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Original article

Regulation of autophagy in cardiomyocytes by Ins(1,4,5)P_3 and IP_3-receptors

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ABSTRACT

Autophagy is a process that removes damaged proteins and organelles and is of particular importance in terminally differentiated cells such as cardiomyocytes, where it has primarily a protective role. We investigated the involvement of inositol(1,4,5)*tris*phosphate (Ins(1,4,5)P_3) and its receptors in autophagic responses in neonatal rat ventricular myocytes (NRVM). Treatment with the IP_3-receptor (IP_3-R) antagonist 2-aminoethoxydiphenyl borate (2-APB) at 5 or 20 μmol/L resulted in an increase in autophagosome content, defined as puncta labeled by antibody to microtubule associated light chain 3 (LC3). 2-APB also increased autophagic flux, indicated by heightened LC3II accumulation, which was further enhanced by bafilomycin (10 nmol/L). Expression of Ins(1,4,5)P_3 5-phosphatase (IP_3-5-Pase) to deplete Ins(1,4,5)P_3 also increased LC3-labeled puncta and LC3II content, suggesting that Ins(1,4,5)P_3 inhibits autophagy. The IP_3-R can act as an inhibitory scaffold sequestering the autophagic effector, beclin-1 to its ligand binding domain (LBD). Expression of GFP-IP_3-R-LBD inhibited autophagic signaling and furthermore, beclin-1 co-immunoprecipitated with the IP_3-R-LBD. A mutant GFP-IP_3-R-LBD with reduced ability to bind Ins(1,4,5)P_3 bound beclin-1 and inhibited autophagy similarly to the wild type sequence. These data provide evidence that Ins(1,4,5)P_3 and IP_3-R act as inhibitors of autophagic responses in cardiomyocytes. By suppressing autophagy, IP_3-R may contribute to cardiac pathology.

1. Introduction

Chronic diseases of the myocardium, including heart failure and valvular heart disease commonly display an increased expression of IP_3-receptors (IP_3-R) [1–4]. No functional significance of this increase has been established, although arrhythmia [5] and hypertrophic growth [6] have been suggested as possible consequences. Another possibility is that IP_3-R influences autophagy in the diseased myocardium and thereby contributes to disease progression.

Cardiomyocytes *in vivo* and in cell culture undergo autophagy, in addition to the well characterized hypertrophic and apoptotic responses. Given that cardiomyocytes are replaced with only minimal efficiency at best, apoptosis is considered to be always detrimental to the myocardium. In contrast, the role of autophagy is less clear, and it is likely that autophagy plays a complex role whereby moderate levels of autophagy protect the myocytes by removal of damaged proteins and organelles, but excessive levels contribute to cardiomyocyte death [7,8]. With this in mind, it is important to define the pathways that culminate in autophagy from different initiating stimuli and to understand the mechanisms by which autophagy can be inhibited.

Autophagy in cardiomyocytes is heightened by glucose depletion as well as by ischemia and post-ischemic reperfusion and there is evidence that different pathways are involved under these different conditions. The pathway best characterized in cardiomyocytes involves inhibition of the mTOR, itself an inhibitor of autophagy, by AMP-activated protein kinase (AMPK) [9,10]. AMPK is activated by nutrient, especially glucose, depletion and by ischemia and both of these initiate autophagic responses [10,9]. Under conditions of post-ischemic reperfusion, however, autophagy is activated by a mechanism independent of AMPK and mTOR, instead involving increased availability of beclin-1 [10]. Beclin-1 forms complexes with class-III PI3kinase (PI3K), Bcl-2 and nutrient activated factor-1 (NAF-1), and in some tissues, these are scaffolded onto IP_3-R [11]. Beclin-1 bound onto this complex is inactive in promoting autophagy, but, once released, beclin-1 initiates autophagy by facilitating the formation of autophagic vesicles [12]. Disruption of the beclin-1-containing complex by IP_3-R antagonists promotes the release of beclin-1 and initiates autophagy [13]. In addition to the role of inhibitory scaffold, IP_3-R, in some cell types, acts as an inhibitor of autophagy by facilitating mitochondrial Ca^{2+} uptake to sustain energy metabolism [14].

While a role for IP_3-R in autophagy has been established in neuronal cells [15,16], there is currently no information concerning a possible involvement in autophagy in the heart. This is important because there is increasing evidence that autophagy is activated in failing myocardium, a condition associated with heightened IP_3-R expression [1,17]. As autophagy is protective under these conditions [18], there is a possibility

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that the increased IP_3-R expression in heart failure might contribute to disease progression by limiting autophagy.

2. Methods

2.1. Preparation of neonatal rat ventricular myocytes (NRVM)

Studies were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee. Ventricular myocytes were prepared from 1 to 2 day old Sprague–Dawley rats using repeated pancreatin/collagenase digestion, followed by separation from non-myocytes using discontinuous Percoll gradients as described previously [19]. Treatments were initiated 1 day after isolation.

2.2. Constructs and adenoviruses

Adenoviruses expressing FLAG-tagged IP_3-5-phosphatase (INPP5A, 43 kDa type 1) have been described previously [20]. The GFP-tagged IP_3-R(1) ligand binding domain (human IP_3-R(1) 224–605; GFP-IP_3-R-LBD) [21] was provided by Dr. Tamas Balla (National Institutes of Child Health, Bethesda, MD). Mutations in GFP-IP_3-R-LBD (K579Q and R582Q, GFP-IP_3-R-LBD-QQ) were generated using GeneTailor™ (Invitrogen, #12397-014) [22]. Adenoviruses were prepared using the Gateway® procedure (Invitrogen), propagated on HEK-293 cells and purified using CsCl gradients. Viruses were used at a multiplicity of infection of 30–50 per cell.

2.3. Confocal microscopy

NRVM were plated onto gelatinized glass bottom confocal dishes (MatTek, USA) and infected with adenovirus or treated, as indicated. After 24 h, NRVM were fixed with PFA (4% w/v paraformaldehyde, 0.1 M Pipes pH 6.8) before permeabilization with Saponin buffer (0.05% w/v, 0.1 M Pipes pH 6.8). The plates were washed in PBS, and incubated with anti-LC3 antibodies (abcam #ab58610) diluted in PBA (PBS + BSA 1% w/v). Plates were then further washed in PBS, and incubated with anti-rabbit-AlexaFluor-633 (Invitrogen). The samples were then further washed in PBS, and mounted using Vectashield mounting media (Abacus ALS, Australia). Images were captured using a Zeiss Meta-510 LSM (excitation 633 nm, emission at 647 nm). LC3-labled puncta were counted in individual cells (4–5/field, 20 fields per experiment, 3 experiments total) and values averaged. Each value represents a total of 200 cells counted.

2.4. Western blotting

For evaluation of LC3-II accumulation, NRVM, treated as described, were incubated in the presence or absence of bafilomycin (10 nmol/L) for 4 h to inhibit autophagy downstream of the autophagosome [23]. Proteins were separated by SDS-PAGE using 16% gels and were transferred to PVDF membranes (Immobilon^{Psq}). Antibodies were used at the following dilutions; LC3 (Cell Signaling # 2775, 1/500), tubulin (abcam, #ab6046, 1/5000). HRP-conjugated secondary antibodies and ECL plus (Amersham Life Sciences) were used to detect proteins of interest. Images were captured and data analyzed using a BioRad ChemiDoc™ XRS + imaging system. Expression of p62 was measured using 10% gels, and anti-p62 antibody (1/1000, Sigma #P0067). Data were normalized relative to tubulin.

2.5. Co-immunoprecipitation of beclin-1 and GFP-IP_3-R-LBD or GFP-IP_3-R-LBD-QQ

NRVM were washed and lysed in RIPA buffer (50 mmol/L pH 7.8, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP40 and protease inhibitors (Roche)) and centrifuged. Supernatants were pre-cleared with Protein A Sepharose (IP beclin-1) or Protein G Sepharose (IP GFP). Primary antibody, anti-GFP (Cell Signaling #2555 1/100) or anti-beclin-1 (Cell Signaling #3738 1/100) was added for 2 h at 4 °C and then Protein A- or Protein G-Sepharose was added and incubated overnight. Pellets were washed 3 times in RIPA buffer containing protease inhibitors and subjected to SDS-PAGE on 9% gels, western blotting and developed with anti-GFP (1/1000) or anti-beclin-1 (1/500) antibodies.

3. Results

3.1. The IP_3-R blocker, 2-aminoethyoxyphenyl borate (2-APB), increases LC3-stained puncta, LC3-II generation and p62 content

Macroautophagy involves the encapsulation of damaged proteins and organelles within a membrane structure, an autophagosome, enriched in microtubule-associated light chain 3 (LC3) subsequent to addition of phosphatidylethanolamine to generate LC3II. Thus, autophagy is commonly measured by the accumulation of autophagosomes identified as LC3-stained puncta or by the generation of LC3II from LC3I. Using these criteria, we examined the effect of the IP_3-R antagonist, 2-aminoethoxyphenyl borate (2-APB), on autophagy in NRVM. FoxO1 is an established autophagic effector in NRVM [24] and we treated cells with adenovirus expressing constitutively active FoxO1 (Ad-CA-FoxO1) as a positive control. NRVM were treated with 2-APB (2-APB, 5 or 20 μmol/L, [25]) for 24 h. Cells were fixed and stained using anti-LC3 antibodies to examine the formation of autophagosomes (punctate LC3 staining). As shown in Fig. 1A, addition of 2-APB (5 or 20 μM) caused the appearance of LC3-labeled vesicles. Autophagosomes were quantified as the average number of clearly defined puncta per cell (Fig. 1B). Data from CA-FoxO1-treated cells are included for comparison because FoxO1 is a well established autophagic factor in cardiomyocytes [24].

We also measured the effect of 2-APB (5 and 20 μM) on the generation of LC3II from LC3I. These experiments were performed in the presence and absence of bafilomycin (10 nmol/L), an inhibitor of autolysosome formation, to ensure that increased LC3II reflected increased generation rather than inhibition of autophagic flux downstream [23]. 2-APB at 5 and 20 μM increased LC3II as shown in Fig. 1C and D. Bafilomycin further increased the response, validating that 2-APB increased flux through the autophagic pathway.

In addition to LC3-II, p62 (sequestosome 1/SQSTM1) associates with autophagosomes and is subsequently degraded in the autolysosome [26]. Content of p62 often decreases during the autophagic process, except in cardiomyocytes where increased p62 content has been reported in response to several different autophagic stimuli [27–29]. In agreement with these studies, p62 expression increased after 24 h treatment with 2-APB (Fig. 2A and B). As a positive control, we examined p62 expression after treatment with Ad-CA-FoxO1 for 24 h, to induce autophagy [9]. As found after 2-APB treatment, expression of CA-FoxO1 caused an increase in p62 expression (Fig. 2C and D).

3.2. Depletion of Ins(1,4,5)P_3 increases LC3-stained puncta, LC3-II generation and p62 content

The finding that the IP_3-R antagonist, 2-APB, induced autophagy in NRVM prompted us to question a possible involvement of the natural IP_3-R ligand, Ins(1,4,5)P_3. To directly assess the contribution of Ins (1,4,5)P_3, NRVM were treated with adenovirus expressing FLAG-IP_3-5-phosphatase (type 1, 43 kDa), a phosphatase that specifically dephosphorylates Ins(1,4,5)P_3 to Ins(1,4)P_2 [30,20], a metabolite that does not bind IP_3-R [31]. As shown in Fig. 3A and B, IP_3-5-phosphatase expression increased autophagosome formation in NRVM, as indicated by an increase in the percentage of cells containing LC3 puncta.

IP_3-5-phosphatase expression increased the LC3II content ratio (Fig. 3C and D) and the content of p62 (Fig. 3E and F). Thus, depletion of Ins(1,4,5)P_3 is sufficient to cause autophagy, implying that Ins(1,4,5)P_3, itself, inhibits autophagy.