Cardiac Myocyte Apoptosis Is Associated With Increased DNA Damage and Decreased Survival in Murine Models of Obesity

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Abstract—Disruption of leptin signaling is associated with obesity, heart failure, and cardiac hypertrophy, but the role of leptin in cardiac myocyte apoptosis is unknown. We tested the hypothesis that apoptosis increases in leptin-deficient ob/ob and leptin-resistant db/db mice and is associated with aging and left ventricular hypertrophy, increased DNA damage, and decreased survival. We studied young (2- to 3-month-old) and old (12- to 14-month-old) ob/ob and db/db mice and wild-type (WT) controls (n=2 to 4 per group). As expected, ventricular wall thickness and heart weights were similar among young ob/ob, db/db, and WT mice, but higher in old ob/ob and db/db versus old WT. Young ob/ob and db/db showed markedly elevated apoptosis by TUNEL staining and caspase 3 levels compared with WT. Differences in apoptosis were further accentuated with age. Leptin treatment significantly reduced apoptosis in ob/ob mice both in intact hearts and isolated myocytes. Tissue triglycerides were increased in ob/ob hearts, returning to WT levels after leptin repletion. Furthermore, the DNA damage marker, 8oxoG (8-oxo-7,8-dihydroguanidine), was increased, whereas the DNA repair marker, MYH glycosylase, was decreased in old ob/ob and db/db compared with old WT mice. Both ob/ob and db/db mice had decreased survival compared with WT mice. We conclude that leptin-deficient and leptin-resistant mice demonstrate increased apoptosis, DNA damage, and mortality compared with WT mice, suggesting that normal leptin signaling is necessary to prevent excess age-associated DNA damage and premature mortality. These data offer novel insights into potential mechanisms of myocardial dysfunction and early mortality in obesity. (Circ Res. 2006;98:119-124.)

Key Words: obesity ▪ apoptosis ▪ DNA damage ▪ mortality

Obesity is a major risk factor for cardiovascular disease and diabetes. It is associated with an increased incidence of left ventricular (LV) hypertrophy,1 cardiomyopathy of obesity,2 heart failure,3,4 and sudden death.5 Leptin, the ob gene product,6 is a neurohormone produced by adipose tissue that regulates food intake, body weight and adipose stores, and energy homeostasis.7 Leptin receptors, the product of the db gene,8 are present in the heart and modulate numerous direct cardiac effects.9–11 Heart failure, hypertension, and obesity itself are all associated with dysregulated leptin signaling, primarily in the form of leptin resistance.12,13

Cardiac myocyte apoptosis is increased in heart failure and normal aging and is a marker of poor cardiovascular outcomes.14 Apoptosis is further associated with increased DNA damage and/or impaired DNA repair systems.15 Although dysregulation of leptin may increase apoptosis through lipotoxic pathways,16,17 whether leptin itself plays a direct role in cardiac myocyte apoptosis is unknown.

Because of the known increases in apoptosis in Zucker Diabetic Fatty (ZDF, fa/fa) rat pancreatic islet cells18 and cardiac myocytes,19 we tested the hypothesis that disruption of leptin signaling would not only result in increased apoptosis in the mouse heart but would also cause increased age-associated DNA damage and decreased long-term survival. To approach this hypothesis, we studied 2 mouse models of obesity with disrupted leptin signaling pathways: the ob/ob mouse, with a naturally occurring leptin deletion; and the db/db mouse, with a nonfunctional leptin receptor.

Materials and Methods

Animals

We studied ob/ob and db/db mice and C57BL6 wild-type (WT) controls. All animals were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were housed in an animal facility with a 12-hour light–dark cycle and allowed water and food ad libitum. Animal treatment and care was provided in accordance with institutional guidelines. The Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine approved all protocols and experimental procedures.

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Weight Loss by Administration of Exogenous Leptin

We induced weight loss in 6- to 8-month-old ob/ob mice by exogenous leptin repletion for 4 weeks. Mice were anesthetized with 2% to 3% isoflurane. Osmotic minipumps (100 µL capacity; Alzet) were filled with recombinant mouse leptin (Amgen, 0.3 mg/kg/day) and implanted subcutaneously in the interscapular area. The incision was closed with two 4-0 silk sutures. Pumps were replaced once after 14 days.

Myocyte Isolation and Fixation

Myocytes were isolated from collagenase digested hearts as described previously. Briefly, myocytes were obtained by mechanically disrupting digested hearts, followed by filtration, centrifugation, and suspension in isolation solution. The Ca²⁺ concentration was slowly increased in a stepwise fashion to a final concentration of 0.5 mmol/L. The cells were then resuspended in minimum essential medium (MEM) with Hanks’ salts, with L-glutamine containing 5% fetal bovine serum (FBS), and were allowed to settle. Pellets were resuspended in MEM without FBS and plated onto laminin-coated coverslips for 1 hour with or without 100 nmol/L leptin at 37°C. The cells were fixed with 4% paraformaldehyde and stored in phosphate buffered saline (PBS) at 4°C.

Evaluation of Cell Death by TUNEL

To quantify the relative numbers of cells with DNA fragmentation, TUNEL assay was performed in heart tissue sections using the In Situ Cell Death Detection Kit Roche, according to the protocols of the manufacturer for each. Briefly, for tissue sections, slides were first deparaffinized and myocyte nuclei were stained by stripping treatments with proteinase K for 10 minutes. Following treatment with 3% H₂O₂ for 5 minutes, slides were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme with TdT buffer and biotin-tagged nucleotides (for tissue sections) or fluorescein-tagged nucleotides (for isolated cells) in a humidified chamber at 37°C. Tagged nucleotides were detected using streptavidin–horseradish peroxidase (HRP) or anti-fluorescein–HRP conjugates, respectively. After washing, sections were stained with diamidino benzadine (DAB)/H₂O₂ solution and counterstained with hematoxylin, dehydrated, and mounted. To determine the percentage of apoptotic cells, TUNEL-positive and TUNEL-negative cells were counted. Results are expressed as number of TUNEL-positive cells/total cells×100%.

Western Blots

Whole mouse hearts were homogenized using a Polytron 3100 and centrifuged to remove debris. The lysates were quantitated with BSA standards (Sigma) using the BCA assay (Pierce Biotechnology). Equal amounts of protein were denatured, reduced, and resolved on 10% Bis-Tris gels (Invitrogen) using 4-morpholinepropanesulfonic acid/sodium dodecyl sulfate running buffer and transferred to 0.45-µm polyvinylidene difluoride membranes (Millipore) using Towbin buffer. The membranes were blocked for 1 hour with 5% nonfat dried milk solution (Dako) and steamed for 40 minutes. Slides were preincubated for 1 hour, followed by rinsing twice. Slides were mounted with mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Immunoblots were incubated with a primary rabbit polyclonal MYH (1:100; EMD Biosciences) and goat polyclonal anti-8oxoG (1:200, EMD Biosciences) in antibody dilution buffer for 1 hour at room temperature. After washing twice in PBST, slides were incubated with the anti-rabbit IgG secondary antibody (diluted 1:300 and mixed with normal bovine serum [1:100] in PBST) for 1 hour. After rinsing twice, slides were incubated in biotin-conjugated donkey anti-goat IgG (diluted 1:250 and mixed with normal bovine serum [1:100]) for 1 hour. After rinsing twice, slides were incubated in streptavidin–HRP conjugates (diluted 1:300 and mixed with normal bovine serum [1:100]) in PBST. After rinsing twice, slides were incubated in 3,3′-diaminobenzidine (DAB)/H₂O₂ solution and counterstained with hematoxylin, dehydrated, and mounted.

Statistics

Data are reported as mean±SEM unless otherwise noted. Statistical analyses were performed with Stata 8, GraphPad InStat, or Microsoft Excel Data Analysis ToolPak. Statistical significance was determined by 2-tailed Student’s t test or ANOVA with Student–Newman–Keuls post hoc analysis where appropriate. Isolated myocyte apoptosis rates were compared using Fisher’s exact test. Kaplan–Meier survival functions were compared using the log-rank test, and hazard ratios were computed from Cox proportional hazard models. Statistical differences were considered significant at P<0.05.

Results

Increased Apoptosis in ob/ob and db/db Mice

We studied the hearts of young (2- to 3-month-old) and old (12- to 14-month-old) ob/ob, db/db, and WT mice and 6-month-old ob/ob mice after 4 weeks of leptin infusion (n=2 to 4). We images using averaged measurements from 3 consecutive cardiac cycles. Fractional shortening (FS) was calculated from the end-diastolic (LVIDd) and end-systolic (LVIDs) diameters using the following equation: FS=100%×(LVIDd−LVIDs)/LVIDd.
assessed apoptosis by TUNEL staining (percentage positive nuclei). TUNEL staining showed low levels of apoptosis in young WT (0.05 ± 0.01%), which increased with age (0.3 ± 0.1%, P < 0.05). Young ob/ob (1.3 ± 0.2%) and db/db (1.1 ± 0.2%, P < 0.05 versus WT for both) showed markedly elevated apoptosis that rose even further with age (old ob/ob 2.6 ± 0.3%, old db/db 1.8 ± 0.3%; P < 0.05 versus WT for both) (Figure 1). In addition, both pro-caspase 3 and activated caspase 3 were upregulated in ob/ob and db/db compared with age-matched WT (n = 5 to 6 for each group) by Western blot (Figure 2).

Reduction of Apoptosis by Leptin in ob/ob Mice In Vivo and In Vitro

Leptin treatment for 4 weeks significantly reduced the rate of apoptosis in ob/ob mice by both TUNEL staining (0.5 ± 0.2%, P < 0.05 versus young ob/ob) (Figure 1G and 1H). To define whether the effect we saw in vivo was attributable to alterations in leptin signaling, rather than simply associated with obesity, we studied collagenase-digested isolated cardiac myocytes. Indeed, ob/ob myocytes had a 9.1% rate of apoptosis by TUNEL staining compared with 0.9% in WT (n = 400 to 750 cells counted; P < 0.001). Preincubation of ob/ob myocytes for 60 minutes with 100 nmol/L leptin significantly reduced the rate of apoptosis to 4.1%, although not to WT levels (P < 0.01 versus both untreated ob/ob and WT).

Cardiac Hypertrophy in Old ob/ob and db/db Mice

Septal wall thickness by echocardiography before death and heart weights were similar among young mice of all strains (data not shown) but increased in old ob/ob and db/db versus old WT, confirming our previous findings of age-related LV hypertrophy in these models (Figure 3).11

Increased Cardiac Triglycerides With Reduction by Leptin in ob/ob Mice

To determine whether increased ectopic lipid overload may be playing a role, we measured total triglycerides extracted from cardiac tissue as a marker of total cardiac lipids in WT, untreated ob/ob, and leptin-treated ob/ob (n = 3 hearts in each group). We found that extracted total triglycerides were increased 1.8-fold in untreated ob/ob compared with WT (P < 0.05). Four weeks of leptin repletion returned cardiac triglycerides back to WT levels (Figure 4).

Increased DNA Damage and Decreased DNA Repair in Old ob/ob and db/db Mice

We next evaluated DNA damage and repair systems in old ob/ob, db/db, and WT (n = 2 to 4; 12- to 14-month-old) mice using immunofluorescence staining and confocal microscopy. Staining for 8oxoG, a marker of DNA damage, was increased in both ob/ob and db/db compared with WT, whereas staining for MYH glycosylase, a marker of DNA repair activity, was decreased in both ob/ob and db/db compared with WT mice (Figure 5).

Increased Mortality in ob/ob and db/db Mice

Survival analysis of ob/ob (n = 174), db/db (n = 87), and WT (n = 342) mice with and average of 2 years of follow up revealed that both obese strains had decreased survival compared with WT mice. Both ob/ob (hazard ratio, 2.7; median survival, 15.3 months; P < 0.05) and db/db (hazard ratio, 2.0; median survival, 13.3 months; P < 0.005) animals had increased mortality compared with WT (median survival, 23.9 months) (Figure 6). There were no gender differences in survival within each strain (data not shown).

Discussion

The major new finding of this study is that leptin-deficient ob/ob and leptin-resistant db/db obese mice demonstrate increased cardiac myocyte apoptosis compared with WT mice, associated with increased DNA damage and decreased long-term survival. This provides an important extension of earlier work showing increased apoptosis in islet cells and cardiac myocytes in ZDF rats, which have a mutation in the leptin receptor (fa/fa), analogous to db/db in mice.18,19 Cardiac myocyte apoptosis, even at low levels, is now well recognized as an important physiologic step in the development of heart failure.14,22,23 Our findings provide a strong association between leptin signaling, a key endocrine system regulating fat, and myocyte apoptosis. These results offer...
new insights into the role of leptin in the pathogenesis of heart failure and obesity-associated cardiovascular disease.

We have previously shown that \textit{ob/ob} and \textit{db/db} mice develop LV hypertrophy with age and that leptin repletion in \textit{ob/ob} can reverse hypertrophy, restore disruptions in the \(\beta\)-adrenergic signal transduction pathway, and improve a number of metabolic abnormalities.\textsuperscript{11,20} Here we have demonstrated that high levels of apoptosis in \textit{ob/ob} mice can be returned toward normal with leptin repletion. This key finding suggests that increased cell death in these models is not merely a reflection of senescence or injury, but rather specific pathways directly related to disruption of leptin signaling.

There is considerable evidence that ectopic lipid overload and ceramide formation are linked to apoptosis, termed lipoapoptosis, which can lead to a lipotoxic cardiomyopathy.\textsuperscript{24} For example, cardiomyocyte-specific acyl coenzyme A synthase expression results in lipotoxic cardiomyopathy very similar to, though more severe than, that of the leptin-deficient and leptin-resistant animals studied here.\textsuperscript{25} Interestingly, it has subsequently been shown that concomitant leptin overexpression in these animals completely prevents the cardiomyopathy caused by acyl coenzyme A synthase overexpression.\textsuperscript{26}

Disruption of leptin signaling itself also increases triglycerides and fatty acids.\textsuperscript{24} Here we have shown that cardiac tissue lipids are increased in \textit{ob/ob} mice and, importantly, that leptin repletion completely reverses this accumulation. It has
been suggested that when lipids accumulate in nonadipose tissues, such as in overnutrition, fatty acids enter deleterious pathways of ceramide production, which through nitric oxide formation causes apoptosis.\textsuperscript{16} Ceramide, a key mediator of apoptosis and the cellular stress response, is 2- to 3-fold higher in ZDF rat hearts by 14 weeks of age. This accumulation of ceramide leads to a 15-fold increase in DNA fragmentation, another independent marker for apoptosis.\textsuperscript{19} Apoptosis has thus been proposed as a possible cause of progressive cardiac dysfunction in the ZDF rat.\textsuperscript{16}

Increasing evidence supports the concept that there is increased oxidative stress in the failing heart, which in turn contributes to the pathogenesis of myocardial remodeling and heart failure.\textsuperscript{27} Chronically elevated oxidative stress has been demonstrated in humans\textsuperscript{28} and animals\textsuperscript{29} and appears to play a central role not only in the development of cellular necrosis but also programmed cellular death or apoptosis.\textsuperscript{30}

Increased oxidative stress can also lead to an imbalance between DNA damage and DNA repair systems, resulting in accumulated oxidative damage with age. Old \textit{ob/ob} and \textit{db/db} mice showed a shift toward greater DNA damage by increased 8oxoG, the most stable product of DNA oxidative damage,\textsuperscript{15} with relatively less DNA repair activity. Conversely, the MYH glycosylase system, a major DNA repair pathway that excises adenine residues misincorporated opposite unrepaired 8oxoG during replication,\textsuperscript{15} was upregulated in the old WT but not in the old obese mice. These findings suggest accumulated DNA oxidative damage as a downstream mechanism for the observed increases in apoptosis in these models, for the age-related component in particular. Furthermore, the apoptosis of aging itself may also be related to leptin. In normal rats, leptin sensitivity virtually disappears with age; thus, a normal old rat is almost as leptin resistant as a young ZDF rat.\textsuperscript{31}

Lipid and lipoprotein metabolism is an important metabolic pathway involving leptin as well as other signaling systems, such as estrogen.\textsuperscript{32} It is interesting to note that \textit{ob/ob} mice of both genders are severely hypogonadal, with infertility and very low estrogen or testosterone levels.\textsuperscript{33,34} Dysregulation within this pathway can lead to excessive apoptosis or programmed cell death.\textsuperscript{35} For example, male mice in which exon 2 of estrogen receptor-\(\alpha\) was deleted showed significant increases in the level

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5}
\caption{Immunofluorescence staining showing DNA damage and repair in old WT (A), old \textit{ob/ob} (B), and old \textit{db/db} (C). Both \textit{ob/ob} and \textit{db/db} hearts show evidence of increased DNA damage and decreased DNA repair activity compared with WT. Green indicates 8oxoG (DNA damage); red, MYH (DNA repair); blue, 4',6-diamidino-2-phenylindole (DAPI) (nucleus).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Increased mortality in \textit{ob/ob} and \textit{db/db} mice compared with WT. Kaplan–Meier survival analysis for colonies of WT (dotted line), \textit{ob/ob} (dashed line), and \textit{db/db} (solid line) mice demonstrate increased mortality in both obese strains compared with WT. See text for hazard ratios and median survival. \(^*P<0.05\) and \(^\dagger P<0.005\) vs WT.}
\end{figure}
of serum triglycerides and free fatty acids,36 in part attributable to increases in lipoprotein lipase.37 The ob/ob and db/db strains each had decreased survival compared with WT mice. These findings correlate with the increased rates of apoptosis, although causation cannot be proven from this study. Interestingly, there were no gender differences in survival within each strain, despite the well-established role of estrogen in modulating leptin signaling in other peripheral tissues.32 Although the mode of increased death has not been firmly established, we believe that cardiovascular death is likely, given the known cardiac hypertrophy that develops, as well as the increased apoptosis and DNA damage we have shown here.

One limitation of this study is that we have not delved into the details of mitochondrial function in these 2 models of obesity. Rather, we have focused on the upstream events that may increase rates of apoptosis, such as lipid accumulation and DNA oxidative damage, and further studies of mitochondrial function are ongoing.

In conclusion, we have shown that normal leptin signaling is necessary to maintain normal low levels of apoptosis in the heart and to prevent excess cardiac lipid accumulation, increased age-associated DNA damage, and premature mortality. These data offer novel insights into potential mechanisms of myocardial dysfunction and early mortality in obesity.

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