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Cholesterol depletion enhances adrenergic signaling in cardiac myocytes

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ABSTRACT

Cardiac myocytes endogenously express α and β adrenergic receptors, prototypes of the G-protein coupled receptor superfamily. Depending upon the dose of norepinephrine (agonist) exposure, hypertrophy and apoptosis are initiated by differential induction of two discrete constituents of the transcription factor AP-1, *i.e.*, FosB and Fra-1. We explored differential adrenergic signaling as a paradigm for understanding how cholesterol dictates cells to choose hypertrophy or apoptosis. For this, we used *fosB* and *fra-1* promoter–reporter constructs for monitoring adrenergic signaling. We show that cholesterol depletion enhances norepinephrine-mediated signaling in cardiac myocytes. Importantly, this increased signaling is reduced to original level upon cholesterol replenishment. We used specific ligands for α and β adrenergic receptors and show that the enhanced signaling upon cholesterol depletion is a combined effect of both α and β adrenergic receptors. These results constitute the first report demonstrating the effect of cholesterol on adrenergic signaling using a direct end-point gene expression.

1. Introduction

Cholesterol is an essential and representative lipid in higher eukaryotic cellular membranes and is crucial in membrane organization, dynamics, function, and sorting [1,2]. Cholesterol is often found distributed nonrandomly in domains in biological membranes [1,3]. Current understanding of the organization of biological membranes involves the concept of lateral heterogeneities in the membrane, collectively termed as membrane domains. Many of these domains (sometimes termed as 'lipid rafts') are thought to be important for the maintenance of membrane structure and function, although characterizing the spatiotemporal resolution of these domains has proven to be challenging [3–6]. The idea of such specialized membrane domains assumes significance in cell biology since physiologically important functions such as membrane sorting and trafficking, signal transduction processes, and the entry of pathogens [3,7–9] have been attributed to these domains. Importantly, cholesterol plays a vital role in the function and organization of membrane proteins and receptors such as G-protein coupled receptors (GPCRs) [10–12].

The GPCR superfamily is the largest and most diverse protein family in mammals, involved in signal transduction across membranes [13,14]. GPCRs are prototypical members of the family of seven transmembrane domain proteins and include >800 members which together constitute ~5% of the human genome [15]. GPCRs regulate physiological responses to a diverse array of stimuli, and mediate multiple physiological processes. For this reason, GPCRs have emerged as major targets for the development of novel drug candidates in all clinical areas [16]. It is estimated that up to 50% of clinically prescribed drugs act as either agonists or antagonists of GPCRs [17]. Adrenergic receptors are important members of the GPCR superfamily and are endogenously expressed in cardiac myocytes. In adult heart, terminally differentiated myocytes are susceptible to discrete pathological consequences like hypertrophy, apoptosis and autophagy, each with immense clinical implications [18,19]. It has been reported that agonists such as angiotensin II and norepinephrine (NE) may cause hypertrophy and apoptosis, depending upon the concentration, thereby providing a framework for understanding the crosstalk between these two pathways [20–22]. NE released from the sympathetic nervous system and its cognate receptors (α and β with several subtypes) play a critical role in cardiac performance and homeostasis [23,24]. In addition, *ex vivo* cardiac myocytes elicit hypertrophic response upon treatment with lower dose (≤10 μM), while higher dose (≥50 μM) of NE induces apoptosis [20,21]. However, in spite of significant enhancement of our knowledge of adrenergic signaling, the role of membrane components such as cholesterol in these processes is not understood yet. Such knowledge is crucial in a better understanding of cardiovascular pathobiology, particularly keeping in mind the close relationship between cholesterol, adrenergic signaling and heart failure [25].

Abbreviations: GPCR, G-protein coupled receptor; MβCD, methyl-β-cyclodextrin; NE, norepinephrine

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It has previously been demonstrated that cholesterol is required for the function of GPCRs such as the serotonin$_{1A}$ receptor [11,12,26] and the oxytocin receptor [27]. In this paper, we have explored differential adrenergic signaling in cardiac myocytes as a paradigm for understanding how cellular cholesterol dictates cells to choose hypertrophic or apoptotic responses. We earlier demonstrated that both *ex vivo* and *in vivo* myoblasts and myocytes elicit hypertrophic and apoptotic responses through respective pathways, demarcated by the induction of FosB and Fra-1, two distinct members of the AP-1 family of transcription factors [21]. These results have unequivocally established a gene-specific framework for delineating differential signaling leading to hypertrophic and apoptotic responses. In the present study, we used *fosB* and *fra-1* genes as the end-point targets for analyzing respective signaling pathways, and monitored the effect of cellular cholesterol on these pathways. Our results show that cholesterol depletion enhances NE-mediated adrenergic signaling in cardiac myocytes. Importantly, we used specific ligands for α and β adrenergic receptors and our results show that signaling, as monitored by activities of *fosB* and *fra-1* promoters, is enhanced in both types of receptors upon cholesterol depletion.

2. Materials and methods

2.1. Materials

All reagents used in this study were from Sigma Aldrich (St. Louis, MO) unless mentioned otherwise. Fetal bovine serum was purchased from Life Technologies (Carlsbad, CA). Luciferase assay reagents were purchased from Promega (Madison, WI). Amplex Red cholesterol assay kit was from Molecular Probes (Eugene, OR). Bradford reagent was purchased from Bio-Rad (Hercules, CA). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

2.2. Methods

2.2.1. Cell culture

H9c2 cells (embryonic rat cardiac myoblasts) were cultured and maintained as monolayer in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose, supplemented with 10% fetal bovine serum (heat inactivated), 90 U/ml penicillin, 90 μg/ml streptomycin and 5 μg/ml amphotericin B at 37 °C in humidified incubator with 5% CO_2 [21].

2.2.2. Generation of promoter–reporter constructs

Fragments (1493 base pair and 2689 base pair) of 5′ upstream regions of *fosB* and *fra-1* genes were isolated by PCR amplification from rat genomic DNA and cloned in pGL3 luciferase basic vector (Promega) using standard recombinant DNA methods [21]. Genomic DNA isolated from H9c2 cells was PCR amplified using primers (forward 5′ GGAATGGCAGGCTTTCAACAC 3′ and reverse 5′ CTGTGACCACGCTGAGGTCTT 3′) spanning −1033 base pair to +460 base pair of the 5′ upstream region of *fosB* gene. This 1493 bp PCR amplified product was further cloned in pGL3 luciferase basic vector (Promega) at SmaI site. Similarly, 2689 base pair PCR amplified product using *fra1* specific primers (forward 5′ GTGCTAGCCCATGCGTGCTTGTGCGTGT 3′ and reverse 5′ CGAGATCTGCTGGATGT TCGGTA 3′) spanning −2474 base pair to +215 base pair region was cloned in pGL3 vector at Nhe I/Bgl II sites.

2.2.3. Transient transfection

H9c2 cardiac myoblasts were grown in 6-well plate up to 60–70% confluency and transiently transfected with reporter plasmids using Escort IV transfection reagent. Briefly, 2 μg of plasmid DNA and 4 μl of transfection reagent (Sigma) were used per well. Cells were incubated with the transfection reagent in serum- and antibiotic-free medium for 10–12 h, followed by incubation in medium containing 20% serum and antibiotics for 6 h. Cells were then kept in 10% serum containing medium for 6 h. Finally, cells were kept in serum-free medium for 12 h, treated with the agonists for 2 and 6 h (~36–40 h post-transfection). The agonists used were NE, phenylephrine (α-adrenergic receptor agonist) and isoproterenol (β-adrenergic receptor agonist). Cells were harvested after 2 and 6 h of treatment for reporter assay.

2.2.4. Luciferase reporter assays

Cells were lysed in reporter lysis buffer. Cell lysates were analyzed for luciferase activity using the Luciferase Reagent Assay Kit and the corresponding luminescence was measured with a luminometer (Turner Scientific, CA). Normalization of transfection efficiency was done by the estimation of total protein used for the luciferase assay [28].

2.2.5. Cholesterol depletion of cells in culture

Cells were grown in 6-well plate and transfected with reporter constructs. Cholesterol depletion was carried out by treating cells with 5 mM MβCD in serum-free medium for 30 min at 37 °C, followed by wash with serum-free medium [29,30].

2.2.6. Replenishment of cholesterol in cholesterol-depleted cells

Cholesterol-depleted H9c2 cells were replenished with cholesterol using a water-soluble cholesterol–MβCD complex. Cholesterol replenishment was carried out by incubating cholesterol-depleted cells with the cholesterol–MβCD complex for 5 min in a humidified atmosphere with 5% CO_2 at 37 °C [31]. The complex was prepared by dissolving required amounts of cholesterol and MβCD in a ratio of 1:10 (mol/mol) in DMEM medium by constant vortexing at room temperature (~23 °C) [26]. Cholesterol–MβCD complex was freshly prepared and filter sterilized before each experiment.

2.2.7. Analysis of cholesterol content

Cholesterol content in cell lysates was estimated using the Amplex Red cholesterol assay kit [32].

3. Results

The water-soluble compound MβCD has earlier been shown to selectively and efficiently extract cholesterol from cellular membranes by including it in a central nonpolar cavity [33]. Fig. 1 shows the cholesterol content in cholesterol-depleted and cholesterol-replenished H9c2 cardiac myocytes. Upon treatment of cells with 5 mM MβCD, the cholesterol content was reduced to ~48% of that of control (without MβCD or agonist treatment) in case of cells transfected with *fosB* plasmid (Fig. 1a). In case of cardiac myocytes that were transfected with *fra-1*, the corresponding reduction in cholesterol content was comparable (~50%; see Fig. 1b). Cholesterol replenishment of cholesterol-depleted cells was carried out by incubating the cholesterol-depleted cells with cholesterol–MβCD complex, as described in Materials and methods. As shown in Fig.1, replenishment of cholesterol with this complex resulted in recovery of cholesterol. Similar results were obtained when cells were stimulated by 2 μM (Fig. 1a) and 100 μM (Fig. 1b) NE.

Low and high doses (2 and 100 μM) of NE selectively induce the transcription of *fosB* and *fra-1* genes (Jindal and Goswami, manuscript in preparation). This is based on the earlier observation that hypertrophic and apoptotic doses of NE (2 and 100 μM, respectively) differentially induce FosB and Fra-1 [21]. As shown in Fig. 2, *fosB* promoter was induced (~1.5 fold) by 2 μM NE in 2 h and *fra-1* promoter was induced (~2.5 fold) by 100 μM NE in 6 h in control (without MβCD or agonist treatment) cells. It should be noted that promoter activity appears to be modulated with cholesterol content, even in the absence of agonist treatment. Interestingly, cholesterol depletion enhances the induction of *fosB* promoter to ~2.4 fold upon