PPARα agonist prevented the apoptosis induced by glucose and fatty acid in neonatal cardiomyocytes

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ABSTRACT. Objective: We investigated the effect of peroxisome proliferator activator receptors α (PPARα) on cardiomyocyte apoptosis induced by glucose and fatty acid, and if high glucose levels could increase fatty acid-induced apoptosis. Methods: Cardiomyocytes were divided in Dulbecco’s Modified Eagle Medium and divided into 5 groups: Group N (control Group); Group G (exposed to 25.5 mmol/l glucose); Group L (exposed to 5 mmol/l glucose, fatty acid); Group H (exposed to 25.5 mmol/l glucose and fatty acid); Group I (exposed to 25.5 mmol/l glucose, fatty acid and Wy14643). Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling. Immunocytochemistry staining detected PPARα’s expressing, and western blotting detected PPARα and nuclear factor κB’s (NF-κB) protein level. Results: Exposure to fatty acid resulted in a significant increase of cardiomyocytes apoptosis, with the extension of NF-κB formation, whereas exposure to 25.5 mmol/l glucose had no influence on the apoptosis rate. However, combination with fatty acid and high glucose concentration had induced more apoptosis with the up-regulation of NF-κB formation. The fatty acid and glucose-induced effects were improved by Wy14643, with down-regulation of NF-κB formation. Conclusion: These results suggested that in neonatal cardiomyocytes, fatty acid and glucose in combination with fatty acid induced apoptosis via NF-κB formation and activation of apoptosis pathways; glucose in combination with fatty acid induce more apoptosis rate for the more NF-κB formation, activation of the PPARα can reverse such apoptosis effect. The results also suggest that gluco-lipotoxicity may play a central role in the development of diabetic cardiomyopathy, and PPARα-agonist may be an effective drug in treating the diabetic cardiomyopathy (J. Endocrinol. Invest. 34: 271-275, 2011) ©2011, Editrice Kurtis

INTRODUCTION

It has long been known that many risk factors of diabetes cardiovascular complication, such as hyperglycemia, hyperlipotoxia, hypertension can increase the diabetes mortality rate; and some studies pointed out that patients with diabetes have a markedly adverse course after myocardial infarction, with high rates of heart failure and death. Several studies supported the hypothesis that diabetic cardiomyopathy is a specific diabetic complication, except for the distance of coronary artery disease and hypertension, or valvular disease (1, 2). The hypertrophy, apoptosis, and necrosis of cardiomyocyte is the main pathological foundation. Several mechanisms for the pathogenesis of diabetic cardiomyopathy have been proposed, such as energy metabolic derangement, endothelial dysfunction, autonomic dysfunction, and interstitial fibrosis. However, the new factors that lead to diabetic cardiomyopathy remain to be investigated. Both glucose and free fatty acids are pathologically elevated in the circulating blood of diabetic patients and have been suggested to have adverse effects on cell function; these conditions are called “glucotoxicity” and “lipotoxicity”, respectively (3, 4), but each of them is not enough to include all the causes of diabetes cardiovascular risk factors and until recently, some scholars have pointed out that glucolipotoxicity, the combination of the two, can induce the diabetic tissue injure. However, the possible role of glucose and fatty acids in apoptosis of neonatal cardiomyocytes has not been addressed (5).

The peroxisome proliferator activator receptors (PPAR), a class of ligand-activated transcription factors, have emerged as master transcriptional regulators of glucose and lipid metabolism. PPARα exert influence on intracellular lipid and glucose metabolism through direct transcriptional control of genes involved in peroxisomal and oxidation pathways, fatty acid uptake, and triglyceride catabolism. Moreover, preclinical data suggest a role for PPARα in body weight control, supporting the use of PPARα agonists to treat obesity (6). Mice deficient in PPARα exhibit a delayed response to inflammatory stimuli (7). Many clinical trials indicated that fibrates might decrease circulatory inflammatory markers and reduce the progression of coronary atherosclerotic lesions. The ability of PPARα to improve symptoms of the metabolic syndrome (visceral obesity, insulin resistance, atherogenic dyslipidemia, and inflammation) suggests that PPARα is conducive to the prevention and treatment of Type 2 diabetes mellitus and associated complications (8). Recently, apoptosis of cardiomyocytes was shown to play a central role in the development of heart failure (7). Saturated fatty acid-induced apoptosis has been demonstrated in neonatal rat cardiomyocytes (9, 10). However, whether fatty acids also can induce apoptosis in adult cardiomyocytes, which are post-mitotic, and the underlying mechanism remain to be investigated. They are de-
dependent on culture conditions, cell type, and genetic background (11-14). The aim of the present study was to investigate the role of fatty acid and elevated glucose concentrations in neonatal cardiomyocyte apoptosis, and whether high glucose levels could increase fatty acid-induced apoptosis, and foremost, the role of activation of PPARα in the glucolipotoxicity-induced cardiomyocytes apoptosis was assessed. In addition, the involvement of nuclear factor κB (NF-κB) pathway was studied.

MATERIALS AND METHODS

Cell culture
Cardiac muscle cells of neonatal rats (Sprague-Dawley-Ivanovas, 1-3 days old) were isolated by type 2 collagenase (Gibecco). After perfusion, the heart tissue was minced and incubated at 37 °C for another 10 min in KB medium containing collagenase (9, 10). Cells were cultured in dishes coated with 0.1% gelatin in Dulbecco’s Modified Eagle Medium (DMEM) (Gibecco, with glucose concentration of 5 mmol/l – hypoglycemic DMEM and 25.5 mmol/l – hyperglycemic DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 1% penicillin/streptomycin, and 20 mmol/l creatine. In order to inhibit growth of contaminating cells, 10 mmol/l 1-b-D-arabinofuranosyl-cytosine was added throughout the culture period. Palmitic and oleic acids (Sigma) were dissolved at 100 mmol/l in DMEM medium containing 11% fatty acid-free BSA (Sigma) under N2 atmosphere, shaken overnight at 37 °C, sonicated for 15 min, and filtrated under sterile conditions (stock solution). For control incubations, 11% BSA concentration was prepared, as described above. The effective free fatty acid concentration was determined before and after sterile filtration with a commercially available kit (Wako Chemicals, Neuss, Germany). A control experiment was performed with fatty acid dissolved first in 10 mol/l NaOH, then diluted to 12 mmol/l in DMEM medium containing 12.5% fatty acid-free BSA (Sigma) under N2 atmosphere, and shaken overnight at 50 °C. The resulting free fatty acid concentration and the apoptotic effect were similar (not shown). The calculated concentration of not albumin-bound free fatty acid was palmitic acid 1.0 mmol/l for a final concentration. The Wy14643 (acknowledged by Cayman) was dissolved in alcohol and a final concentration of 20 mg/l.

Groups
To mimic the circumstances as normal, hyperglycemia, fatty acid, hyperglycemia and fatty acid, and PPARα intervention hyperglycemia and fatty acid, the cultured cardiomyocytes were maintained in DMEM containing 5 mmol/l glucose and supplemented with 10% FBS, after incubation for 2 days, we used DMEM which without any serum to meet the cell cycle synchronization for 1 day in each group and were divided into 5 groups: Group N (Exposure of neonatal cardiomyocytes for 3 days to hypoglycemia type DMEM); Group G (Exposure of neonatal cardiomyocytes for 3 days to hyperglycemia type DMEM); Group L (Exposure of neonatal cardiomyocytes for 3 days to hypoglycemia type DMEM and treated with palmitic acid); Group H (Exposure of neonatal cardiomyocytes for 3 days to hyperglycemia type DMEM and fatty acid); Group I (Exposure of neonatal cardiomyocytes for 3 days to hyperglycemia type DMEM and fatty acid, and Wy14643).

Immunohistochemistry for PPARα and α-actin
Cardiomyocytes were identified by immunohistochemical staining with a rat monoclonal antibody against goat PPARα and α-actin (1:200, Santa Cruz, goat anti-rat) followed by a biotinylated rabbit anti-goat IgG (1:400, Santa Cruz) and then incubated with streptavidin-HRP; finally, the sections underwent DAB staining. The staining of cardiomyocytes were detected at 400× and 100× magnification in light microscopy.

Determination of apoptosis by TUNEL
The free 3-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique. Myocardial apoptosis was detected in paraffin-embedded cardiac sections. In situ labeling of fragmented DNA was performed by the TUNEL method. DNA fragments were identified with an in situ cell detection kit (Kygen) according to the manufacturer’s instructions. Briefly, sections were treated with 0.1% TritonX-100 for 3 min at 4 °C. After 3 washes with PBS, sections were mixed in a solution of TdT with Biotin-11-dUTP mixture. Next, the sections were counterstained with SSC to stop the reaction, for localization of nuclei, then incubated with Streptavidin-HRP, and at last, DAB stained. All the sections were detected by light microscopy. A total of 7 visual fields (total 200 nuclei) in which a cross section of cardiomyocyte was clearly visible were randomly selected, and the number of TUNEL-positive nuclei contained with DAB, which was regarded as cardiomyocyte apoptosis, was counted at 250× magnification. The apoptosis rate (TUNEL-positive nuclei in the section/200) is used to count the cardiomyocytes apoptosis.

Western blot analysis
Mitochondrial fractions were diluted 1:1.5 in SDS-Laemmli buffer (30) and boiled for 5 min. Equivalent amounts of each treatment group were run on 10% SDS polyacrylamide gels. Proteins were transferred electrically (12V, 30 min) to PVDF membrane and incubated with a rat anti-PPARα, NF-κB (P65), and β-actin polyclonal antibody (1:300, Santa Cruz Biotechnology, Santa Cruz, CA; 2 h at 37 °C, sonicated for 15 min, and filtrated under sterile conditions (stock solution). For control incubations, 11% BSA concentration was prepared, as described above. The effective free fatty acid concentration was determined before and after sterile filtration with a commercially available kit (Wako Chemicals, Neuss, Germany). A control experiment was performed with fatty acid dissolved first in 10 mol/l NaOH, then diluted to 12 mmol/l in DMEM medium containing 12.5% fatty acid-free BSA (Sigma) under N2 atmosphere, and shaken overnight at 50 °C. The resulting free fatty acid concentration and the apoptotic effect were similar (not shown). The calculated concentration of not albumin-bound free fatty acid was palmitic acid 1.0 mmol/l for a final concentration. The Wy14643 (acknowledged by Cayman) was dissolved in alcohol and a final concentration of 20 mg/l.

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Table 1 - The expression of peroxisome proliferator activator receptors α (PPARα), nuclear factor κB (NF-κB) in protein level and apoptosis rate in each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>The expression of cardiomyocytes (%)</th>
<th>The expression of PPARα (no.=3)</th>
<th>The expression of NF-κB (no.=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2.2143±0.8591</td>
<td>0.9282±0.1699</td>
<td>1.3258±0.2353</td>
</tr>
<tr>
<td>G</td>
<td>3.0000±1.1420</td>
<td>0.9492±0.088</td>
<td>1.2435±0.2856</td>
</tr>
<tr>
<td>L</td>
<td>56.7857±8.1336&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1.6659±0.051&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1968±0.060&lt;sup&gt;α,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>H</td>
<td>73.0000±11.4164&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>1.8815±0.2725&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.7749±0.2139&lt;sup&gt;α,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>I</td>
<td>13.1428±1.7728</td>
<td>3.4057±0.5634</td>
<td>1.4745±0.341</td>
</tr>
</tbody>
</table>

*vs N: p<0.01; vs I: p<0.01, p<0.05; vs L: p<0.01.*