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RESEARCH NOTE

Adrenomedullin 2 activates extracellular-signal-regulated kinase in endothelial cells via a protein kinase C α -independent pathway [version 1; peer review: 2 approved with reservations]

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Abstract

Adrenomedullin 2 plays diverse physiological roles such as regulating cardiovascular functions and blood pressure. It was reported that adrenomedullin 2 can activate protein kinase C in murine ventricular myocytes to augment cardiomyocyte contractile function. Using a protein kinase C α knockout mouse model, we show here that adrenomedullin 2 activates extracellular-signal-regulated kinase in a protein kinase C α -independent mechanism in endothelial cells.

Keywords

adrenomedullin 2 (ADM2), intermedin (IMD), protein kinase C (PKC), endothelial cells, signal transduction

Open Peer Review

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06 Jan 2016	view	view

1. **James G. Taylor VI**, National Institutes of Health, Bethesda, USA
2. **Hajime Takizawa**, Kyorin University, Mitaka, Japan

Any reports and responses or comments on the article can be found at the end of the article.

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Competing interests: There is no disclosure of competing interests.

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Introduction

Adrenomedullin 2 (ADM2), also known as intermedin, is a secreted peptide that belongs to the calcitonin gene-related-peptide family^{1,2}. It has been reported that ADM2 regulates intracellular calcium levels and contractile function in protein kinase C (PKC) - and protein kinase A (PKA) - dependent mechanisms in cardiomyocytes³. ADM2 activates the cAMP/PKA signaling pathway, which mediates inactivation of contractility and strengthening of cell-cell adhesion in endothelial cells⁴. ADM2 activates extracellular-signal-regulated kinase (ERK), a key signaling molecule for cell proliferation in endothelial cells⁵. To investigate whether ADM2 activates ERK through PKC α , which is a major upstream activator of ERK in endothelial cells we examined the effect of phosphorylation of ERK on ADM2 stimulation in endothelial cells isolated from PKC α null mice or wild type (wt) counterpart mice.

Methods

Animal care and experimental procedures were performed under protocol # CC0004 approved by the Institutional Animal Care and Use Committees of Yale University. Endothelial cells were isolated from wild type (C57BL/6J, The Jackson Laboratory, Cat # 000664) and PKC α -/- mice (*Prkca*^{tm1Jmk}, The Jackson Laboratory, Cat # B6;129-Prkcatm1Jmk/J) and maintained as previously described⁶. Briefly, the arteries of both wild-type and knockout mice were harvested, finely minced with scissors, and digested with 25 ml collagenase (2 mg/ml) at 37°C for 45 min under gentle agitation. The crude preparation was triturated, passing it 12 times through a cannula needle, and was then filtered on a 70- μ m sterile cell strainer. The filtered preparation was spun at 400 $\times$ g, and the pellet was resuspended in 2 ml of 0.1% BSA. Magnetic beads (Invitrogen) coated with anti-mouse CD31 (BD Biosciences) were added to the cell suspension and incubated with rotation at room temperature for 15 min. The bead-bound cells were recovered with a magnetic separator and washed with DMEM containing 20% FBS. Cells were suspended in 10 ml of complete DMEM and seeded on cell culture plates (Catalog # 353003, Corning Inc., Corning, NY). Subconfluent cells were serum-starved for 16h followed by incubation with 10 ng/ml ADM2 peptide (Pheonix Pharmaceuticals, Burlingame, CA) for the indicated time length: 0, 5, and 30min. Cells were lysed in RIPA buffer (Catalog # R0278, Sigma-Aldrich, St Louis, MI), supplemented with protease inhibitor cocktail (Catalog # 11 873 580 001, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktails (Catalog # P0044 and P5726, Sigma-Aldrich, St Louis, MI) as instructed by manufactures. Total cell lysates were subjected to immunoblotting analysis as described previously². The membranes were hybridized with antibodies recognizing phospho-ERK (at 1:2,000 dilution of Catalog # 4370, Cell Signaling Technologies, Danvers, MA), total ERK (at 1:1,000 dilution of Catalog # 4695, Cell Signaling Technologies, Danvers, MA), PKC α (at 1:500 dilution of Catalog # 610108, BD BioSciences, San Jose, CA), and β -actin (at 1:10,000 dilution of Catalog # sc-47778, Santa Cruz Biotechnology Inc., Dallas, Texas). Following incubation with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Zymed Laboratories Inc., San Francisco, CA). Western signals were visualized with enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA).

Results and conclusion

Dataset 1. Gel images for 'Adrenomedullin 2 activates extracellular-signal-regulated kinase in endothelial cells via a protein kinase C α -independent pathway' by Guo X., et al.

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As shown in Figure 1, ADM2 increased phosphorylation of ERK in endothelial cells. However, there was no difference in ERK phosphorylation levels in wt versus PKC α null endothelial cells (Figure 1). Our results indicate that ADM2 activates ERK in endothelial cells via a PKC α - independent pathway.

Figure 1. ERK, but not PKC α , mediates ADM2 signaling in endothelial cells. Representative immunoblot showing that ADM2 increased phosphorylation of ERK, via a PKC α -independent pathway, in endothelial cells. Mouse endothelial cells (mEC) were isolated from wild type (wt) and Protein kinase C α knockout mice (PKC α ^{-/-}). Cells were serum-starved overnight followed by stimulation with ADM2 synthetic peptide (10ng/ml) for indicated time and cell lysates were analyzed by immunoblotting for ERK activation.

Data availability

F1000Research: Dataset 1. Gel images for 'Adrenomedullin 2 activates extracellular-signal-regulated kinase in endothelial cells via a protein kinase C α -independent pathway' by Guo X., et al., 10.5256/f1000research.2420.d110689⁷

Author contributions

XG cultured and treated cells and performed Western analyses, and prepared the manuscript; RJ isolated endothelial cells from mice, CC and MS directed the study.

Competing interests

There is no disclosure of competing interests.

Grant information

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The purpose of the current study was to investigate whether ADM2 activates ERK through PKC α , which is a major upstream activator of ERK in endothelial cells. The authors examined the effect of phosphorylation of ERK on ADM2 stimulation in endothelial cells isolated from PKC α null mice or wild type (wt) counterpart mice. The findings obtained in their experiments were clear. However, they studied by only a single method. They need to study, for example, by siRNA method to certify the independency on PKC α . Another concern is that the authors are expected to, at least, discuss the crucial signal pathway to activate ERK in this cell system. In this context, they need to study Ras-Raf-MAPK/ERK kinase (MEK).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 19 February 2016

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James G. Taylor VI

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The manuscript "Adrenomedullin 2 activates extracellular-signal-regulated kinase in endothelial cells via a protein kinase C α -independent pathway" presents an elegant mutational analysis of cellular activation by the enigmatic adrenomedullin 2. Endothelial cells harvested from PKC- α knock out mice still demonstrate ERK phosphorylation, suggesting that the current paradigm of ADM2 intracellular activation (ADM2 signalling occurring through a PKC receptor pathway) is not completely understood. While this work is limited in scope, it does set a foundation for needed work in this field. There are some technical and context specific elements which would significantly strengthen this research note.

Major points:

1. Which specific ADM2 peptide was used? It would be helpful to mention this in the methods; ADM2-47 is believed to be the more potent agonist compared to the -40 peptide. Perhaps it would also be appropriate to test both ADM2 peptides (both -47 and -40) for comparison.
2. The abstract and introduction sets the context for this work by describing ADM2 signalling in cardiomyocytes, but this research note presents data for arterial endothelial cells. The addition of the same phospho-Westerns for the same PKC- α knock out mouse cardiomyocytes would address this criticism.
3. The results section is clearly presented, but a brief discussion and conclusion to put these results in a physiologic context would again enhance the impact of this research note. Specifically, this reviewer could cite a review by [Hong *et al.* \(2012\)](#) where it is stated that the receptors that mediate ADM2 action are not completely understood (i.e. the authors results suggest that perhaps the calcitonin like receptor pathway for ADM2 might activate endothelial cells independent of protein kinase). Presenting an updated version of Figure 4 from this review would also further work in this field and put the results in context.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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