Alterations in nitric oxide-cGMP pathway in ventricular myocytes from obese leptin-deficient mice

Jun Su, Shengjun Zhang, James Tse, Peter M. Scholz, and Harvey R. Weiss. Alterations in nitric oxide-cGMP pathway in ventricular myocytes from obese leptin-deficient mice. Am J Physiol Heart Circ Physiol 285: H2111–H2117, 2003. First published July 17, 2003; 10.1152/ajpheart.00316.2003.—Leptin is a regulator of body weight (31), and is a well-recognized controller of body weight (31), and is a well-recognized controller of energy metabolism and appetite regulation and studied the effects on contraction of NO donors in obesity. The aim of the present study was to examine contractile responses to stimulation of the NO-cGMP signal transduction system in ventricular myocytes from genetically obese (ob/ob) mice and their lean controls. Ob/ob mice are known to bear a mutation that eliminates the production of leptin, which is a major controller of body weight (31), and are a well-recognized model for investigation of profound obesity (24, 29). We monitored myocyte shortening with video edge detection and studied the effects on contraction of S-nitroso-

myocyte shortening; soluble guanylyl cyclase; protein kinase

Obesity, which is classically defined as a body mass index of 20% above the desirable level (25 kg/m²), is epidemic in the United States. More than 50% of US adults are now overweight based on this criterion (5). Obesity plays a pivotal role in the pathophysiology of metabolic and cardiovascular disease (2, 8). It can initiate a cluster of cardiovascular, renal, and metabolic disorders such as cardiomyopathy, hypertension, impaired glucose tolerance, Type 2 diabetes, and dyslipidemia. However, the mechanisms are currently poorly understood. It has been observed that lack of leptin or functional leptin receptors in rodents results in severe obesity. Leptin is the product of an obesity gene (31). It is a 16-kDa plasma protein known to be a regulator of food intake and energy expenditure mainly via its hypothalamic effects. Recently, a significant amount of evidence has accumulated (13, 16, 17) to show that leptin has the ability to stimulate production of nitric oxide (NO). These findings suggest that NO levels might be altered in obesity that is induced by this gene mutation.

NO is a lipophilic and highly reactive gaseous molecule that is involved in a variety of physiological and pathological functions in many tissues. It plays an important role as a negative regulator in myocardium with regard to basal myocardial function, β-adrenergic responses, and energy supply (1). It is widely accepted that in cardiac myocytes, soluble guanylyl cyclase-generated cGMP mediates most of the negative inotropic effects of NO. cGMP acts through effects on cGMP-dependent protein kinase and cGMP-regulated cAMP phosphodiesterases (14). Previous work has demonstrated that cGMP protein kinase is a major pathway to mediate the negative inotropic effects of NO donors in myocardium, and a few protein targets were identified such as L-type calcium channels, protein phosphatases, and troponin I (9, 11, 12). NO biosynthesis is blunted in obese humans due to impaired endothelial function (28). The reduced bioavailability of NO may have an impact on the downstream signal transduction pathways. However, it is unknown how the NO-cGMP signal transduction pathway is altered in cardiac myocytes in obese individuals.

The aim of the present study was to examine contractile responses to stimulation of the NO-cGMP signal transduction system in ventricular myocytes from genetically obese (ob/ob) mice and their lean controls. Ob/ob mice are known to bear a mutation that eliminates the production of leptin, which is a major controller of body weight (31), and are a well-recognized model for investigation of profound obesity (24, 29). We monitored myocyte shortening with video edge detection and studied the effects on contraction of S-nitroso-
N-acetylpenicillamine (SNAP), a NO donor, and 8-br-cGMP (8-Br-cGMP), a membrane-permeable cGMP analog. We also examined whether the changes were related to changes in the activity of soluble guanylyl cyclase or cGMP-dependent protein kinase. Our results demonstrate that increased cGMP produces more prominent effects in obese than lean mice and that the alteration is associated with changed cGMP protein kinase activity. NO administration, however, does not produce differential effects in lean and obese mice, but this is not related to changes in the activity of soluble guanylyl cyclase. Our data also suggest that the cGMP-independent NO pathway might be enhanced in corpulent mice.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey. Ob/ob B6.5-Lepob1 and age-matched (7 wk old) control C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12:12-h light-dark illumination cycle and allowed food and water ad libitum. Plasma was collected when the animals were killed, and glucose levels were determined (Polytron homogenizer) at 20,000 rpm for 15 s in buffer (5 mM Tris-HCl, 1 mM MgCl2, and 0.25 M sucrose, pH 7.4) and then centrifuged at 15,500 rpm for 20 min at 4°C. The supernatant was the cell extract, and it was aliquoted and stored at −80°C for later use. Protein concentration was determined by the Bradford dye-binding procedure using a spectrometer set at 595 nm.

Soluble guanylyl cyclase activity. The activity of soluble guanylyl cyclase in isolated ventricular myocytes was evaluated with a modified assay used by Sadoff et al. (21). Briefly, a reaction system was established with total volume of 0.2 ml that contained 50 mM Tris-HCl (pH 7.6) and 10 mM theophylline and a GTP regeneration system that contained 10 mM creatine phosphate, 10 μM creatine kinase, 1 μM UTP, 4 μM MgCl2, and 1 mM GTP in the absence or presence of 0.5 mM nitroprusside (a donor of NO). Reactions were initiated by the addition of the above enzyme-containing cell extract and were maintained at 37°C for 10 min. Sodium acetate (0.8 ml, 50 mM, pH 4.0) was added, and water bathing at 90°C for 3 min was performed to completely terminate the reaction. Measurements were obtained for maximal activity after stimulation of nitroprusside.

In vitro phosphorylation assay. The 8-Br-cGMP (2.5 × 10−4 M) and KT-5823 (7.5 × 10−4 M; Calbiochem), which is an inhibitor of cGMP protein kinase, were added to 10 μl of cell extract from the ob/ob or control animals, in which the concentration of the total protein was 0.5 mg/ml. The reagents were placed at room temperature for a 10-min equilibration. They were then cooled on ice, and 0.5 μl of [γ-32P]ATP (10 μCi/μl; Amersham) were added to initiate the reaction. An equal volume of reducing sample buffer was added after 15 min to terminate the phosphorylation reaction. The samples were then warmed to 95°C for 5 min, and electrophoresis was performed with miniature 12% SDS-polyacrylamide slab gels. Subsequently, the gels were stained with Coomassie brilliant blue, destained overnight, dried with gel drier (Bio-Rad), and exposed to X-ray film at −20°C for 36 h. The assay was repeated five times. The developed films were used for phosphoprotein analysis. Molecular weights of protein bands on films were calibrated with ONE-Dscan 2.05 software (Scanalytics).

Experimental protocol. The following protocol was used for the measurements of myocyte shortening. After the myocytes were loaded in the measurement system, a stabilization period of 4 min was used before the contraction data for the individual ventricular myocyte were recorded. A 4-min interval was allowed between reagent treatments, and 10 consecutive contractions were used for analysis. In the first group, 8-Br-cGMP was added at a final concentration of 10−6 M, which was followed by a higher dose of 8-Br-cGMP (final concentration of 10−5 M). In the second group, the NO donor SNAP was added at a final concentration of 10−6 M, which was followed by a higher dose of SNAP (final concentration of 10−5 M). Each group was repeated on three ventricular myocytes from each animal, either obese or control.

Statistical analysis. Repeated-measure ANOVA was used to compare variables measured in the experiment. Duncan’s multiple-range test was used to compare differences between the baseline and various treatments. A value of P < 0.05 was
used as the level of statistical significance. All values are expressed as means ± SE.

RESULTS

Compared with lean control mice, the obese mice had a greater body weight (obese, 58.3 ± 1.2 g vs. lean, 23.0 ± 0.6 g) and heart weight (obese, 148.3 ± 4.0 g vs. lean, 90.0 ± 8.2 mg) but a significantly lower heart weight-to-body weight ratio (obese, 254.9 ± 8.5 vs. lean, 391.7 ± 33.9 mg/g). Nonfasting plasma glucose levels were also significantly higher in the obese mice (obese, 315.9 ± 42.9 mg/dl vs. lean, 201.1 ± 7.1 mg/dl). However, the baseline mechanical parameters of the ventricular myocytes from both groups of animals were similar. There were no significant differences in any of the measured parameters such as cell length, maximal rate of shortening, maximal rate of relaxation, percent shortening, time to peak shortening, time to 50% relaxation, and time to 90% relaxation (Table 1).

The cGMP analog 8-Br-cGMP significantly decreased the percentage shortening in a dose-dependent pattern in ventricular myocytes from both groups of animals (Fig. 1A). These data are presented as percentages of the baseline value in Fig. 1. The percent shortening of the myocytes from lean mice was reduced by 39%, whereas it was significantly more depressed in cells from obese mice, where it was reduced by 47%. In a similar manner, the maximal rate of shortening and the maximal rate of relaxation were also depressed by 8-Br-cGMP at doses of 10^{-4} and 10^{-5} M (Fig. 1, B and C), but these decrements were significantly greater in obese mice. Specifically, in the lean group, the maximal rate of shortening was reduced by 36% and the maximal rate of relaxation by 34%. In contrast, the values were decreased by 46 and 43%, respectively, in the obese group in the presence of 10^{-5} M 8-Br-cGMP. There were no significant effects of 8-Br-cGMP on the time to peak shortening or rate of relaxation parameters.

The NO donor SNAP also significantly decreased the percent shortening, maximal rate of shortening, and maximal rate of relaxation in a dose-dependent manner in ventricular myocytes from both the obese group and the control lean group (Fig. 2). However, there were no statistically significant differences in the responses of the lean and obese group myocytes to the administered SNAP. The average decrement in all parameters was ~38%. There were no significant effects of SNAP on the time to peak shortening or time of relaxation parameters.

The activity of guanylyl cyclase was examined in ventricular myocytes from four lean and four obese mice. We examined the basal activity and also stimulated the enzyme with 0.5 mM sodium nitroprusside, which is a dose that is sufficient to obtain maximal activity (21). There was no significant difference in the basal soluble guanylyl cyclase activity between the two groups (obese, 1.6 ± 0.6 vs. lean, 2.1 ± 0.6 pmol.

### Table 1. Baseline mechanical characteristics in ventricular myocytes from adult lean and obese mouse hearts

<table>
<thead>
<tr>
<th></th>
<th>Mean Length, μm</th>
<th>+dL/dt, μm/s</th>
<th>−dL/dt, μm/s</th>
<th>PCS, %</th>
<th>TPS, ms</th>
<th>TR50, ms</th>
<th>TR90, ms</th>
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<tbody>
<tr>
<td>Lean</td>
<td>127.2 ± 0.6</td>
<td>53.7 ± 4.1</td>
<td>55.1 ± 4.5</td>
<td>3.6 ± 0.2</td>
<td>302.0 ± 13.0</td>
<td>74.5 ± 3.2</td>
<td>319.7 ± 13.2</td>
</tr>
<tr>
<td>Obese</td>
<td>127.2 ± 0.6</td>
<td>52.3 ± 2.3</td>
<td>53.1 ± 2.4</td>
<td>3.4 ± 0.1</td>
<td>325.0 ± 17.4</td>
<td>73.6 ± 5.2</td>
<td>319.4 ± 14.2</td>
</tr>
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Values are means ± SE. +dL/dt, maximal rate of relaxation; −dL/dt, maximal rate of shortening; PCS, percent shortening; TPS, time to peak shortening; TR50, time to 50% relaxation; TR90, time to 90% relaxation.

![Fig. 1. Effects of 8-bromo-cGMP (8-Br-cGMP) on percent shortening (A), maximal rate of shortening (B), and maximal rate of relaxation (C) of ventricular myocytes from hearts of lean and obese mice. Basal levels were similar in the two groups of myocytes, and the values are presented as percentages of the baseline values. Note that 8-Br-cGMP treatment significantly decreased the values from the baseline across all groups, but it had significantly greater effects in obese mice than in lean controls. *P < 0.05, significantly different from lean myocytes treated with the same dose of 8-Br-cGMP; n = 7 animals.](http://ajpheart.physiology.org/)

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During stimulation with sodium nitroprusside, the maximal activity of guanylyl cyclase was also not significantly altered (obese, 24.2 ± 3.3 vs. lean, 24.6 ± 4.0 pmol cGMP·mg protein⁻¹·min⁻¹).

To further study the downstream effects of the NO-cGMP pathway in cardiac myocytes from obese and lean mice, the pattern of protein phosphorylation activated by cGMP protein kinase was examined after stimulation with 8-Br-cGMP. In the control myocytes, there was a basal level of phosphorylation (Fig. 3, lane 1). The addition of 8-Br-cGMP enhanced the labeling of protein bands, especially those ranging from 34 to 14 kDa (lane 2). The phosphorylation of these proteins was significantly decreased (lane 3) when KT-5823, a specific inhibitor of cGMP protein kinase, was present, which indicates that the phosphorylation was specific for cGMP protein kinase. In ventricular myocytes from obese mice, the same group of proteins demonstrated a similar pattern when the reagents were applied (lanes 4–6). However, the phosphorylation increments for some protein bands were greater. Specifically, the effect of 8-Br-cGMP on four proteins bands at about 34, 22, 16, and 14 kDa were greatly enhanced in obese mice. Similar results were obtained from ventricular myocytes from five control and five obese animals.

**DISCUSSION**

The contractile responses of cardiac myocytes to activation of the NO-cGMP signaling pathway in obesity have not been fully characterized. In the present study, we found that both the NO donor SNAP and the cGMP analog 8-Br-cGMP dose-dependently reduced the contractility of ventricular myocytes from both obese and lean mice. The major finding of this study was that 8-Br-cGMP induced a much greater negative effect in myocytes from obese mice compared with controls.

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**Fig. 2.** Effects of 
S-nitroso-N-acetyl-penicillamine (SNAP) on percent shortening (A), maximal rate of shortening (B), and maximal rate of relaxation (C) of ventricular myocytes from lean and obese mouse hearts; n = 7 animals. Basal levels were similar in the two groups of animals, and the values are presented as percentages of the baseline values. Note that SNAP treatment significantly decreased the values from the baseline across all groups, and the decrement was similar in both lean and obese mice.

**Fig. 3.** Effects of cGMP-dependent protein phosphorylation on ventricular myocytes from lean (lanes 1–3) and obese (lanes 4–6) mice. Equal amounts of protein (5 μg) were loaded in each lane. Basal phosphorylation level is shown in lane 1 (lean) and lane 4 (obese). Lanes 2 and 5 show protein phosphorylation in the presence (+) of 8-Br-cGMP in the lean and obese animals, respectively. Lanes 3 and 6 show phosphorylation of proteins from the myocytes of the two groups of animals in the presence of both cGMP and the cGMP-dependent protein kinase inhibitor KT-5823. Molecular weight standards are indicated (left). Note that the level phosphorylation was increased in the presence of cGMP and was reduced once KT-5823 was added. In obese mice, 8-Br-cGMP more significantly increased phosphorylation of four proteins at 34, 22, 16, and 14 kDa.
was associated with an alteration in the phosphorylation profile (increased phosphorylation of some proteins) of cGMP protein kinase, which may account for the enhanced effect in obese mice. In contrast, the effect of NO was similar in obese mice and lean controls. We also observed that there was no change in the activity of the soluble guanylyl cyclase (the major target of NO in this system), which suggests that some of the functional effects of NO with obesity were due to cGMP-independent pathways. Those non-cGMP pathways might reduce the negative effects induced by the downstream effects of cGMP.

We examined myocardial contractile responsiveness using an isolated cardiac myocyte preparation. One advantage of using isolated ventricular myocytes is the elimination of heterogeneous cell types that are associated with heart tissue. The yield of isolated myocytes was high with 50–70% of rod-shaped healthy cells and was similar between the two groups of animals used in this study. Cell viability was constant over the experimental duration, and cell contraction measurements were obtained on random cells in each preparation. The cells used in contraction measurements were rod shaped and reacted to different reagents throughout the entire time course of the experiment. In preliminary studies, untreated cells contracted at a roughly constant rate over the time course of the experiment. For the soluble guanylyl cyclase activity assay and the phosphoprotein analysis, cells from each experimental animal were kept frozen at −70°C to prevent protein degradation. The sizes of the myocytes were not different between the obese and lean groups.

It is well recognized that obesity induces a spectrum of metabolic derangements including elevated nonesterified fatty acids, adipose tissue, and cytokines; altered leptin; and abnormalities of low-density lipoproteins and the renin-angiotensin system (28). Obesity is commonly associated with impaired myocardial contractile function (15). There are reports of decreased diastolic compliance and prolonged relaxation (20, 25). The contractile responsiveness to isoproterenol, which is a potent stimulator of cardiac function via β-adrenergic activation, was also reported to be depressed. Basal myocardial contractility was impaired in obese rats (32) and rabbits (4). It has also been reported for obese rats that the baseline contraction profile of ventricular myocytes was changed with decreased peak shortening and prolonged duration of relaxation (20). In contrast, we did not observe any difference in basal contraction parameters in ventricular myocytes from obese vs. lean mice such as percent shortening, maximal rate of shortening or relaxation, duration of shortening, and duration of relaxation. Consistent with our findings is a recent study that reports that the basal contractility was similar in lean and corpulent rats (3). The above discrepancy may in part be attributed to specific experimental manipulations as well as species differences.

Leptin is strongly implicated in the development of obesity. Although the most common causes of obesity are dependent on overfeeding, which subsequently leads to an increased leptin plasma level and leptin resistance, both leptin deficiency and defects in the leptin receptor are sufficient to generate obesity. In the study, we used the ob/ob B6.V-Lepob mouse as an obesity animal model. This animal has a relatively clear biological background. Besides having a phenotype of profound obesity due to a defect of the appetite-controlling hormone protein leptin, the ob/ob mouse also possesses the features of insulin resistance, hyperglycemia, and fatty liver. Therefore, it serves as a good model for the study of obesity and obesity-associated disease. Nonfasting plasma glucose levels measured in the experiment confirmed that the ob/ob mice were hyperglycemic (31). Leptin is a 16-kDa plasma protein that is known as a regulator of food intake and energy expenditure mainly via its hypothalamic effects. There is strong evidence that leptin can stimulate the production of NO (13, 16, 17). This would suggest potential changes in the NO-cGMP signal transduction system in leptin-deficient mice.

Significant negative functional effects of cGMP have been observed in ventricular myocytes (6, 23). Consistent with previous studies, our results demonstrate that cGMP had negative inotropic effects in both the obese and lean groups in a dose-dependent pattern. Previous work also demonstrates that cGMP-dependent protein kinase was one of the main myocardial targets for cGMP and that it mediates its negative metabolic and functional effects by phosphorylating an array of proteins. These include proteins that directly or indirectly decrease Ca2+ influx such as L-type Ca2+ channels (11) and protein phosphatases (9), proteins that prolong action potentials such as ATP-sensitive potassium channels (7), and proteins that sense Ca2+ concentration variance such as troponin I (12). Recently it was also demonstrated that PKG-1α, which is one subtype of cGMP protein kinase, is expressed in the mouse heart (19). We found that cGMP was significantly more effective in reducing myocyte function in obese mice than in lean controls. The profound contractile response to cGMP in ventricular myocytes from ob/ob mice suggests that cGMP protein kinase activity might be augmented. We observed that the phosphorylation of some specific proteins rather than all of the protein targets was substantially increased in these obese mice. This suggests that part of the greater negative functional effects of cGMP might be explained by the changed phosphorylation profile. In the present study, we found four proteins with phosphorylation levels that were specifically enhanced. These proteins need to be further identified to determine their relationship to the functional effects. Some of these proteins may play an important role in NO-cGMP signaling in the control of cardiac function of ob/ob mice.

It has been well documented that NO is generated endogenously by a family of NO synthases in myocardium. NO plays an important role not only in the pathophysiology of several cardiac disease states but also in modulating physiological myocardial function including basal myocardial contraction, force development, and O2 consumption (22). Leptin can activate
NO synthase to increase production of NO (13, 16, 17). There is evidence that NO operates through two basic types of pathways: cGMP-dependent and -independent pathways (14). It appears that many of the cardiac actions of NO are mediated through elevation of intracellular cGMP content that is secondary to the activation of soluble guanylyl cyclase upon binding of NO to the heme moiety of the enzyme (22). In this study, although NO demonstrated negative inotropic effects similar to those of cGMP, it did not show any differential effects in comparisons between the two animal models. Our results were consistent with a previous clinical study where an exogenous NO donor produced similar negative hemodynamic responses between obese and lean subjects (26).

In this study, the effects of cGMP but not NO were enhanced in the leptin-deficient mouse. One possible explanation is a reduction in the activity of soluble guanylyl cyclase in the obese mice. However, we found that the activity of soluble guanylyl cyclase was not attenuated in the obese mice. This suggests that the cGMP-independent pathways of NO may play a role in obesity to counteract the increased effects of cGMP. Recent work suggests the possibility that some positive inotropic effects of NO donors may be involved. There is some evidence for cGMP-independent pathways, e.g., stimulation of adenyl cyclase (27) or altering of Ca2+ flux by nitrosylation of L-type Ca2+ channels (10) or ryanodine receptors (30). It is possible that one of these pathways was enhanced in obese mice. Furthermore, there is mounting evidence that in obesity, mitochondrial formation of reactive oxygen species is enhanced (18). Augmented production of reactive oxygen species may scavenge NO and thereby decrease its bioavailability. In addition, peroxynitrite, which is a resultant molecule of the interaction between NO and reactive oxygen species, may nitrosylate proteins and exert positive inotropic effects (18) that might offset the negative functional effects of cGMP in obese cardiomyocytes. The effects of these reactive oxygen species in obese mice have not been investigated, but this mechanism may be involved in this model. Thus it appears that the effects of cGMP and its protein kinase are enhanced in cardiac myocytes from obese mice. However, it also appears that some non-cGMP-dependent effect of NO prevents its negative functional effects from being enhanced in obese cardiac myocytes.

In summary, we have demonstrated that basal myocyte contraction was not altered in ventricular myocytes from an obese mouse model compared with lean mouse controls. Activation of the NO-cGMP signal transduction system with a cGMP analog and an NO donor decreased contractility in myocytes from both lean and obese mice. We found a significantly enhanced effect of cGMP in obesity that might be explained by an altered phosphorylation profile for cGMP protein kinase. In contrast, the effects of NO in obese mice were not increased. In addition, soluble guanylyl cyclase activity was not altered with obesity. This suggests that some cGMP-independent pathway for NO might also be enhanced to offset the greater effects of cGMP in obese mice.

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DISCLOSURES
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REFERENCES


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