539)	TTCTTTCGGACCTCTGCGGG
P67 ^{phox}	AB_002664	F (307-326)	GCAGTGGCCTACTTCCAGAG
		R (464-445)	ACCTCACAGGCAAACAGCTT
P47 ^{phox}	AB_002663	F (408-427)	ACCTGAAACTGCCCCTGAC
		R (557-538)	CTGTTCCCGAACTCTTCTCG
NOS1	NM_008712	F(2196-2215)	CTCACCCCGTCCTTTGAGTA
		R(2366-2347)	GGTCGCTTTGACTCTCTTGG
NOS3	BC_052636	F(3341-3360)	TCTGCGGCGATGTCACATG
		R(3414-3398)	CATGCCGCCCTCTGTTG
GAPDH	XM_146433	F (639-660)	GGTTGTCTCCTGCGACTTCAAC
		R (741-717)	ATACCAGGAAATGAGCTTGACAAAG