Insulin Cannot Induce Adipogenic Differentiation in Primary Cardiac Cultures

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Abstract

Cardiac tissue contains a heterogeneous population of cardiomyocytes and nonmyocyte population especially fibroblasts. Fibroblast differentiation into adipogenic lineage is important for fat accumulation around the heart which is important in cardiac pathology. The differentiation in fibroblast has been observed both spontaneously and due to increased insulin stimulation. The present study aims to observe the effect of insulin in adipogenic differentiation of cardiac cells present in primary murine cardiomyocyte cultures.

Oil Red O (ORO) staining has been used for observing the lipid accumulations formed due to adipogenic differentiation in murine cardiomyocyte cultures. The accumulated lipids were quantified by ORO assay and normalized using protein estimation. The lipid accumulation in cardiac cultures did not increase in presence of insulin. However, addition of other growth factors like insulin-like growth factor 1 and epidermal growth factor promoted adipogenic differentiation even in the presence of insulin and other inhibitory molecules such as vitamins. Lipid accumulation also increased in cells grown in media without insulin after an initial exposure to insulin-containing growth media. The current study adds to the existing knowledge that the insulin by itself cannot induce adipogenic induction in the cardiac cultures. The data have significance in the understanding of cardiovascular health especially in diabetic patients.

Keywords

- ▶ primary cardiomyocyte culture
- ▶ fibroblasts
- ▶ adipogenic differentiation
- ▶ insulin
- ▶ growth factors

The primary cultures of cardiomyocyte as a model have enabled researchers to study and understand the morphological, biochemical, and electrophysiological characteristics of the heart.^{1,2} They are a valuable tool for various pharmacological, toxicological, and regeneration studies, besides being used in the study of cellular-level functions such as regulation of electrophysiology, intracellular calcium fluxes, contractile mechanics, and protein expression.^{1,2} Besides cardiomyocytes, a large number of nonmyocyte cell populations exist in the heart and among them the most important is cardiac fibroblasts (CFs).^{3,4} CFs are recognized for their fundamental roles in both development and disease through structural, paracrine, and electrical interactions with cardiomyocytes.^{3–5}

The heart is surrounded by an extensive layer of fat called the epicardial adipose tissue (EAT), which generates various bioactive molecules and is important in cardiac pathology.⁶ EAT originates from the epicardium induced by mesenchymal transformation and peroxisome proliferator-activated receptor activation.⁷ In cases of ventricular cardiomyopathy, fibroadipose substitution and adipose replacement of functional cardiomyocytes have been observed.^{8–10} Since cardiomyocytes barely differentiate or proliferate in postnatal and adult cultures, any differentiation in primary cardiomyocyte cultures is due to CFs differentiation.^{1,11} Fibroblasts are biologically dynamic and show mesenchymal stem cell-like properties.^{12,13} Fibroblast progenitors show preadipocytic

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lineage choice abnormalities in older heart which are similar to those associated with increased insulin stimulation.¹⁴

The differentiation of stem cells derived from blood, adipose tissue, and pulp tissue into cardiomyogenic lineage with and without inducing agents has been studied extensively.^{15,16} It has been observed that adipose tissue-derived stem cells undergo more differentiation into spontaneously beating cell clusters due to the changes at transcriptome levels.¹⁷ Interestingly, explant cultures of heart showed development of cells similar to those of adipogenic lineage.¹⁸ The forward and backward transition of cells between cardiogenic and adipogenic lineages has not yet been observed. In this study, we intend to elucidate whether differentiation of cardiac cells into cells of adipogenic lineage occurs spontaneously or through specific induction with insulin. For this investigation, we have used primary neonatal mice cardiomyocyte cultures and grew them in media with and without insulin.

Methodology

Primary Cardiomyocyte Culture

Neonatal murine cardiomyocyte culture (NMCC; primary culture derived from murine heart) was used for studying adipogenic induction. Two- to six-day-old CD-1 Swiss albino mice pups were sacrificed in accordance with the norms provided by the Institutional Animal Ethics Committee, University of Saskatchewan. The hearts were immediately removed and cultured on 0.02% gelatin-precoated cell culture flasks as per the protocol described previously.^{19,20}

Adipogenesis Induction

NMCC were grown in cardiomyocyte maintenance media (CMM) along with insulin (~0.3 IU/mL) as previously described^{19,20} till the cultures attained ~60% confluency (~2–3 weeks). Treatments were performed by maintaining the cells in the media as described below for various time periods—1 month, 2 months, 4 months, and 6 months, following which the cells were assayed for adipogenic induction.

- NMCC maintained in CMM and insulin—control media with insulin (CMI)
- NMCC maintained in CMM without insulin—insulin-deprived media (IDM)
- NMCC maintained in Claycomb media²¹ containing 0.1 mM norepinephrine (in 0.3 mM ascorbic acid)—induction media with insulin and other factors (IOF)

Oil Red O Staining and Assay

Adipogenic differentiation of NMCC was assessed in three independent experiments. Oil Red O (ORO) staining was performed as previously reported to monitor lipid accumulation with slight modifications.^{22,23} ORO (0.3 g; Sigma Aldrich, United States) was dissolved in 100 mL of isopropanol and left overnight at room temperature (RT). The solution was filtered; 40 mL of deionized H₂O was then added to 60 mL of the filtered solution (to make 0.1% working solution) and left overnight at 4°C. The working solution was filtered twice

before addition to cells. Adherent cells were rinsed with phosphate buffered saline and fixed in 4% paraformaldehyde for 1 hour at RT, followed by rinsing with Dulbecco phosphate buffered saline once for 1 minute at RT and with MQ H₂O (MilliQ water) twice at RT for 1 minute each. After the rinsing, staining with 0.1% ORO was performed for 10 minutes at RT. Plates were washed with 60% isopropanol for 3 minutes at RT to eliminate nonspecific staining and then rinsed thrice with MQ H₂O at RT for 1 minute each. Photographs of cells on the plates were taken in MQ H₂O. For quantification, the dye was extracted from dried wells with 100% isopropanol for 10 minutes at RT and absorbance was determined at 520 nm. Controls used were ORO stained wells (without cells). Values were normalized to total protein levels determined with a BCA assay kit (Thermo Scientific, United States) performed in microtiter plates as per the manufacturer's instructions. The amount of ORO expression was quantified as relative ORO absorbance/unit of protein.

Statistical Analysis

Statistical analysis on the data obtained from the various assays was performed using an analysis of variance (ANOVA) (Sigma Plot version 10 software package, Systat Software Inc., United States). The significance is represented as “*,” “**,” and “***” in ▶Fig. 1 for *p*-values ≤0.05, ≤0.001, and ≤0.0001, respectively, to indicate significant differences.

Results and Discussion

Adipogenesis was observed in plates containing NMCC treated with IDM using ORO stain (▶Fig. 2).²³ Following treatment with IDM, cells containing fat globules were observed to form after 2 months (▶Fig. 2E). The number of fat cells increased with time (4 and 6 months) (▶Fig. 2G, I). On the contrary, NMCC maintained in CMI did not differentiate into cells containing fat globules even after 6 months (▶Fig. 2A, B). The staining was further confirmed by ORO staining quantification assay, values of which were normalized using BCA assay using duplicate wells of treatment.²³ ▶Fig. 1 clearly shows the difference in adipogenesis following treatment with CMI and IDM. The slight expression observed in cells grown in CMI can be attributed to the slight nonspecific staining of NMCC by ORO, as there were no observable fat cells after 6 months (▶Fig. 2A, B). A sixfold increase in adipogenic cells was observed in NMCC grown in IDM compared with those grown in CMI (▶Fig. 1A).

The observed data show that the presence of insulin in the media does not induce adipogenic differentiation in cardiac cultures, contrary to the previous studies using fibroblast or their precursor.^{14,24–26} Deprivation of insulin subsequent to initial insulin exposure increases adipogenic differentiation. Insulin deprivation induced the redistribution of glucose transporters and thus attempts to increase glucose transport into the cells.²⁷ High glucose levels in muscle-derived stem cells have been known to induce adipogenic differentiation.²⁸ It is expected that adipogenic differentiation could have resulted in the few fibroblast

Fig. 1 Graphical representation of Oil Red O dye quantification showing fold level lipid accumulation represented as arbitrary units after various treatments for different time periods (1, 2, 4, and 6 months) in neonatal murine cardiac cultures (NMCC). (A) Comparison of normalized Oil Red O staining in NMCC grown in CMI (control media with insulin; blue bars) and IDM (insulin-deprived media; green bars) and (B) between NMCC grown in CMI (blue bars) and IOF (induction media with insulin and other factors; red bars). The relative presence of lipid accumulation in treated cells is shown in B. *Significant p -values ≤ 0.05 ; ***significant p -values ≤ 0.0001 .

precursor cells due to higher glucose levels following insulin deprivation.

Claycomb media has been originally used to maintain HL-1 cardiac muscle cell line. Since HL-1 cells were originally derived from the AT-1 mouse atrial cardiomyocyte tumor lineage, we investigated whether primary cardiac cultures could be maintained in Claycomb media, which contains insulin besides a variety of other factors including steroids and vitamins.²¹ Therefore, Claycomb media used in the study was abbreviated as “IOF” for media with insulin and other factors. Interestingly, NMCC maintained in Claycomb media showed signs of adipogenic differentiation. We observed that NMCC grown in IOF showed enhanced adipogenic differentiation. Treatment with IOF for 1 month was sufficient to initiate adipogenic differentiation as observed by ORO staining (→Fig. 2D). Subsequent treatment of 2, 4, and 6 months enhanced the number of differentiated adipogenic cells, as observed as colonies of fat cells in →Fig. 2F, H, and J. A >30-fold increase in number of adipogenic cells was observed in NMCC grown in IOF compared with those grown in CMI (→Fig. 1B).

Norepinephrine inhibits preadipocyte proliferation²⁹ but at the same time stimulates the expression of fibroblast growth factor 2,³⁰ which in turn stimulates adipogenic differentiation.³¹ Insulin-like growth factor 1 (IGF-1) activates the insulin receptor in preadipocytes and thus along with insulin acts synergistically to induce adipocyte differentiation.³² Epidermal growth factor (EGF) stimulates proliferation in preadipocytes, but EGF action in differentiation is based on concentration.³³ EGF concentrations in IOF was

<1 nM and thus promoted differentiation. Fetuin impairs adipocyte function effecting insulin resistance³⁴ and transferrin increased lipolysis in adipogenic cells through increased insulin resistance.³⁵ Ascorbic acid inhibits preadipocyte cell line differentiation in a dose-dependent manner³⁶ and retinoic acid inhibits adipocyte differentiation by activating the cellular retinoic acid binding proteins-II/retinoic acid receptor- γ pathway in preadipose cells.³⁷ Linoleic acid (~25 μ M in IOF) at concentrations of 100 μ M prevents adipogenic differentiation.³⁸ The remaining components in IOF (i.e., fetal bovine serum, glutamine, penicillin-streptomycin, bovine albumin) have been included in the media used for growing NMCC (CMI) and hence do not influence the outcome of the study. γ -Oleoyl- β -pal- α -phosphatidylcholine and cholesterol are important lipid molecules and their effect on adipogenic differentiation cannot be ascertained since these molecules are required for normal cell membrane integrity.

To conclude, the current study demonstrates that insulin alone cannot induce adipogenic differentiation in cardiac cultures. The initial insulin induction followed by deprivation can induce adipogenic differentiation. However, addition of other factors especially growth factors such as IGF-1 and EGF can promote differentiation even in the presence of insulin and other inhibitory molecules especially vitamins (retinoic acid and ascorbic acid). These preliminary findings have potential significance in the cardiovascular health of diabetic patients. Validation of the current data requires use of diabetic animal model studies. NMCC used were grown in batches and could be used only for a particular set of experiments, since NMCC being primary cultures cannot be

Fig. 2 Photographs of fat droplets stained by Oil Red O dye in treated neonatal murine cardiac cultures (NMCC). NMCC were grown in different media—control media with insulin (CMI), insulin-deprived media (IDM), and induction media with insulin and other factors (IOF)—and stained by Oil Red O dye (fat globules stained red) at the end of treatment time periods (1, 2, 4, and 6 months). NMCC grown in CMI after treatment for 1 month (A) and 6 months (B); NMCC grown in IDM for 1 month (C), 2 months (E), 4 months (G), and 6 months (I); NMCC grown in IOF for 1 month (D), 2 months (F), 4 months (H), and 6 months (J). Magnification scale bar: 25 μ m.

maintained like cell lines. Therefore, further in-depth investigations are required to understand the mechanisms involved.

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