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Leptin-induced Cardiomyocyte Hypertrophy Reveals both Calcium-dependent and Calcium-independent/RhoA-dependent Calcineurin Activation and NFAT Nuclear Translocation

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ABSTRACT

Leptin, a product of the obesity gene, has been shown to produce cardiac hypertrophy. Although leptin's mechanism of action is poorly understood activation of the RhoA/ROCK pathway has been proposed as a contributing mechanism. The Ca^{2+}-dependent phosphatase calcineurin plays a critical role in the hypertrophic program although it is not known whether leptin can activate this signaling pathway or whether there is a relationship between RhoA activation and calcineurin. Accordingly, we determined the effect of leptin on calcineurin activation and assessed the possible role of RhoA. Experiments were performed using cultured neonatal rat ventricular myocytes exposed to 50 ng/ml leptin for 24 h which resulted in a robust hypertrophic response. Moreover, leptin significantly increased intracellular Ca^{2+} and Na^{+} concentrations which was associated with significantly reduced activity of the $3Na^{+}$-2 K^{+}ATPase. The hypertrophic response to leptin were completely abrogated by both C3 exoenzyme (C3), a RhoA inhibitor as well as the reverse mode $3Na^{+}$-$1Ca^{2+}$ exchange inhibitor KB-R7943 ((2-[2-[4-(4-nitrobenzyloxy)phenyl] ethyl]isothiourea methanesulfonate), however only the effect of the latter was associated with attenuation of intracellular Ca^{2+} concentrations whereas Ca^{2+} concentrations were unaffected by C3. Similarly, C3 and KB-R7943 significantly attenuated early leptin-induced increase in calcineurin activity as well as the increase in nuclear translocation of the transcriptional factor nuclear factor of activated T cells. The hypertrophic response to leptin was also associated with increased p38 and ERK1/2 MAPK phosphorylation and increased p38, but not ERK1/2, translocation into nuclei. Both p38 responses as well as hypertrophy were abrogated by KB-R7943 as well as the calcineurin inhibitor FK-506 although ERK1/2 phosphorylation was unaffected. Our study therefore demonstrates a critical role for the calcineurin pathway in mediating leptin-induced hypertrophy. Moreover, we report a novel RhoA-dependent leptin-induced calcineurin activation which acts independently of changes in intracellular Ca^{2+} concentrations.

1. Introduction

Leptin, a 16-kDa pleiotropic peptide encoded by the *ob* gene has been suggested to be a link between obesity and increased cardiovascular risk [1–4]. Although plasma levels of leptin are closely related to the degree of adiposity [5,6] plasma leptin levels are also elevated in cardiac disease states including heart failure as well as coronary artery disease [1,2,7,8] and leptin has been shown to be a significant predictor for the development of cardiovascular events [9]. Leptin is synthesized primarily in white adipocytes however studies from our laboratory have demonstrated the ability of the heart and cardiomyocytes to synthesize leptin [10–12]. Interestingly as well, leptin levels are also elevated in epicardial adipose tissues in patients with coronary artery disease [13]. Therefore, when taken together leptin may regulate cardiac function as a result of its production from peripheral adipose tissue as well as locally produced leptin functioning in either a paracrine or autocrine manner.

While the direct cardiac effects of leptin are not completely understood there is substantial evidence that its direct administration to cardiac tissue, either under in vitro or in vivo conditions can produce a robust hypertrophic response [14–17]. We have previously documented a critical role for RhoA/ROCK signaling in mediating the hypertrophic response to leptin and have shown that a potential critical

Abbreviations: BSA, bovine serum albumin; C3, Clostridium botulinum C3 exoenzyme; DTT, dithiothreitol; ELIPA, enzyme linked inorganic phosphate assay; ERK1/2, extracellular signal regulated kinases 1/2; FBS, fetal bovine serum; FK-506, Tacrolimus; HBSS, Hank's balanced salt solution; KB-R7943, ((2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate; MAPK, mitogen activated protein kinase; MCIP, modulatory calcineurin-interacting protein; NFAT, nuclear factor of activated T cells; NCX, 3Na + − 1Ca2 + exchange; NKA, 3Na + − 2 K + ATPase; PBS, phosphate-buffered saline; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated, coiled-coil containing protein kinase.

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mechanism involves a RhoA/ROCK-dependent selective translocation of p38 MAPK into nuclei [18,19]. The selectivity of this translocation was evident by the fact that only p38 and not ERK1/2 were translocated following leptin administration [19].

Although RhoA activation likely plays an important role in mediating the pro-hypertrophic effect of leptin, the pathological hypertrophic program represents a well-choreographed multiplicity of intracellular signaling pathways resulting in the hypertrophic phenotype [reviewed in 20]. Among the many cellular pathways involved in the development of pathological cadiomyocyte hypertrophy, there is extensive evidence that the calcineurin pathway plays a critical role by virtue of its ability to dephosphorylate the transcriptional factor nuclear factor of activated T-cells (NFAT) thereby allowing the translocation of the latter in the nucleus [reviewed in 21–23]. Calcineurin, a calcium/calmodulin-activated serine threonine protein phosphatase is activated by various prohypertrophic factors including α- and β adrenergic agonists, angiotensin II as well as endothelin-1 [24–29]. To our knowledge there are no studies potentially linking leptin to calcineurin in the heart although the peptide has been demonstrated to increase calcineurin activity in the rat hypothalamus [30]. The present study had therefore three principal goals. First, we wished to determine whether leptin can stimulate calcineurin activity in cultured cardiomyocytes. Secondly, we assessed whether any effects on calcineurin are related to potential changes in intracellular ion homeostasis. Lastly, we set out to determine any potential relationship between the RhoA pathway and leptin-induced changes in calcineurin activity or calcineurin-related intracellular changes, in view of strong evidence linking the RhoA pathway to leptin-induced hypertrophy.

2. Materials and Methods

2.1. Primary culture of cardiomyocytes

The procedures followed for cardiomyocyte isolation were in accordance with the University of Western Ontario animal care guidelines and conform to the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada). Primary cultures of cardiomyocytes were prepared from 1-day old Sprague–Dawley neonatal rat heart as described previously [31]. Briefly, rats were sacrificed by decapitation and hearts were excised and rinsed in buffer containing 1X HBSS. Ventricles were minced and the tissue was subjected to 6 sequential digestions using a solution containing 0.4% type 2 collagenase, in a water-jacketed Erlenmyer flask maintained at 37 °C. After each extraction, the supernatant was added to a tube having an equal volume of buffer solution containing 20% fetal bovine serum to stop collagenase activity. All extractions were pooled, filtered using a 70 μm cell strainer and centrifuged at 500 x g for 5 min at 4 °C. The pellet was resuspended in cell culture medium supplemented with 10% FBS and subjected to two 30 min periods of preplating to enrich the cardiomyocyte population. Cardiomyocyte were plated on Primaria dishes and cultured for 48 h followed by washing with PBS and culturing for 24 h in FBS free culture medium before starting treatment. Our method of preparation resulted in less than 5% contamination by non-cardiomyocytes as demonstrated by myosin staining.

2.2. Experimental protocol and determination of cell surface area

Myocytes were treated with leptin (Sigma-Aldrich, Oakville, Ontario, Canada; 50 ng/ml, 3.1 nM) alone or leptin in the presence of RhoA inhibitor C3-exoenzyme (C3, Alexis Biochemicals, San Diego, California, USA; 30 ng/mL), the reverse mode $3Na^+$-$1Ca^{2+}$ exchanger (NCX) inhibitor KB-R7943 (Tocris Bioscience, Minneapolis, Minnesota, USA; 10 μM) or the calcineurin inhibitor FK-506 (Cell Signaling Technology, Danvers, Massachusetts, USA; 2 nM). After 24 h of treatment cell surface area was analyzed using a Leica inverted microscope equipped with an Infinity 1 camera at 100 x magnification. Cell surface area was measured using SigmaScan Software (Systat, Richmond, California, USA). Time points for determinations of other parameters are provided in the individual sections below.

2.3. Determination of intracellular calcium concentrations

Cells were loaded with 0.5 μM FURA-2 (Life Technologies, Burlington, Ontario, Canada) by incubating for 30 min at 37 °C followed by washing with PBS and incubating for 30 min at 37 °C in culture medium. Myocytes were treated with leptin in the presence or absence of C3 or KB-R7943. Fluorescence was read at 340 nm excitation and 540 nm emission using a Molecular Devices (Sunnyvale, California, USA) Spectromax M5 with Softmax Pro plate reader software. Readings were standardized against mg of protein. Fluorescence determinations were made early (10 min, 1 h) after leptin addition as well at 24 h after leptin addition as shown in Results.

2.4. Determination of intracellular sodium concentration

For determination of intracellular Na^+ concentrations myocytes were loaded with 1.0 μM CoroNa Red Na^+ indicator (Life Technologies) using an identical procedure as described for determination of calcium concentrations. Fluorescence was read at 545 nm excitation and 575 nm emission using identical procedures and time periods as for FURA-2. Determinations were made at identical time points as for Ca^{2+} determinations as shown in Results.

2.5. $3Na^+$-$2\ K^+$-ATPase (NKA) activity

Following treatments, myocytes were washed with TBS and lysed using lysis buffer (50 mM Tris, pH 7, 5, 20 mM EGTA, 1 mM DTT, 0.2% NP-40, protease inhibitors cocktail). ATPase assays were performed on 5 μg protein in presence or absence of 1 mM ouabain using an ELIPA kit (Cytoskeleton, Denver, Colorado, USA). The ATPase activity at 300, 600, 900 and 1200 sec was averaged for each sample and the difference between total ATPase activity and the activity in presence of ouabain represented the NKA activity. Determinations were made at identical time points as for Ca^{2+} determinations as shown in Results.

2.6. RNA isolation, reverse transcription and real-time PCR analysis for MCIP1, α-skeletal actin, and 18 s rRNA genes

After appropriate treatments total RNA was extracted from myocytes using Trizol (Life Technologies) according to the manufacturer's protocol. Reverse transcription reaction was performed to synthesize first strand of cDNA using 5 μg of RNA and M-MLV (Life Technologies) according to the manufacturer's instructions. For gene analyses real-time polymerase chain reaction was carried out in a 10 μl reaction volume containing 2 X EvaGreen qPCR Mastermix-S (Applied Biological Materials, Richmond, British Columbia, Canada), 0.5 μM primers and cDNA and the gene expression was quantified with an MJ Research DNA Opticon 2 continuous fluorescence detector in conjunction with the Opticon Monitor analysis software by BioRad (Hercules, California, USA). The following primer sequences were used: 5'-ATTCGAAGAGGGGGTGACTT-3' (forward) and 5'-GCCATC TTCCGAGACTTCTG-3' (reverse) for NCX-1, 5'-GCCCAATCCAGACAAA CAGT-3' (forward) and 5'-TGATTTTTGGCTTGGGTCTC-3' (reverse) for MCIP1, 5'-CACGGCATTATCACCAACTG-3' (forward) and 5'-CCGGAGG-CATAGAGAGACAG-3' (reverse) for 18 s rRNA, and 5′-CACGGCATTAT-CACCAACTG-3′ (forward) and 5′-CCGGAGGCATAGAGAGACAG-3′ (reverse) for α-skeletal actin. PCR conditions used to amplify genes were 30s at 94 °C, followed by annealing at 58 °C for 20s for NCX-1, MCIP1 and α-skeletal actin , and 50 °C for 20s for 18 s rRNA followed by elongation at 72 °C for 30s. NCX-1, MCIP1, and α-skeletal actin