Leptin-induced cardiomyocyte hypertrophy involves selective caveolae and RhoA/ROCK-dependent p38 MAPK translocation to nuclei

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Received 14 June 2007; revised 25 August 2007; accepted 12 September 2007; online publish-ahead-of-print 19 September 2007

Time for primary review 38 days

Aims Leptin-induced cardiomyocyte hypertrophy is dependent on both RhoA and p38 mitogen-activated protein kinase (p38 MAPK) activation. The present study investigated the role of lipid raft/caveolae in these responses and assessed the nature of p38 MAPK activation in mediating leptin-induced hypertrophy.

Methods and results Studies were carried out using cultured neonatal rat ventricular myocytes. Pharmacological, molecular, microscopy, and confocal imaging techniques were used to assess the role of caveolae in leptin-induced hypertrophy and to study the underlying cellular mechanisms. Leptin (3.1 nmol/L) treatment for 24 h significantly increased caveolae number two-fold and increased expression of caveolin-3 to 278 +14% of control values. These effects were associated with increased cell surface area by 29±5% and leucine incorporation by 40+6%. The hypertrophic effect of leptin was associated with significant activation of RhoA (422±26%) and a decrease in the G-actin-to-F-actin ratio from 3.1±0.2 to 0.9±0.1. Caveolae disruption with methyl-beta-cyclodextrin (MβCD) potently attenuated leptin-induced cell hypertrophy and the associated signalling. RhoA was detected in caveolae fraction of a sucrose gradient after treatment with leptin for 5 min, indicating subcellular translocation of RhoA: this effect was inhibited by MβCD, the RhoA inhibitor C3 exoenzyme, and by disruption of actin filaments with latrunculin B. Furthermore, leptin-induced hypertrophy was associated with p38 MAPK but not with extracellular signal-regulated kinase (ERK1/2) translocation to nuclei, which was inhibited by MβCD, C3 exoenzyme, and the Rho kinase inhibitor Y-27632.

Conclusion Our results indicate that p38 import into nuclei represents a key mechanism for leptin-induced hypertrophy acting through lipid raft/caveolae and a RhoA-dependent pathway.

KEYWORDS Cardiomyocytes; Leptin; Hypertrophy; RhoA; Caveolae

1. Introduction

The satiety factor leptin, a product of the ob gene, is generally considered to be an adipose-derived protein. However, recent evidence has shown that leptin is produced by a multiplicity of tissues including the heart, vascular smooth muscle (VSM), digestive epithelia, and gastric mucosa. Leptin was first described as a neurohormone whose primary function was to regulate energy balance and food intake via the hypothalamus. However, studies have revealed a variety of other effects including re-epithelialization, and platelet aggregation.

The concept that leptin acts as a direct stimulant for cellular growth is supported by studies showing an increase in cell size and protein synthesis in neonatal cardiomyocytes and VSM as well as enhanced proliferation of pediatric ventricular myocytes and a murine HL-1 cell line. Others have shown an antihypertrophic effect of leptin infusion in obese leptin-deficient mice, thereby suggesting a complexity of leptin-induced effects which may be dependent on experimental model.

Although the precise molecular mechanisms underlying the hypertrophic effect of leptin are unknown, recent studies from a number of laboratories have suggested that both mitogen-activated protein kinase (MAPK) and the small monomeric GTPase, RhoA, may be involved. Indeed, leptin has been shown to stimulate both RhoA and MAPKs in cardiac myocytes, and emerging evidence suggests that these systems are important for initiating leptin-induced myocyte hypertrophy or proliferation. With respect to MAPK, leptin-induced cardiomyocyte hypertrophy appears to be p38 MAPK-dependent, as the hypertrophic

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response can be completely prevented by p38 MAPK inhibition. However, the ability of leptin to increase proliferation of both HL-1 cells and pediatric human ventricular myocytes is likely dependent on ERK1/2 activation. The complexity of leptin’s hypertrophic effect is further enhanced by the finding that RhoA/ROCK activation plays a critical role in leptin-induced hypertrophy most likely due to changes in actin dynamics.

Caveolae have been recently proposed as important mediators contributing to the regulation of cell signalling. Caveolae are a subset of lipid rafts, characterized by flask-shaped invaginations that are rich in sphingolipids, cholesterol, and caveolin proteins. Caveolae are present in many types of mammalian cells, including cardiomyocytes, and are important for numerous cell functions including cholesterol homeostasis, protein trafficking, and signal transduction (reviewed in Razani et al.14). Caveolin-3 (cav-3) is a major constituent of cardiac caveolae14 and is a critical protein for normal muscle function and the development of T-tubule systems.15 Although it has been suggested that caveolins negatively regulate the signalling molecules involved in cardiomyocyte hypertrophy such as ERK1/2, heterotrimeric G proteins, and PKC,16,17 their role in the regulations of p38 MAPK and RhoA signalling especially in leptin-induced cardiomyocyte hypertrophy has not been established. Accordingly, the present study was carried out with two major objectives. First, we wished to determine the effect of leptin on caveolae content in cardiac myocytes and whether caveolae play any role in the hypertrophic response to leptin. Secondly, we determined whether the participation of the MAPKs and RhoA/ROCK systems on leptin-induced hypertrophy are interrelated or whether these represent separate and distinct mechanistic pathways and the role of caveolae in these processes.

2. Methods

2.1. Neonatal cardiomyocyte cell culture

Neonatal cardiomyocytes were prepared from the ventricles of 1- to 3-day-old Sprague–Dawley rats (Charles River Canada, Montreal, Quebec, Canada), as described previously. The investigation conforms to 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).

2.2. Treatments

The myocytes were treated with or without 3.1 nmol/L leptin (Sigma-Aldrich, Oakville, Canada) in the presence or absence of the following inhibitors for 24 h: leptin receptor antibody (OBR-Ab, 166 ng/mL; Alpha Diagnostic International, Inc., San Antonio, TX, USA), C3 exoenzyme (30 ng/mL; Alexis Biochemicals, Carlsbad, CA, USA), a clostridial toxin that selectively inhibits Rho, Y-27632 (10 μmol/L; Sigma-Aldrich, Oakville, Canada), a selective ROCK inhibitor, latrunculin B (10 nmol/L; Calbiochem, San Diego, CA, USA), an actin depolymerization agent. All agents were added 1 h before leptin administration and were present throughout the leptin treatment period.

2.3. Measurement of cell surface area

Cell surface area was measured and analysed as described previously. Briefly, cardiomyocytes were plated at a density of 1 × 10^6 cells and cultured for 48 h in serum-containing media followed by 24 h serum starvation. At the end of the treatment period, the cardiomyocytes were visualized with a Leica inverted microscope, and surface area was quantified by imaging the complete boundary of 45–50 individual cells, using Mocha software (SPSS Inc., Chicago, IL, USA). This process was replicated in 6–9 independent experiments and the data were combined. The cell surface area in control cells was normalized to 100% and results were expressed as the percentage of control values.

2.4. Leucine incorporation

Protein synthesis was examined by analysis of [3H]-leucine incorporation. Briefly, cardiomyocytes were plated at 1 × 10^6 cells per well into 24-well Primaria culture plates and cultured for 48 h in serum-containing media. After incubation in serum-free medium for 24 h, cardiomyocytes were treated with the indicated treatments in the presence of 1 μCi of [3H]-leucine for 24 h. After three washes with ice-cold PBS, proteins were precipitated with 5% trichloroacetic acid for 30 min at 4°C. Following two washes with ice-cold 5% TCA, precipitates were then dissolved in 0.2 N NaOH. After neutralizing with 0.5 N HCl, the total radioactivity of incorporated [3H]-leucine into proteins was measured by liquid scintillation counter.

2.5. Immunoblotting

Immunoblotting was performed as described previously. Antibodies used in this study were against total and phosphorylated forms of ERK1/2, total and phosphorylated forms of p38 MAPK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), proliferation cell nuclear antigen (PCNA), actin (Cytoskeleton Inc., Denver, CO, USA), and RhoA (Upstate, Lake Placid, NY, USA).

2.6. RNA isolation, reverse transcription, and real-time polymerase chain reaction analysis of OBRa, OBRb, and 18S rRNA

Total cellular RNA was extracted from cultured cardiomyocytes, using the Trizol Reagent (Invitrogen), following the manufacturer’s instructions. For reverse transcriptase polymerase chain reaction (RT–PCR), first strand of complementary DNA (cDNA) was synthesized from 1 mg of total RNA, using random hexamer primers (Invitrogen) and SuperScript II RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. The following primer sequences were used: 5’-TGATATCGCCAAACAGCAAA-3’ (forward) and 5’-AGTGTCCGCTCTTTTGGAG-3’ (reverse) for leptin receptor isoform a (OBRa), 5’-TGACCATCAGGATCCACA-3’ (forward) and 5’-CCACGTGGTTACGTTGCTG-3’ (reverse) for leptin receptor isoform b (OBRb), and 5’-GTAACCCGTGTGAAACCCATT-3’ (forward) and 5’-CCATCAGATCCAGTGGCC-3’ (reverse) for 18S rRNA. PCR conditions and cell cycle number were optimized for each set of primers. PCR cycle conditions involved 40 cycles for OBRa and OBRb or 35 cycles for 18S rRNA of denaturation (94°C for 30 s), followed by annealing at 54°C for 20 s for 18S rRNA, 58°C for 20 s for OBRa and OBRb, followed by elongation at 72°C for 30 s. All genes were amplified for 40 cycles except 18S rRNA, which was amplified for 35 cycles. The housekeeping gene 18S rRNA was used to normalize expression data.

2.7. Electron microscopy of cardiomyocytes

Myocytes were cultured in collagen-coated dishes (Becton Dickinson). Samples were fixed and dehydrated by a standard method. For quantitation of caveolae, 17–25 cells were photographed at a magnification of 26 000×. Only distinctly flask-shaped, non-coated vesicles (50–100 nm in diameter) found on the plasma membranes were scored as caveolae. Total caveole counts were normalized to the unit length of plasma membrane (10 μm) and measured using commercially available software (ImageJ).
2.8. Detergent-free purification of caveolin-rich membrane fragments

Caveolae fractions were separated by a previously optimized method, using sodium carbonate gradient. Equal volumes of each fraction were loaded in SDS-poly-acrylamide gels, followed by immunoblotting with anti-cav-3 (Santa Cruz Biotechnology, Inc.), anti-OBR, or anti-RhoA antibodies.

2.9. Extraction/replenishment of cholesterol

To determine the effects of cholesterol on leptin-induced signalling, cardiomyocytes were pre-treated with or without the cholesterol chelator (methyl-beta-cyclodextrin, MβCD, 5 mmol/L; Sigma-Aldrich) for 1 h. Following pre-treatment, the cells were treated with leptin, homogenized, and fractionated by sucrose density gradient. For cholesterol repletion, cells were incubated for 3 h at 30°C with 5 mg/mL cholesterol balanced with MβCD (MβCD-Chol, water-soluble cholesterol; Sigma-Aldrich).

2.10. Immunofluorescent cell staining

Cardiomyocytes were grown on collagen-pre-coated glass covers and maintained in serum-containing medium for 48 h followed by incubation in serum-free media for 24 h. Following the appropriate treatments, cardiomyocytes were fixed for 10 min with freshly prepared 3.7% (w/v) paraformaldehyde in PBS (pH 7.2). After fixation, cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 15 min and washed twice with PBS. Non-specific binding sites were blocked with blocking solution (1% BSA, 0.1% Triton X-100 in PBS) for 10 min and washed twice with PBS. The cardiomyocytes were then incubated with anti-cav-3 and/or anti-OBR antibodies (1:100) for 1 h at room temperature. After that cardiomyocytes were washed three times with PBS and then incubated for 1 h at room temperature with Alexa Fluor 488 conjugated goat anti-mouse and/or Alexa 594-conjugated goat anti-rabbit secondary antibodies (1:250; Molecular Probes). Immunofluorescence was assessed with a Zeiss LSM 510 microscope (Carl Zeiss, Oberkochen, Germany).

2.11. RhoA activity assay

The Rho-GTP pull-down assay was performed using the Rho activation assay kit (Upstate).

2.12. Measurement of G/F-actin ratio

F-actin content was compared with free G-actin content, using the G-actin/F-actin in vivo assay kit (Cytoskeleton Inc.), according to the manufacturer’s instructions.

2.13. Preparation of subcellular fractions

Cells were homogenized in buffer A [pH 7.4, 20 mM Tris, 2 mM EDTA, 137 mM NaCl, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM 4-(2-aminophethyl)-benzenesulfonyl fluoride, and 10 μg/mL leupeptin]. The homogenate was clarified by centrifugation at 750 g for 20 min at 4°C. Thereafter, the cytosolic fraction was obtained by subjecting the supernatant to a 10 000 g centrifugation for 20 min at 4°C. The pellet was washed with buffer A and then lysed with buffer B (buffer A containing 2% SDS). After sonication, this solution was used as the nuclear fraction.

2.14. Statistics

Values are presented as mean ± SE. Data were analysed using one-way ANOVA, followed by a post hoc Student’s t-test. P < 0.05 was considered statistically significant.

Figure 1  Effect of leptin on caveolae number and cav-3 expression. Serum-starved cardiomyocytes were treated with or without 3.1 nmol/L leptin for 24 h. Cells were prepared for electron microscopic analysis as described in the Methods section. Representative photographs of caveolae on the surface of cardiomyocytes (A). The number of caveolae on the surface was quantified by transmission electron microscopy (B). Arrows in all panels indicate caveolae-abundant regions. Results were means ± SE for 25–35 slices (17–25 cells) from three independent experiments. Scale bars 100 nm. Effect of leptin on cav-3 expression in cardiomyocytes was examined using immunostaining (C) or western blot (D) techniques. Cardiomyocytes were fixed, permeabilized, and immunostained with anti-cav-3 antibody and FITC-conjugated secondary antibody. *P < 0.05 vs. without leptin.

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3. Results

3.1 Effect of leptin on caveolae

To determine the effect of leptin on caveolae status, we used a multifaceted approach. First, we determined caveolae number visualized by electron microscopy. As illustrated in electron micrographs (Figure 1A) and quantified in Figure 1B, cardiomyocytes treated with leptin demonstrated a two-fold increase in the number of caveolae. We next studied the effects of leptin on cav-3 protein expression, using confocal microscopy and western blotting. The former revealed an intense increase in cav-3 labelling in leptin-treated cells compared with control cells (Figure 1C). Moreover, western blot analysis of lysates prepared from cardiomyocytes incubated with leptin for 24 h revealed that cav-3 protein expression significantly increased to 270 ± 20% of control values (Figure 1D).

3.2. Requirement for lipid rafts/caveolae in leptin-induced hypertrophic responses

To assess the role of lipid rafts/caveolae in mediating the hypertrophic effect of leptin, cardiomyocytes were treated with the cholesterol-chelating agent MβCD on the basis of the fact that the structure and function of caveolae are sensitive to the amount of cholesterol in the membrane. MβCD completely abrogated the effects of leptin on cardiomyocyte surface area (Figure 2A) and protein synthesis (Figure 2B) compared with control cardiomyocytes treated with MβCD. To determine whether the loss of leptin-induced cardiomyocyte hypertrophy and the loss of integrity of caveolin-enriched membrane domains were due to the depletion of cholesterol, cardiomyocytes were first treated with MβCD for 60 min at 37°C followed by incubation for 3 h in the absence or presence of soluble complexes of cholesterol and MβCD which can mediate the incorporation of cholesterol into membranes. As shown in Figure 2, the increased cell surface area and protein synthesis produced by leptin were restored by the concomitant addition of cholesterol to MβCD-treated cells.

3.3. Co-localization of leptin receptors and lipid raft/caveolae

Lipid rafts/caveolae were purified using an established equilibrium sucrose density gradient system that separates these detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins. Western blotting showed that cav-3, the primary caveolae protein in cardiomyocytes, was identified only in the low-density cholesterol-rich fractions (fraction 5; Figure 2C, top panel). Moreover, leptin receptors OBR-a and OBR-b with approximate molecular masses of 80 and 130 kDa (short and long receptor isoforms, respectively) were detected in the same fraction (fraction 5; Figure 2C, bottom panel), suggesting the co-localization of leptin receptors and lipid raft/caveolae.

The incubation of cardiomyocytes with 5 mmol/L MβCD for 1 h had no effect on OBRa or OBRb location in lipid raft/caveolae (Figure 2C). The co-localization of cav-3 with OBR was observed by using fluorescence confocal microscopy after double staining, using anti-cav-3 (green) and anti-OBR (red) antibodies (Figure 2D); MβCD had no effect on cav-3 and OBR co-localization (Figure 2D).

3.4. Effect of cholesterol depletion on leptin-induced increase in OBR expression

To explore the role of leptin on OBR expression, we examined the effect of 24 h incubation of cardiomyocytes with leptin on OBRa and OBRb gene expression. Leptin significantly increased the expression of OBRa and OBRb by two- and three-fold, respectively. MβCD had no effect on control cardiomyocyte OBRa and OBRb expression. However, 1 h treatment with MβCD significantly attenuated leptin-induced increased OBRa and OBRb mRNA expression, indicating that leptin-induced OBR expression is mediated through lipid raft/caveolae-dependent pathway.

3.5. Effect of cholesterol depletion on leptin-induced Rho activation and actin dynamics

To investigate the possible role of caveolae in leptin-induced RhoA activation and changes in actin dynamics, cells were
incubated in the absence or presence of MβCD prior to leptin stimulation for 5 min. The disruption of caveolae by MβCD significantly inhibited leptin-induced RhoA activation (Figure 3A) and completely prevented the ability of leptin to decrease the G/F-actin ratio (Figure 4).

We next sought to determine whether cholesterol repletion following MβCD treatment could reverse the inhibitory effects of MβCD. Cardiomyocytes were treated with MβCD for 60 min, washed twice with PBS, allowed to recover either in culture medium or in culture medium supplemented with MβCD-conjugated cholesterol for 3 h, and then treated with leptin for 5 min or 24 h to measure RhoA activation or G/F-actin ratio respectively. There was a significant RhoA activation (Figure 3A) and a decrease in G-actin-to-F-actin ratio (Figure 4) when cardiomyocytes were allowed to recover in culture medium with MβCD-conjugated cholesterol compared with cholesterol-depleted cells.

3.6. Effect of leptin on caveolar localization of RhoA

Figure 3B (top panel) shows that cav-3 was localized primarily in fraction 5. Under control conditions, RhoA was localized primarily in cytosolic fractions (7–10), whereas treatment of cardiomyocytes with leptin induced a translocation of RhoA to fraction 5 5 min after leptin administration which reversed to control localization 60 min after leptin addition. As expected, treatment with MβCD prevented RhoA translocation. Because of the apparent requirement for active Rho to translocate to intact caveolae, we pre-incubated cardiomyocytes with C3 exoenzyme, followed by 5 min of leptin treatment. This resulted in complete inhibition of leptin-induced Rho translocation, suggesting that functional caveolae are a prerequisite for translocation and signalling of RhoA. Moreover, the ability of latrunculin B to exert a similar inhibitor effect on RhoA translocation to lipid raft/caveolae suggests the involvement of the actin cytoskeleton in leptin-induced RhoA translocation (Figure 3B).

3.7. Cholesterol depletion inhibited leptin-induced p38 mitogen-activated protein kinase, but not ERK1/2 phosphorylation

A previous study from our laboratory showed that the hypertrophic effect of leptin in cardiomyocytes was associated with increased phosphorylation of both p38 MAPK and ERK1/2; however, hypertrophy was selectively abrogated by a p38 MAPK inhibitor, whereas an ERK1/2 inhibitor was without effect. Taken together, those findings suggested that p38 MAPK activation represents a critical component for leptin-induced hypertrophy despite an ability of leptin to activate both p38 MAPK and ERK1/2. To address this issue, we next determined whether selective p38 MAPK vs. ERK1/2 activation could be demonstrated on the basis of caveolae dependency. As shown in Figure 5A, MβCD significantly inhibited leptin-induced p38 MAPK phosphorylation,
an effect which was reversed after treatment with the MβCD-conjugated cholesterol. MβCD, on the other hand, significantly increased ERK1/2 phosphorylation in the absence of leptin (Figure 5B). However, as for p38 MAPK, no further ERK1/2 phosphorylation was observed following leptin administration (Figure 5B).

3.8. Leptin-induced p38 mitogen-activated protein kinase but not ERK1/2 nuclear translocation

Taken together, our results have shown that leptin can produce increased phosphorylation of both p38 MAPK and ERK1/2 through a mechanism involving intact caveolae. It is known however that, upon activation, MAPKs translocate from the cytoplasm to the nucleus, where they activate several transcription factors (reviewed in Treisman20). Thus, nuclear translocation is critical for successful relay of the signals and evocation of cell responses. Accordingly, we next examined whether the phosphorylated p38 MAPK and ERK1/2 were translocated by leptin in cardiomyocytes. Interestingly, as shown in Figure 6A, based on western blotting of nuclear preparations, leptin significantly increased translocation of p38 MAPK within 5 min after its addition, which increased and persisted throughout the treatment period. In contrast, leptin was without effect on ERK1/2 translocation to nuclei as shown in Figure 6B.

We also assessed whether OBR-Ab or MβCD could prevent the nuclear accumulation of p38 MAPK. Figure 7 shows that the dramatic translocation of p38 MAPK by leptin was attenuated when cells were pre-treated with OBR-Ab or MβCD, the latter suggestive of caveolae involvement in the translocation process.

3.9. Rho/ROCK and actin cytoskeleton regulate leptin-induced p38 mitogen-activated protein kinase translocation to the nucleus

The final set of experiments was performed to determine the role of RhoA/ROCK and cellular actin cytoskeleton on leptin-induced p38 MAPK nuclear translocation by examining the effect of C3 exoenzyme (RhoA inhibitor), Y-27632 (ROCK inhibitor), or latrunculin B (inhibitor of actin polymerization) on leptin-induced p38 MAPK nuclear translocation. As shown in Figure 8, the nuclear translocation of p38 MAPK induced by leptin was completely abrogated by pretreatment with all three agents.
probed with antibody to p38 mitogen-activated protein kinase and proliferating cell nuclear antigen (PCNA; nuclear marker). Each bar represents mean ± SE value obtained from six independent experiments. *P < 0.05 vs. without leptin. † P < 0.05 vs. with leptin.

4. Discussion

The major novel findings in the present study can be summarized as follows. First, we show that leptin upregulates caveolae content as determined by increased cav-3 protein expression as well as an increase in caveolae number. Secondly, our study shows that OBR co-localizes within caveolae and that caveolae are likely critical for the induction of both cardiac hypertrophy and RhoA activation by leptin. Lastly, and of primary importance, our results show that leptin induces a selective translocation of p38 MAPK from the cytoplasmic fraction to the nuclear fraction, which is dependent on intact caveolae and activity of the Rho/ROCK pathway and actin dynamics. In contrast, although ERK1/2 phosphorylation occurred similarly as shown for p38 MAPK, this was associated with only minimal and insignificant nuclear translocation.

In this study, we used 3.1 nmol/L leptin, as this concentration falls well within plasma levels of leptin found in obese individuals. Moreover, we have shown previously that this concentration is devoid of any cardiotoxic effects and that higher leptin concentrations failed to increase the degree of hypertrophy in cultured neonatal ventricular myocytes. In the present study, we observed that increased caveolae number was associated with cav-3 and OBR upregulation in hypertrophied cardiomyocyte induced by leptin. Indeed, different cell types that contain numerous caveolae (such as endothelial cells and VSMC) usually have high levels of caveolin mRNA and protein expression. Furthermore, overexpression of caveolin in caveolin-negative cell lines results in the correct targeting of caveolin to caveolin-enriched membrane fractions and drives the de novo formation of caveolae. These results indicate the importance of cav-3 as a structural protein for directing the formation of caveolae in membranes (reviewed in Cohen et al.).

The phenomenon of increased caveolae in cardiac hypertrophy has been previously found in spontaneously hypertensive rats in an early stage of hypertension as well as in neonatal cardiomyocyte treated with the hypertrophic α₁ adrenoceptor agonist phenylephrine. A growing body of evidence has demonstrated that different signalling molecules important for cardiac hypertrophy, such as G protein-coupled receptors, are found to be enriched in cardiomyocyte caveolae. From our study, we propose that OBR constitutes another family of pro-hypertrophic factors localized within caveolae, and intact caveolae are required for functional OBR. Although our study suggests that OBR localization and RhoA translocation to caveolae are important for the hypertrophic effect of leptin to materialize, the role of caveolae in the hypertrophic programme is very likely complex. This is particularly evident from studies using caveolin knockout mice which are associated with increased cardiac hypertrophy and a myopathy phenotype. Thus, although the role of caveolae in mediating hypertrophy may be complex, our results appear to be conceptually similar to the cardiac hypertrophic effect of ouabain, in which the effect of the glycoside was shown to be caveolae-dependent, reflecting the ability of pro-hypertrophic cell-signalling molecules to localize within caveolae in response to ouabain. Our study demonstrates a relatively similar phenomenon with respect to RhoA and supports the concept of RhoA translocation from cytoplasm to cell membrane. Thus, by using sucrose gradient fractionation, we have shown that leptin can transiently and relatively rapidly (within 5 min) cause the translocation of RhoA to caveolae. Moreover, we found that cholesterol depletion of cardiomyocytes significantly reduced the leptin-induced hypertrophic response in terms of both the increased cell surface area and protein synthesis which was associated with abolition of RhoA activation and its translocation to caveolae, whereas replenishment of plasma-membrane cholesterol resulted in a reversal of these effects. Other investigators have proposed that RhoA is linked to caveolin-enriched membrane domains. Taken together, our results strongly suggest that intact caveolae are necessary for the
translocation of active RhoA for the transduction of leptin signalling-induced cardiomyocyte hypertrophy.

Various hypertrophic factors including phenylephrine, endothelin-1, and leptin have been shown to activate MAPKs in cardiac myocytes. Recent studies have shown the necessity of p38 MAPK but not ERK1/2 activation for leptin-induced hypertrophy in cardiac and VSM cell on the basis of the ability of the p38 MAPK inhibitor SB202190 to prevent the hypertrophic response, with similar results shown for both endothelin-1 and phenylephrine. On the other hand, ERK1/2 activation may be important for leptin-induced myocytes proliferation as shown for both cultured HL-1 cells as well as pediatric human ventricular myocytes. A major goal of our study therefore was to address the question of selective p38 MAPK involvement in leptin-induced hypertrophy despite leptin-induced phosphorylation of both p38 MAPK and ERK1/2. Activation of MAPKs occurs in the cytoplasm, but to exert many of its actions, they must translocate into the nucleus to interact with transcriptional factors, thus producing the hypertrophic response. Our results clearly show that leptin-induced hypertrophy is associated with p38 MAPK nuclear translocation while ERK1/2 was unaffected. Indeed, it has been shown that, in the nucleus, p38 MAPK phosphorylates Elk-1, c-fos, and ternary complex factors. Many studies have shown the involvement of ROCK in nucleocytoplasmic trafficking of several signalling molecules (reviewed in Aplin and Juliano) but not p38 MAPK. In this report, we have shown for the first time that p38 MAPK translocation was dependent on RhoA/ROCK activity and actin cytoskeleton dynamics remodelling, as p38 MAPK translocation was completely inhibited by inhibitors of RhoA and ROCK as well as latrunculin B. Our finding that p38 MAPK nuclear translocation was dependent on OBR and RhoA/ROCK activation coupled with the inhibitory effect of MβCD on p38 MAPK translocation suggests the importance of intact caveolae for signalling molecules that lead to p38 MAPK nuclear translocation following leptin administration.

In conclusion, this article demonstrates for the first time that leptin-induced hypertrophy occurs subsequent to selective p38 MAPK translocation from cytosol to nucleus. We further show that p38 MAPK nuclear import as well as the hypertrophic response is dependent on intact caveolae, Rho/ROCK signalling, and subsequent actin polymerization. This study explains the involvement of p38 MAPK in the hypertrophic response to leptin although the nature of the transcriptional regulation associated with p38 MAPK nuclear translocation needs to be determined.

Conflict of interest: none declared.

Funding
This study was supported by the Canadian Institutes of Health Research. A.Z. was supported by the Heart and Stroke Foundation of Ontario Program in Heart Failure. M. K. holds a Canada Research Chair in Experimental Cardiology.

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