Suppression of endothelin-1-induced cardiac myocyte hypertrophy by PPAR agonists: role of diacylglycerol kinase zeta

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Aims
Ligand activation of peroxisome proliferator-activated receptors (PPARs) prevents cardiomyocyte hypertrophy, but the underlying signalling mechanisms remain unknown. We previously reported that the anti-hypertrophic effect of the dietary polyunsaturated fatty acid, conjugated linoleic acid (CLA), was associated with the upregulation of diacylglycerol (DAG) kinase (DGK). DGK catalyses phosphorylative conversion/attenuation of DAG, thereby modulating protein kinase C (PKC) and G-protein signalling. As the anti-hypertrophic effects of CLA were attenuated by inhibitors of PPARs, the present aim was to investigate the involvement of DGK in the anti-hypertrophic actions of bona fide selective PPAR agonists.

Methods and results
Endothelin-1 (ET1)-induced hypertrophy of neonatal, and then adult, Sprague–Dawley rat cardiomyocytes served as experimental paradigms. Expression of DGKζ, the predominant DGK isoform in myocytes, was stimulated by ligands of PPARγ (troglitazone) or PPARα (fenofibrate) and was accompanied by increased DGK activity. Troglitazone or fenofibrate prevented hypertrophic indicators elicited by ET1, including myocyte size augmentation, de novo protein synthesis, hypertrophic gene expression, and activation of the pro-hypertrophic signal, PKC₁. shRNA knockdown of DGKζ abolished the growth-inhibitory effects of PPARs and restored all ET1-induced aspects of hypertrophy. Importantly, the involvement of DGK in the ability of troglitazone and fenofibrate to block ET1-induced hypertrophy and PKC₁ signalling was verified in adult rat myocytes.

Conclusion
Collectively, these findings show that the anti-hypertrophic actions of PPARs require DGKζ. Thus, within the cardiomyocyte, there exists a PPAR-DGK signalling axis that underpins the ability of PPAR ligands to inhibit ET1-dependent hypertrophy.

Keywords
Cardiac hypertrophy • Myocytes • PPAR • Endothelin • Diacylglycerol kinase

1. Introduction
Cardiac hypertrophy is the increase in myocardial mass provoked by haemodynamic stress or myocardial injury. At the myocyte level, it is characterized by increases in cell size, protein synthesis, and changes in gene expression.¹ As hypertrophy leads to decompensation and heart failure,²–⁴ attenuation of hypertrophy is considered a promising therapeutic target to prevent cardiac failure.⁵

There is evidence that peroxisome proliferator-activated receptors (PPARs) act as protective signals in hypertrophy. PPARs belong to the nuclear receptor family of transcription factors that regulate fatty acid and triglyceride metabolism.⁶ All three isoforms are expressed in cardiomyocytes,⁷,⁸ and activation of PPARs α,⁹–¹¹ β/δ,¹²–¹⁴ or γ¹⁵–¹⁷ prevents hypertrophy in response to mechanical strain or growth agonists such as endothelin-1 (ET1).⁹ However, the anti-hypertrophic mechanisms invoked by PPARs remain poorly understood.

The Gₛₐₗ protein-coupled receptor (GPCR) pathway, which includes diacylglycerol (DAG) and protein kinase C (PKC), plays a critical role in hypertrophy.¹⁸,¹⁹ GPCR ligands such as ET1²⁰ and angiotensin II²¹ stimulate phospholipase C-mediated hydrolysis of

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phosphatidylinositol 4,5-bisphosphate to produce inositol trisphosphate and DAG. DAG kinases (DGKs) are intracellular lipid kinases that phosphorylate DAG to produce phosphatidic acid (PA). The resultant decrease in the availability of DAG attenuates translocation and activation of PKC, thereby terminating an arm of G protein signalling. Ten DGK isoforms have been identified in mammals, but only three are found in cardiomyocytes: α, ε, and ζ. The predominant isoform expressed in myocytes, DGKζ, reportedly plays a role in the regulation of cardiac hypertrophy.

This study is predicated on our previous report on the ability of conjugated linoleic acid (CLA), a dietary polyunsaturated fatty acid, to suppress hypertrophy. First, the anti-hypertrophic actions of CLA were blocked by inhibitors of PPARs α and γ. Second, CLA upregulated DGKζ expression and activity, and third, CLA attenuated ET1-induced activation of PKCζ, a key pro-hypertrophic signal. Although others have reported on the anti-hypertrophic properties of DGKζ, a limitation of that initial report was that it did not demonstrate a ‘causative’ link between DGKζ upregulation and the anti-hypertrophic actions of CLA. Nonetheless, those findings collectively led to conjecture that DGK may mediate the anti-hypertrophic actions of PPARs. We therefore address this remaining question by determining whether PPAR-DGK cross-talk occurs within the cardiomyocyte, and whether DGK contributes to the anti-hypertrophic actions of PPAR agonists.

2. Methods

See Supplementary material online for expanded methods and supplementary data.

2.1 Materials

ET1, troglitazone, fenofibrate, PA, and antibodies against sarcomeric α-actinin and β-actin were from Sigma-Aldrich (Oakville, Canada). DGKζ, PPARα, and PPARγ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PKCζ antibody was from Millipore (Temecula, CA, USA). R59022 was from EMD Biosciences, Inc. (Gibbstown, NJ, USA). –1595 hBNP-luciferase was kindly provided by Dr David Gardner (University of California, San Francisco, CA, USA).

2.2 Cell culture of neonatal and adult rat cardiomyocytes

The study 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). Approval was given by the University of Manitoba Animal Care Committee. Ventricular myocytes were isolated from neonatal (1-day-old) or adult (~250 g) Sprague–Dawley rats by alternate cycles of 0.05% trypsin and mechanical disruption or enzymatic digestion or enzymatic digestion. Ventricular myocytes were isolated from neonatal (1-day-old) or adult (~250 g) Sprague–Dawley rats by alternate cycles of 0.05% trypsin and mechanical disruption or enzymatic digestion of cell lysates by western blotting, as described previously.

2.3 Treatment

As applicable, myocytes were subjected to lentiviral infection or transfection. Then, unless otherwise indicated, cells were serum-deprived for 24 h and treated with vehicle, troglitazone (10−5 mol/L), or fenofibrate (10−5 mol/L). Hypertrophy was stimulated by addition of ET1 (10−7 mol/L).

2.4 Transfection and luciferase assay

Myocytes were co-transfected with –1595 hBNP-luciferase and Renilla luciferase, as described previously.

2.5 DGKζ expression

To measure DGKζ mRNA, real-time PCR was performed using primers for rat DGKζ and normalized to GAPDH mRNA. DGKζ protein was assayed by western blotting and normalized to β-actin, as described previously.

2.6 PA formation

Cellular lipids were extracted, and PA was measured using a previously described fluorescence-based enzymatic assay.

2.7 Lentiviral preparation and infection

Lentiviral vectors expressing shRNAs against DGKζ and PPARα α and γ (DGKζ: V2LMM_80143, V2LMM_61844, V2LMM_65193; PPARα: TRCN0000025967; PPARγ: TRCN0000025394, TRCN0000025395, TRCN0000025398 (Open Biosystems, Ottawa, Canada)) were prepared. Scrambled sequences served as non-silencing controls. Lentivirus vector plasmids were co-transfected with pSPAX2 (packaging) and pMD2.G (enveloping) vectors using FuGENE6 Reagent (Roche, Indianapolis, IN, USA). High-titre lentiviral stock was produced in HEK-293T cells 48 h after transfection. Myocytes were infected for 24 h by application of the lentivirus to the culture medium. Specificity and degree of isoform knockdown were confirmed by western blotting.

2.8 Cell size

Myocyte size was assessed by immunofluorescent microscopy and computer-assisted planimetry, as described previously. Each experiment consisted of 30 cells analysed per experimental group (n = 3–6).

2.9 Protein synthesis

[3H]-Leucine incorporation into acid-insoluble protein is a measure of protein synthesis that is commonly used as evidence of hypertrophy. Four hours prior to harvesting, cells were pulsed with 1 μCi/mL [3H]-leucine. [3H]-Leucine incorporation was measured as described previously. Each experiment was done in triplicate.

2.10 Hypertrophic gene expression

Activation of the hypertrophic gene programme was assessed by real-time PCR to detect three foetal genes [brain natriuretic peptide (BNP), β-myosin heavy chain (βMHC), and skeletal muscle α-actin (SMαA)] that are usually only expressed in late embryonic and early neonatal life, but are reactivated during hypertrophic growth. We also examined a contractile protein gene expressed following exposure to hypertrophic stimuli [cardiac muscle α-actin (CMαA)]. Hypertrophic gene mRNAs were normalized to GAPDH mRNAs.

2.11 PKCζ translocation

PKCζ levels were detected in soluble and particulate fractions of cell lysates, as described previously.

2.12 Statistics

n-Values are indicated in legends. Error bars represent SEMs. Data were subjected to one-way ANOVA and the Newman–Keuls multiple comparison test or unpaired Student’s t-test, as appropriate.

3. Results

3.1 PPAR agonists upregulate DGKζ

Selective ligands of PPARs (troglitazone) and PPARγ (fenofibrate) upregulated DGKζ mRNA and protein levels (Figure 1). DGK activity was also increased by troglitazone and fenofibrate. Uptake of DGKζ was abolished by actinomycin D (20 μg/mL; Figure 2).
3.2 DGKζ contributes to the anti-hypertrophic actions of PPARs

The involvement of DGK (all isoforms) was first assessed using the non-selective DGK inhibitor, R59022. ET1 increased BNP promoter activity, an indicator of hypertrophy, and this was attenuated by troglitazone or fenofibrate (Figure 3). However, the ability of troglitazone or fenofibrate to prevent ET1-dependent BNP promoter activity was abolished by R59022.

We next narrowed our query to the role of DGKζ specifically, the predominant isoform in myocytes and a regulator of hypertension. Targeted shRNA against DGKζ achieved significant knockdown of DGKζ to 12 ± 1% vs. control (Figure 4). ET1 induced increases in three indicators of hypertrophy (Figure 4, Table 1): myocyte size, [3H]-leucine incorporation, and hypertrophic gene expression, all of which were suppressed by troglitazone or fenofibrate. This ability of troglitazone or fenofibrate to prevent ET1-dependent hypertrophy was unaffected by non-silencing control shRNA, but was abolished by shRNA knockdown of DGKζ.

3.3 Suppression of pro-hypertrophic PKCε signalling by PPARs involves DGKζ

ET1 increased particulate:total PKCε, and this translocation of PKCε to the particulate fraction was abolished by PPAR ligands (Figure 5). The ability of PPAR ligands to prevent ET1-dependent PKCε activation was unaffected by non-silencing control shRNA, but was abolished by shRNA knockdown of DGKζ.

3.4 Anti-hypertrophic PPAR/DGK signalling in adult rat cardiomyocytes

Consistent with our findings in neonatal rat cardiomyocytes, ET1 increased the size of adult rat cardiomyocytes, and this cell growth was abolished by PPAR ligands (Figure 6). Furthermore, the ability of
PPAR ligands to prevent ET1-dependent growth of adult myocytes was blocked by inhibition of DGK using R59022.

Likewise, in adult rat myocytes, ET1 increased particulate:total PKC\(^{1}\), and this translocation of PKC\(^{1}\) to the particulate fraction was abolished by PPAR ligands (Figure 6). The ability of PPAR ligands to prevent ET1-dependent PKC\(^{1}\) activation in adult myocytes was blocked by inhibition of DGK using R59022.

4. Discussion

These findings provide evidence that DGK\(\zeta\) plays an important role in the anti-hypertrophic effects of PPAR\(\alpha\) and \(\gamma\) ligands. We showed that blocking DGK\(\zeta\), whether using a non-selective chemical inhibitor of DGK or by knockdown of the DGK\(\zeta\) isoform, abolishes the anti-hypertrophic effects of these PPARs. The ability of PPAR\(\alpha\) and \(\gamma\) ligands to block PKCe, a key pro-hypertrophic signal, was also abrogated by DGK\(\zeta\) knockdown, which is consistent with the mechanistic role of DGK\(\zeta\) in terms of attenuating DAG levels.

This work is predicated on our previous report of the anti-hypertrophic actions of CLA, which we were able to attribute to the activation of PPAR\(\alpha\) and PPAR\(\gamma\) but not PPAR\(\beta/\delta\). Thus, the present study highlights PPAR\(\alpha\) and \(\gamma\). However, anti-hypertrophic properties have also been reported for PPAR\(\beta/\delta\), although these other reports refer to the suppression of hypertrophy of neonatal rat cardiomyocytes elicited by angiotensin II or phenylephrine.
To our knowledge, there is no evidence that PPAR\(b/d\) can prevent hypertrophy elicited by ET1. We conducted preliminary experiments to assess a possible role for DGK in the growth-inhibitory effects of PPAR\(b/d\) (see Supplementary material online). In the context of ET1-dependent hypertrophy, the selective PPAR\(b/d\) agonist, GW501516, exerts a partial, but statistically significant, attenuating effect. The ability of GW501516 to partially attenuate ET1-induced hypertrophy is abolished by R59022 (DGK inhibitor). In addition, we found that GW501516 also increased expression of DGK\(z\). Therefore, the role that DGK\(z\) plays in the growth-inhibitory effects of PPARs likely extends to the anti-hypertrophic actions of PPAR\(b/d\), a possibility that warrants further study.

Work by others has led to the suggestion that the anti-hypertrophic actions of PPAR\(a\) might be related to the suppression of AP-1 DNA-binding activity, since ET1-induced PKC\(\varepsilon\) signalling leads to the activation of the AP-1 transcription factor. Our data are consistent with this notion, since forced overexpression of DGK\(z\) in cardiomyocytes blocks not only ET1-dependent hypertrophy, but also AP-1 signalling. Combined with our data showing first, that PPARs increase DGK\(z\) expression, and second, that DGK\(z\)...

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/90/2/267/282062/Downloaded-from-https://academic.oup.com/cardiovascres/article-abstract/90/2/267/282062)
Table 1 Effects of DGKz knockdown on PPAR-mediated suppression of hypertrophic genes

<table>
<thead>
<tr>
<th>Hypertrophic gene</th>
<th>shRNA</th>
<th>ET1</th>
<th>Troglitazone</th>
<th>Troglitazone + ET1</th>
<th>Fenofibrate</th>
<th>Fenofibrate + ET1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNP: GAPDH</td>
<td>Non-silence</td>
<td>256 ± 34*</td>
<td>126 ± 11**</td>
<td>167 ± 8**</td>
<td>136 ± 10**</td>
<td>179 ± 43**</td>
</tr>
<tr>
<td>(mRNA, %control)</td>
<td>DGKz</td>
<td>253 ± 16*</td>
<td>107 ± 2**</td>
<td>248 ± 22**</td>
<td>113 ± 7**</td>
<td>243 ± 36*</td>
</tr>
<tr>
<td>βMHC: GAPDH</td>
<td>Non-silence</td>
<td>230 ± 27*</td>
<td>107 ± 2**</td>
<td>145 ± 8**</td>
<td>122 ± 23**</td>
<td>146 ± 33**</td>
</tr>
<tr>
<td>(mRNA, %control)</td>
<td>DGKz</td>
<td>238 ± 26*</td>
<td>124 ± 7**</td>
<td>271 ± 35**</td>
<td>114 ± 7**</td>
<td>237 ± 31**</td>
</tr>
<tr>
<td>SMuA: GAPDH</td>
<td>Non-silence</td>
<td>214 ± 28*</td>
<td>120 ± 4**</td>
<td>146 ± 3**</td>
<td>129 ± 4**</td>
<td>155 ± 17**</td>
</tr>
<tr>
<td>(mRNA, %control)</td>
<td>DGKz</td>
<td>227 ± 24*</td>
<td>119 ± 6**</td>
<td>268 ± 32**</td>
<td>121 ± 6**</td>
<td>239 ± 33**</td>
</tr>
<tr>
<td>CMyA: GAPDH</td>
<td>Non-silence</td>
<td>280 ± 54*</td>
<td>128 ± 6**</td>
<td>171 ± 36**</td>
<td>123 ± 4**</td>
<td>162 ± 26**</td>
</tr>
<tr>
<td>(mRNA, %control)</td>
<td>DGKz</td>
<td>248 ± 22*</td>
<td>113 ± 4**</td>
<td>255 ± 28**</td>
<td>126 ± 5**</td>
<td>280 ± 7**</td>
</tr>
</tbody>
</table>

Mean ± SEM; n = 3; *P < 0.01 vs. control; **P < 0.05 and ***P < 0.01 vs. ET1.

Of the three DGK isoforms found in myocytes, α, β, and γ,23 anti-hypertrophic activity has so far been linked only to DGKα and DGKγ. DGKz overexpression blocked ET1-induced hypertrophy of cultured myocytes.24 and in vivo, cardiac-restricted overexpression of DGKz in mice blocked angiotensin II- and phenylephrine-induced hypertrophy.25 Expression of DGKz mRNA in the left ventricular myocardium is reduced in aortic-banded vs. sham rats.26 Cardiac-specific overexpression of DGKz in mice also prevented hypertrophy in response to phenylephrine or pressure overload.37 Our experiments focused on DGKz because it is the predominant isofrom expressed in myocytes.25 Given that upregulation of DGKz by troglitazone and fenofibrate was abolished by the transcriptional inhibitor, actinomycin D (Figure 2), and that PPARs can signal as nuclear receptor transcription factors,6 one possibility is direct binding of PPARs to the DGKz gene promoter. Although the PPRE consensus sequence does not appear in the DGKz gene promoter, this does not necessarily preclude direct binding of PPARs. Nakachi et al.38 detected 167 gene promoters with strict PPARγ binding during adipocyte differentiation in 3T3-L1 cells, yet PPRE consensus sequences were present in only 15 of these target genes. Three of these genes were activated by PPARγ, lack consensus PPREs, and instead contain imperfect or atypical PPREs that vary in the binding site and/or spacer sequences.39 PPARs might also regulate the DGKz promoter indirectly through complex formation with other transcription factors such as Sp1. The DGKz promoter contains Sp1 sites, and PPAR-dependent modulation of numerous genes involves Sp1.40–43 Whatever the proximal signalling mechanism, the nature of the putative interaction between PPARs and the DGKz promoter requires further study.

Our finding that DGKz mediates the ability of PPARs to suppress PKCe activity is noteworthy, since PKCe is considered to be a key effecter of hypertrophy in the cardiomyocyte.44 Of the six PKC isoforms found in myocytes,45 only PKCe dramatically translocates to the membrane fraction following hypertrophic stimuli such as pressure overload in vivo46 and ET1 in vitro.47–50 Also, mice overexpressing constitutively active PKCe develop hypertrophy.51,52 Notably, the anti-hypertrophic actions of DGKγ have been linked with the inhibition of PKCe. Both in vitro and in vivo studies implicade termination of PKCe signalling vis-à-vis depletion of DAG levels as the mechanism that underpins blockade of hypertrophy by DGKγ overexpression.24,25

Our data imply that upregulation of DGKz is rapid and possibly transient. Indeed, after 1 h of treatment, a trend was observed in which troglitazone and fenofibrate appear to double DGKz protein...
levels, leading to a sustained increase that was statistically detectable by 2 h. It is important to note, however, that the rapid/transient nature of DGKz upregulation is not inconsistent with the mechanistic role of DGKz that we propose. Binding of ET1 to the ETA receptor causes a conformational change that favours activation of phosphoinositide-specific phospholipase C-b (PLCb). PLCb in turn hydrolysates phosphatidylinositol-4,5-bisphosphate, thereby generating DAG and IP3. This occurs within seconds. Virtually immediately, as sarcolemmal DAG increases, PKCe translocates from the soluble to the membrane fraction, where it is activated by binding of DAG. DAG-dependent translocation of PKCe is detectable within minutes by immunoblotting and two-photon microscopy. Although the distribution of PKCe to the membrane fraction by ET1 is only sustained for a matter of minutes, activated PKCe initiates signalling cascades that collectively confer the hypertrophic response to ET1. Target downstream effectors include small G proteins such as Ras, and Ras-dependent extracellular-signal-regulated kinases (ERKs), which is important particularly since Marshall et al. recently report that ERK1/2 regulates the majority of genes involved in the early stages of cardiomyocyte hypertrophy. Therefore, if signalling by ET1 is disrupted at a pivotal, early point such as PKCe activation, this might have numerous downstream effects that will collectively prevent the hypertrophic response. Here, we show that despite the rapid and/or transient nature of PPAR-induced upregulation of DGKz, it occurs during a window of time when PKCe activation is indeed being suppressed, such that the hypertrophic response per se (cell size augmentation, protein synthesis, and hypertrophic gene expression) is ultimately blocked.

Figure 6 Anti-hypertrophic PPAR/DGK signalling in adult rat cardiomyocytes. Adult rat myocytes were pre-treated with vehicle, troglitazone (10^-6 mol/L), or fenofibrate (10^-5 mol/L), followed by ET1 (10^-7 mol/L) for 24 h (cell size) or 5 min (PKCe). (A and B) Cell surface areas of individual cells were quantified as described in Methods and presented as percent of myocyte size (μm²) vs. vehicle-treated controls. n = 3 sets of 30 cells analysed. (C and D) PKCe levels in particulate and soluble fractions of cell lysates were determined by western blotting and presented as percent of particulate/total PKCe vs. vehicle-treated controls, where total PKCe equals soluble plus particulate fractions. The ability of troglitazone and fenofibrate to (A and C) prevent ET1-dependent cell growth and PKCe translocation was (B and D) abolished by pharmacological inhibition of DGK by R59022. n = 3–4. ns, not significant. *P < 0.05 and **P < 0.01 vs. vehicle-treated controls or as indicated. §P < 0.01 vs. ET1-treated cells.
It bears mentioning that, by inhibiting PKCε activation in ET1-treated myocytes, PPAR-DGKζ signalling might be modulating ERK activity. A role for the ERK cascade in the hypertrophic response of cardiomyocytes was proposed over a decade ago. Numerous hypertrophic stimuli activate ERK, including ET1 and isoproterenol, and mechanical strain, and disrupting ERK signalling abrogates the hypertrophic response to ET1 and isoproterenol. T-cells derived from DGKζ-deficient mice exhibit aberrant augmentation of both ERK activation and cell proliferation. This suggests that, at least in T-cells, DGKζ acts as an endogenous inhibitor of pro-growth ERK signalling. We know that in cardiomyocytes, PKCε participates in the activation of the ERK cascade by ET1, and we have shown that DGKζ suppresses PKCε signalling. Thus, it is possible that at least part of the anti-hypertrophic effect of PPARs involves downstream suppression of ERK, via DGKζ-dependent inhibition of PKCε. Indeed, we found that the ability of ET1 to activate ERK in cardiomyocytes is attenuated, at least in part, by troglitazone and fenofibrate, and again, this attenuation occurs in a DGK-dependent manner (see Supplementary material online).

DGK-mediated phosphorylation of DAG leads to the generation of PA, which is known to trigger hypertrophic processes such as protein synthesis and activation of mitogen-activated protein (MAP) kinases. However, DGK is only a minor contributor; most PA is actually generated through the hydrolysis of phosphatidylcholine by phospholipase D. Thus, overexpression of DGK or PPAR-dependent upregulation of DGK probably contributes only negligibly to the pool of PA and its signalling potential. The ability of troglitazone and fenofibrate to suppress ERK activity (see Supplementary material online) supports this notion since, in contrast, PA activates MAP kinases. Thus, the growth-inhibitory effects of liganded PPARs and γ are more consistent with the ability of DGK to suppress PKCε via depletion of DAG rather than the ability of DGK to generate PA.

ET1 elicited an ~7-fold increase in PKCε translocation in neonatal myocytes vs. only an ~1.75-fold increase in adult myocytes (Figure 6). This might be due to inherent differences between neonatal and adult myocytes. For example, ET1 stimulated phosphorylation of myristoylated, alamine-rich protein kinase C substrate (MARCKS) as well as praline-rich tyrosine kinase (PYK2). Both of these events were PKCε-dependent, and they occurred in neonatal and adult myocytes. However, similar to our findings, the magnitude of these ET1/PKCε-dependent responses is smaller in adult myocytes than in neonatal myocytes. Varying experimental conditions might also play a role. We synchronize cultured myocytes by serum deprivation in neonatal myocytes. Varying experimental conditions might also play a role. We synchronize cultured myocytes by serum deprivation in neonatal myocytes. Varying experimental conditions might also play a role. 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