Tumor suppressor p53 pathway mediates glucolipotoxicity-induced apoptosis of the rat cardiomyoblast cell

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Directed by Professor Hyun Chul Lee

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<ABSTRACT>

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Reactive oxygen species (ROS) generated by exposure to excessive levels of glucose and free fatty acids (glucolipotoxicity) have been reported to induce apoptosis. Here, we investigated the glucolipotoxic effects of high glucose and palmitic acid (C16:0) on the rat cardiomyoblastic cell line, H9c2, focusing on the role of the tumor suppressor, p53. Combined exposure to high levels of glucose (30 mM) and 250 μM palmitate resulted in a 2.2-fold increase in ROS generation compared to 30 mM glucose only. This increase in ROS was associated with upregulation of p53 and increased cleavage of

caspase-3, as well as increased expression of nuclear factor erythroid 2-related factor 2 (Nrf2). Somewhat unexpectedly, the Nrf2 target, NAD(P)H:quinine oxidoreductase-1 (NQO1), a detoxifying enzyme, was downregulated. Pifithrin-α, an inhibitor of p53, attenuated glucolipotoxicity-induced ROS generation and p53 expression, restoring p53 to normal levels. Chromatin immunoprecipitation analysis revealed that upregulated p53 interacted with the Nrf2-binding ARE region of the *NQO1* promoter, and blocked Nrf2–mediated NQO1 expression, causing greater myoblast apoptosis. Pretreatment with the ROS scavenger, N-acetyl-L-cysteine decreased glucolipotoxicity-induced p53 expression and normalized NQO1 levels. These results indicate that glucolipotoxicity-induced p53 elevation mediates apoptosis of rat cardiomyoblast cells through a dual mechanism: stimulation of a pro-apoptotic caspase signaling pathway and inhibition of the anti-apoptotic Nrf2-NQO1 signaling pathway.

Key work: p53, NQO1, Glucolipotoxicity, ARE region, Reactive oxygen species(ROS), palmitate, hyperglycemia, apoptosis

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I. INTRODUCTION

The incidence of diabetes and its complications has reached epidemic proportions and is increasing steadily. Plasma levels of glucose and free fatty acids in diabetic patients are pathologically elevated. A number of studies have confirmed that hyperglycemia and hyperlipidemia can induce intracellular toxicity[1-3]. The saturated long chain fatty acid palmitate, in particular, induces cell death in many cell types[4-7]. Although glucotoxicity[8] or lipotoxicity[9] alone can induce apoptosis, when combined they synergize, and the resulting glucolipotoxicity causes a more robust apoptotic response[10].

Glucolipotoxicity-induced oxidative stress is reported to play a key role in apoptosis in cardiac myocytes, but the underlying molecular mechanisms remain unclear.

Increased levels of reactive oxygen species (ROS) associated with oxidative stress induce an increase in nuclear factor erythroid-related factor 2 (Nrf2). Nrf2 binds to antioxidant response elements (AREs) in the promoters of ROS scavenging and detoxification enzymes, including NAD(P)H:quinine oxidoreductase-1 (NQO1) and glutathione S-transferases, increasing their expression[14]. The resulting Nrf2-induced upregulation of antioxident enzymes is reported to protect cells against ROS[15]. The tumor suppressor, p53, which is upregulated in response to genotoxic stress and causes cell-cycle arrest and cell death, is also upregulated by ROS[11]. Like Nrf2, p53 is also known to bind to the AREs of ROS scavenging and detoxification enzymes that are induced by Nrf2[13]. Thus, under ROS stress, p53 can inhibit Nrf2-induced transcription of antioxidant enzymes through competitive binding to AREs[13].

Because p53 is a key apoptotic regulator and responds to ROS, we examined whether p53 is involved in glucolipotoxicity-induced cell death in H9c2 cells, a rat cardiomyoblast cell line. Specifically, we hypothesized that the p53-Nrf2/ARE pathway might be an additional mechanism that contributes to glucolipotoxicity-induced apoptosis in these cells. Here, we demonstrate

that glucolipotoxicity-induced apoptosis of H9c2 cells is mediated through dual p53-dependent mechanisms: stimulation of a pro-apoptotic caspase signaling pathway by elevated ROS, and inhibition of the anti-apoptotic Nrf2-NQO1 signaling pathway through binding of p53 to *cis* regulatory ARE sites in the *NQO1* promoter.

II. MATERIALS AND METHODS

1. Cell culcure

H9c2 rat cardiomyoblasts were maintained at 37°C, 5% CO_2 in DMEM/F-12 (WelGENE, Daegue, South Korea) supplemented with 10% bovine calf serum (WelGENE, Daegue, South Korea), penicillin (100 U/ml), and streptomycin (100 μg/ml).

2. Treatment H9c2 cells with palmitic acid.

H9c2 cells were incubated for 18 h in DMEM/F12 medium containing normal (5 mM) or high (30 mM) glucose with 0.25% (wt/vol) BSA alone (control) or 0.25 mM palmitate (C16:0) complexed to 0.25% BSA. The 0.25 mM palmitic acid medium was prepared as previously described[16]. Briefly, a 20 mM solution of palmitic acid in 0.01 mol/l NaOH was incubated at 70°C for 30 min. Then, 900 μl of 5% BSA and 300 μl of the palmitic acid/NaOH solution was mixed together. It has been shown that the addition of BSA or a palmitic acid/BSA mixture does not affect the pH of the medium.

3. Preparation of Nuclear extracts for Nrf2

To prepare nuclear extracts, 10cm culture dish cell rinsed 2 times using PBS and harvested cell pellets. The pellets were suspended in 1ml buffer A (10mM Hepes pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, and 1mM dithiothreitol) and incubated on ice for 15min. After incubation, added 15μl 10% NP-40 and centrifugation at 12,000g for 5min. The supernatant was removed and the pellet was resuspended in 200μl buffer C [20mM Hepes pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM ditiothreitol, and protease inhibitor cocktail (Roche applied science, Indianapolis, USA)] by vortexing for 5s, and then shaking incubated 4℃ for 30min. After centrifugation at 12,000g for 10min on 4℃, the supernatant was collected and quantified protein concentrations with the Bradford's reagent (Sigma-Aldrich, St. Louis, USA)

4. Western blot analysis

For western blot analysis, H9c2 cells were rinsed with ice-cold PBS and scraped on ice into lysis buffer that contained 50mM Tris-Cl (pH8), 120mM NaCl, 1mM EGTA, 1mM EDTA, 0.5% NP-40, protease inhibitor cocktail (Roche applied science). The cell lysates were then shaking for 30 min in 4℃. Cell debris were removed by centrifugation (12,000g for 15 min in 4℃), the supernatant was collected and quantified protein concentrations with the

Bradford's reagent (Sigma-Aldrich). Protein samples were boiled for 5min in Laemmli sample buffer, fractionated by SDS-polyacrylamide gel in a 12% polyacrylamide gel and electrotransferred onto PVDF membrane (Millipore Corporation, Bedford, USA). Membrane were incubated 2h at 4℃ in a blocking solution of 5% non-fat powdered milk in T-phosphate-buffered saline (phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20). p53, NQO1, Nrf2 (Santa-Cruz biotechnology, CA, USA), cleaved caspase3 (Cell signaling) and β-actin (Sigma-aldrich) antibodies were used as primary antibodies, diluted 1:1000. Horseradish peroxidase-conjugated goat anti-mouse IgG, anti-rabbit IgG and anti-goat IgG (Santa-Cruz biotechnology, CA, USA) were used as secondary antibodies (at dilution of 1:5000). Development of the western blot was performed using an enhanced chemioluminisence western blotting analysis system (Animal Genetics, Inc., South Korea). To confirm p53 involvements, we used pifithrin-α (PFT-α) (Chemicon, MA, USA), as a p53 inhibitor at the same time with palmitic acid.

5. DNA fragmentation

After treating with glucose (5mM or 30mM) with or without 250μM palmitic acid for 18 h, H9c2 cells were harvested into ice-cold PBS, and an equal volume of 2X lysis buffer (200 mM HEPES [pH 7.5], 2% Triton X-100, 400 mM NaCl, 20 mM EDTA [pH 8]) was added. The cells were then

incubated on ice for 30 min and microcentrifuged at maximum rpm for 15 min at 4°C. After removing the supernatant, the cells were treated with 0.1 mg/ml RNase A for 1 h at 37ºC and then with 0.2 mg/ml proteinase K for 3 h at 56ºC. Genomic DNA was then extracted with phenol/chloroform and dissolved in TE buffer (10 mM Tris–Cl, 1 mM EDTA, pH 8.0). DNA fragmentation was analyzed by electrophoresis in 1.8% agarose gels.

6. Detection of ROS

Accumulation of ROS in H9c2 cells treated with normal glucose, high glucose and palmitic acid (for 3hrs) was monitored using the fluorescence-generating probe H_2DCFDA (Invitrogen, Oregon, USA). Cells ($2X10^5$ cells/60mm dish) were treated with 5μM H_2DCFDA. After 30-min incubation at 37°C, cells were examined under a fluorescence microscope set at 488 nm for excitation and 530 nm for emission, and sorted by flow cytometry.

7. Cell survival assays.

H9c2 cells were dispensed in 24-well plates at a density of $5X10^4$ cells/well. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay was used to determine cell viability following the manufacturer's protocols. The MTT labeling reagent (5 mg/ml MTT in PBS) was added to the culture to a final concentration of 0.5 mg/ml, and the culture

was incubated for 4 hrs. After discard of media 250µl DMSO was added to stained cells to solubilize the purple crystals at a 1:1 ratio, and 200µl samples moved to 96-well microplate. Absorbance of the samples was read using a microplate reader at 490 nm.

8. Chromatin immunoprecipitation assay

H9c2 cells were grown in 150-mm dishes and treated normal glucose, high glucose and palmitate (for 18hrs). The cells were fixed using 1% formaldehyde in medium for 10 min at room temperature. The cells were treated with 0.125M glycine for 5 min to stop fixation and washed twice with ice-cold PBS. The cells were scraped in PBS, collected by centrifugation, and lysed in lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, 1 mM PMSF supplemented with protease inhibitors). After centrifugation, nuclear pellet was resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.8% SDS, 1 mM PMSF supplemented with protease inhibitors) and sonicated for four cycles (30-s pulse and 30-s rest on ice). The sonication conditions were optimized to determine generation of DNA fragments between 500 and 1000 base pairs in length. Sheared chromatins were immunocleared with protein A/G-agarose slurry (Santa-Cruz biotechnology, CA, USA) for 1 h at 4 °C. A portion of the precleared chromatin was stored and labeled as “input DNA.” The remaining chromatin

was immunoprecipitated with IgG (control), p53 antibody. The immunoprecipitates were washed sequentially with wash low salt buffer; with high salt buffer; with LiCl buffer; and TE buffer for 5min each. Protein-DNA complexes were resuspended in TE buffer for reversal of cross-links by heating at 65 °C overnight. Then, DNA was purified using proteinase K treatment at 50 °C for 3 h followed by phenol extraction and ethanol precipitation. PCR was performed using 1:100-diluted inputDNA and 3μl of immunoprecipitated DNA from a 30μl sample. The association of p53 with endogenous NQO1 promoter ARE region in H9c2 cells was measured by PCR on immunoprecipitated chromatin using the following primers spanning the NQO1 promoter containing AREs: F;5'-GCAGTTTCTAAGAGCAGAATC-3' R; 5'-TTAGTCCTTGGTCAGATGTGG-3'. The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

9. Annexin V-PI staining

Early apoptosis and necrosis were identified by means of double fluorescence staining with Annexin V-FITC and propidium iodide(PI) stainings. Apoptotic cells translocate phosphatidylserine from the inner site of the plasma membrane to the outer surface while the membrane remains physically intact. Apoptotic cells were therefore stained with Annexin V-FITC, which binds with high affinity to phosphatidylserine, resulting in a red

fluorescence when excited at 450− 480 nm, and exclude PI, a DNA dye unable to cross the plasma membrane. Necrotic cells lose the physical integrity of their plasma membrane and therefore stained with both annexin V-FITC and PI. Cells that were neither apoptotic nor necrotic were not stained with either dye. Samples (5×10^5 cells per sample) were double stained according to the manufacturer's instructions, and immediately analyzed on a FACScan (Becton Dickinson Heidelberg, Germany).

III. RESULTS

1. Combined treatment with high glucose and palmitate induces apoptosis in H9c2 cells.

H9c2 cells were treated with 5 or 30 mM glucose alone or together with 250 μM palmitic acid for 18 h. High glucose treatment alone did not decrease cell viability (Fig. 1A) or induce apoptosis (Fig. 1B, C, D). However, palmitate in combination with either normal or high glucose reduced cell viability, as assessed by MTT (Fig. 1A), and increased apoptosis, as measured by both DNA fragmentation (Fig. 1B) and annexin V-propidium iodide (PI) staining (Fig. 1C and D). The combination of high glucose and palminate was particularly effective, significantly reducing viability ($P < 0.001$) and promoting the most marked apoptosis.

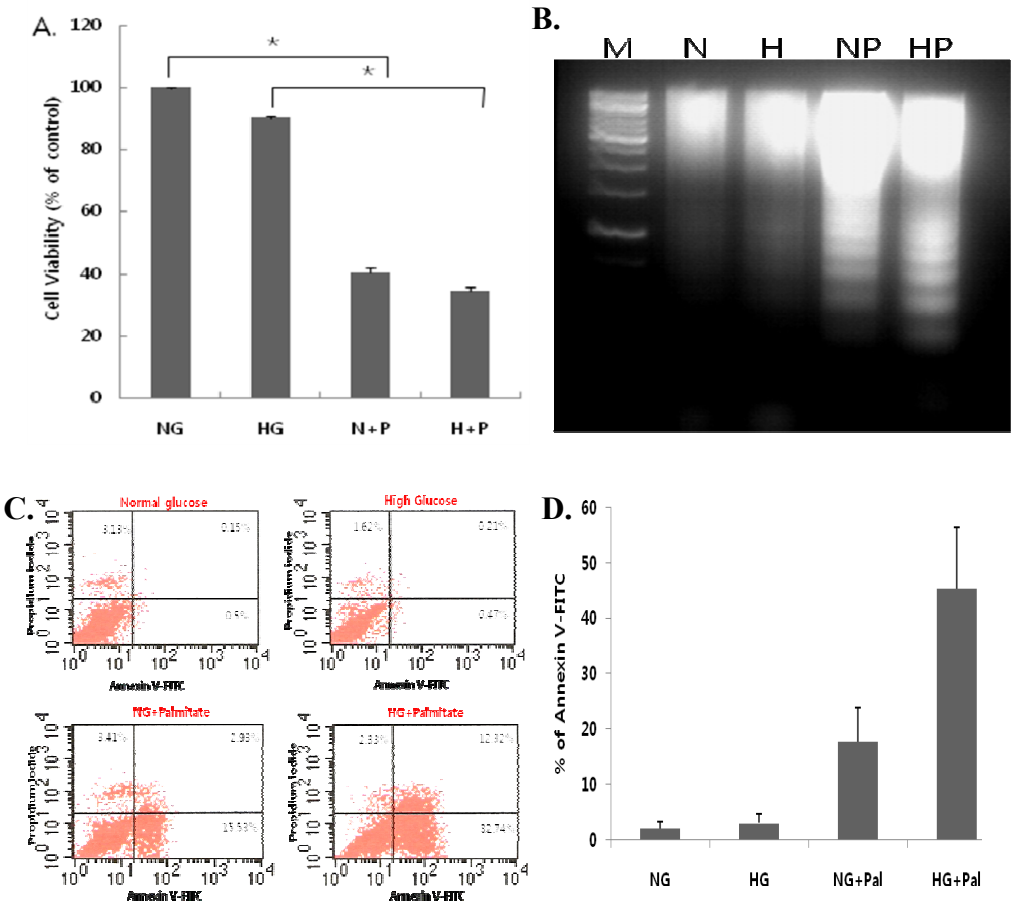

Fig 1. Combined treatment with high glucose and palmitate (glucolipotoxicity) induces apoptosis in cardiomyocytes. H9c2 cells were treated with 5 or 30 mM glucose alone or in combination with 250 μM palmitate for 18 h. High glucose (HG or H) combined with palmitate (A) reduced cell viability, as measured by MTT assays (***$P < 0.001$), and (B-D) increased apoptosis, as measured by DNA fragmentation (B) and annexin V-PI staining (C, D). High glucose alone did not affect viability or apoptosis. The data are presented as means ± SEs of three independent experiments.

2. Combined treatment with high glucose and palmitate generates intracellular ROS.

To investigate the generation of intracellular ROS, we incubated H9c2 cells for 3 h in medium containing normal glucose (5 mM), high glucose (30 mM), or 250 μM palmitic acid alone, or with a combination of glucose (low and high) with 250 μM palmitic acid. After 3 h, cells were incubated with the fluorescent ROS probe, H_2DCFDA, for 30 min, and fluorescence was detected by flow cytometry (Fig. 2A) and fluorescence microscopy (Fig. 2B). As shown in Figure 2A, 250 μM plamitate alone significantly increased ROS levels in H9c2 cells compared with 5 mM glucose, whereas 30 mM glucose alone did not. Under glucolipotoxic conditions (30 mM glucose and 250 μM palmitate), ROS generation was increased 2.2-fold compared with normal glucose ($P < 0.05$). Thus, of all incubation conditions used, the combination of high glucose and palmitate induced the greatest increase in ROS production.

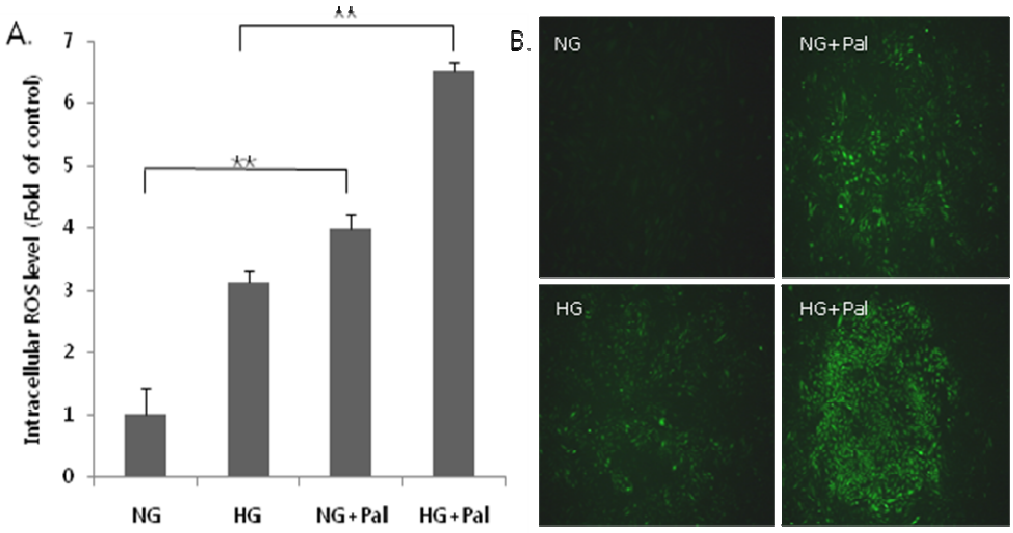

Fig 2. Glucolipotoxicity increases ROS content in cardiomyocytes. Total ROS content in H9c2 cells was assayed using the fluorescent ROS probe, DCF-DA, after a 3-h exposure to palmitate and glucose. ROS fluorescence was measured by (**A**) flow cytometry and (**B**) fluorescence microscopy. Note that palmitate (Pal) with high glucose (HG) led to a significant increase in ROS (*$P < 0.05$). The data are presented as the mean (±S.E) of three independent experiments.

3. Glucolipotoxicity upregulates p53, Nrf2 and cleaved caspase-3, and downregulates NQO1 expression.

Whole-cell lysates were used for detection of p53, NQO1 and cleaved caspase-3 by western blotting, and nuclear extracts were used for Nrf2 detection. As shown in Figure 3, p53 levels were increased approximately 4-fold in cells incubated with palmitate and high glucose compared to cells in normal glucose ($P < 0.001$), and cleaved caspase-3 levels were increased about 4.5-fold ($P < 0.001$). Nrf2 expression was also increased in the presence of high glucose plus palmitate; but interestingly, expression of the Nrf2 target, NQO1, was decreased ($P < 0.05$). These results demonstrate that glucolipotoxicity increased p53 and Nrf2 expression but reduced expression of NQO1.

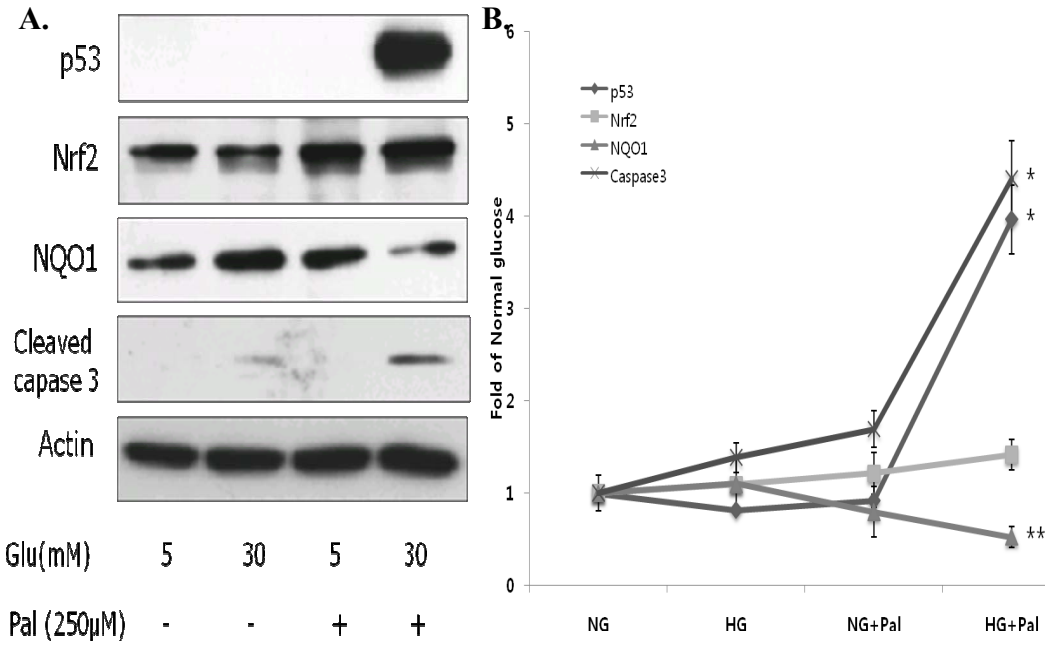

Fig 3. Glucolipotoxicity differentially affects expression of p53, Nrf2, and NQO1 proteins, and cleaved caspase-3 levels. H9c2 cells were exposed to glucose and palmitate for 18 h, and p53, Nrf2, NQO1 and cleaved caspase-3 levels were determined by western blotting. p53, cleaved caspase-3, and Nrf2 protein levels were increased only under high glucose (30 mM) plus palmitate (250 μM) conditions. Although Nrf2 levels were increased, the levels of its target, NQO1, were decreased. The data are presented as means ± SEs of three independent experiments.

4. p53 interacts with the ARE region of the NQO1 gene promoter.

It was recently reported that p53 binds to the ARE region of the promoters of several antioxidant enzymes in mouse hepatocarcinoma cells[13]. To determine whether p53 interacts with endogenous ARE-containing regions of the *NQO1* promoter in H9c2 cells under glucolipotoxic conditions, we used ChIP assays. As shown in Figure 4, anti-p53 antibodies immunoprecipitated the *NQO1* promoter only from chromatin prepared from H9c2 cells incubated with high glucose and palmitate, indicating that p53 interacts with this region under glucolipotoxic conditions. Because Nrf2 also binds to ARE regions of antioxidant enzymes, including NQO1, these results imply that p53 competition for these sites could modulate Nrf2-mediated induction of NQO1.

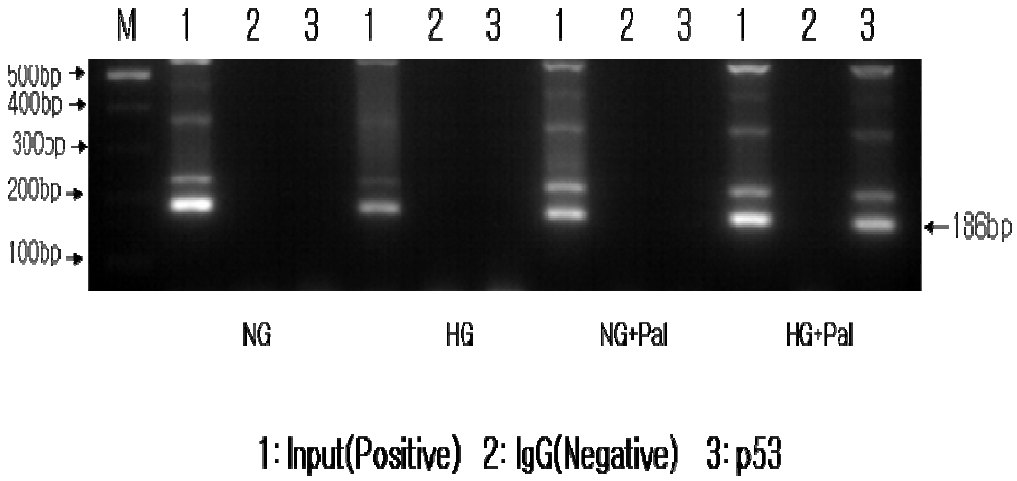

Fig 4. p53 interacts with the *NQO1* gene promoter. p53 binding to the ARE-containing region of the endogenous *NQO1* promoter was determined using ChIP assays. Chromatin prepared from H9c2 cells treated with high glucose and palmitate was immunoprecipitated with mouse IgG or p53 antibodies, as indicated. Immunoprecipitated chromatin was amplified by PCRs using a primer pair bracketing the ARE-containing proximal promoter region (-379 to -533) of the rat *NQO1* gene (NQO1[ARE]).

5. The p53 inhibitor, pifithrin-α, protects H9c2 cells from glucolipotoxicity-induced apoptosis.

To investigate the involvement of p53 in this apoptosis pathway, we examined the effects of pifithrin-α (PFT-α), a p53 inhibitor. Whole-cell lysates were prepared from H9c2 cells co-treated for 18 h with 100 μM PFT-α, 30 mM glucose, and 250 μM palmitate. Apoptosis and viability were analyzed by western blotting (Fig. 5A) and MTT assays (Fig. 5B), respectively. The increases in p53 expression and cleaved caspase-3 levels induced by combined exposure to palmitate and high glucose were prevented by co-treatment with PFT-α (Fig. 5A). PFT-α also partially, but significantly, reduced the level of apoptosis induced by glucolipotoxic conditions (Fig. 5B). Note that PFT-α did not affect cell viability when added to cultures containing normal or high glucose alone. Interestingly, PFT-α markedly increased NQO1 expression compared to palmitate-treated cells (Fig. 5A), suggesting that endogenous expression of NQO1 is highly sensitive to p53. These results further suggest that p53 acts through inhibition of NQO1 to play a key role in glucolipotoxicity-induced apoptosis.

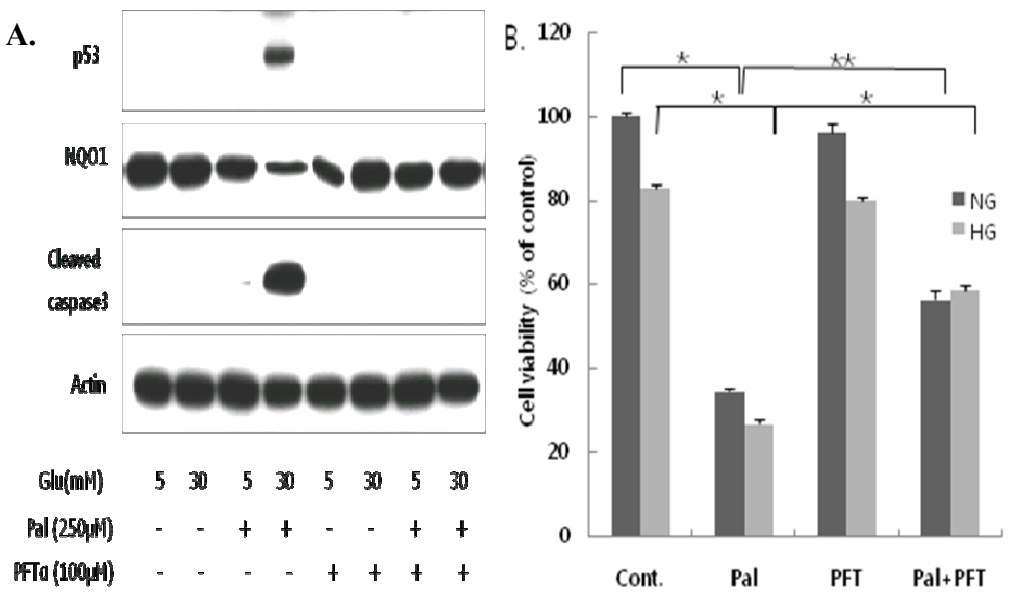

Fig 5. Inhibition of p53 with PFT-α protects H9c2 cells from glucolipotoxicity-induced apoptosis. H9c2 cells were incubated with high glucose (30 mM) and palmitate (250 μM) in the absence and presence of PFT-α (100 μM) for 18 h. (A) Cells were harvested for western blot analysis of p53, NQO1, and cleaved caspase-3. (B) Cell viability was assessed by MTT assays. PFT-α increased survival in H9c2 cells incubated with both high glucose and palmitate (***$P < 0.001$, *$P < 0.05$). Cells treated with normal or high glucose only were unaffected by PFT-α.

6. PFT-α inhibits glucolipotoxicity-induced intracellullar ROS generation.

To investigate the effect of PFT-α on glucolipotoxicity-induced intracellular ROS generation, we incubated H9c2 cells for 3 h with glucose alone or in combination with palmitate in the absence and presence of 100 μM PFT-α. The increased levels of ROS induced by high glucose ($P < 0.05$, high vs. low glucose) or high glucose and palmitate ($P < 0.001$, Pal vs. Cont), which were similar to those shown in Figure 1, were significantly reduced by PFT-α (Fig. 6). These results suggest that p53 plays a role in mediating the glucolipotoxicity-induced elevation of intracellular ROS levels.

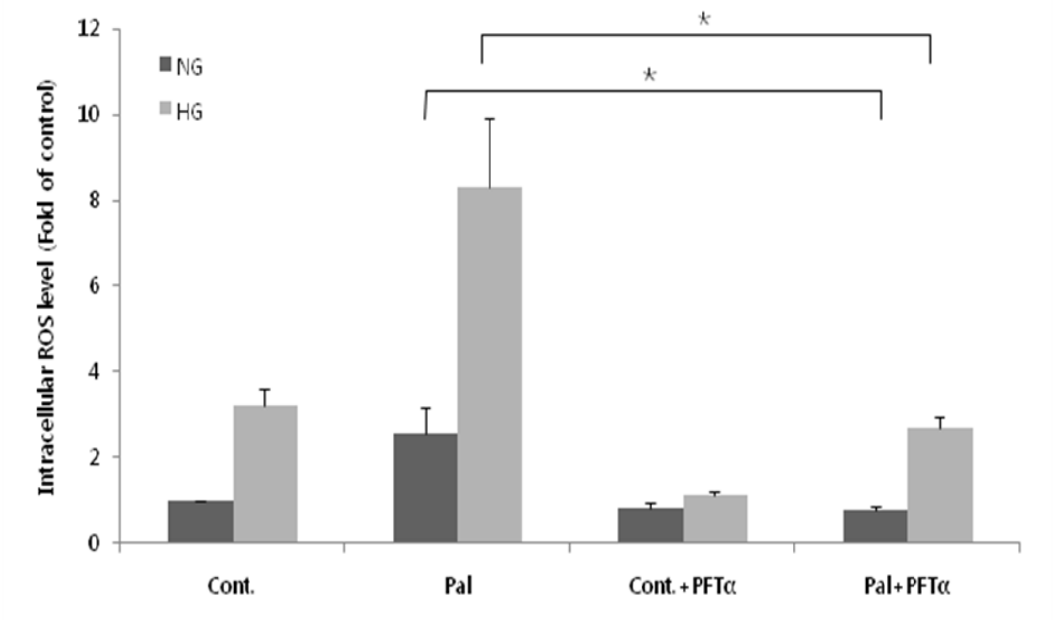

Fig 6. Inhibition of p53 with PFT-α attenuates glucolipotoxicity-induced intracellular ROS generation. Total ROS content in H9c2 cells was assayed after a 3-h exposure to glucose alone or glucose together with palmitate in the absence and presence of PFT-α using the fluorescent ROS probe, DCF-DA. Fluorescence due to intracellular ROS was measured by flow cytometry. The increase in ROS induced by palmitate (Pal) and high glucose (HG) was significantly inhibited by PFT-α (***$P < 0.001$). The data are presented as means ±SEs of three independent experiments.

7. Scavenging of ROS by N-acetyl-L-cysteine decreases glucolipotoxicity-induced p53 expression and normalizes NQO1 levels.

To investigate the relationship between p53 expression and ROS, we repeated the experiments shown in Figure 4 in the presence of the ROS scavenger, N-acetyl-L-cysteine (NAC), preincubating with NAC for 1 h and incubating with normal/high glucose with or without palmitate for 3 h. As shown in Figure 7A, NAC markedly attenuated the increase in ROS levels induced by high glucose and palmitate. As expected, glucolipotoxic conditions increased p53 expression and cleaved caspase-3 levels, and decreased NQO1 expression. Co-treatment with NAC under these conditions significantly decreased p53 expression and cleaved caspase-3 levels; it also completely restored NQO1 expression (Fig. 7B), which had been reduced to undetectable levels in the absence of NAC. Similar effects of NAC on caspase-3 levels and NQO1 expression were observed in cells cultured with the combination of low glucose and palmitate (Fig. 7B), whereas only minimal and variable effects on p53 expression were observed under these conditions. NAC also improved cell viability in the presence of palmitate, but did not affect the viability of cells treated with normal or high glucose alone (Fig. 7C). Taken together, these results suggest that increased p53 expression might interfere with Nrf2-mediated induction of ARE-dependent NQO1 expression.

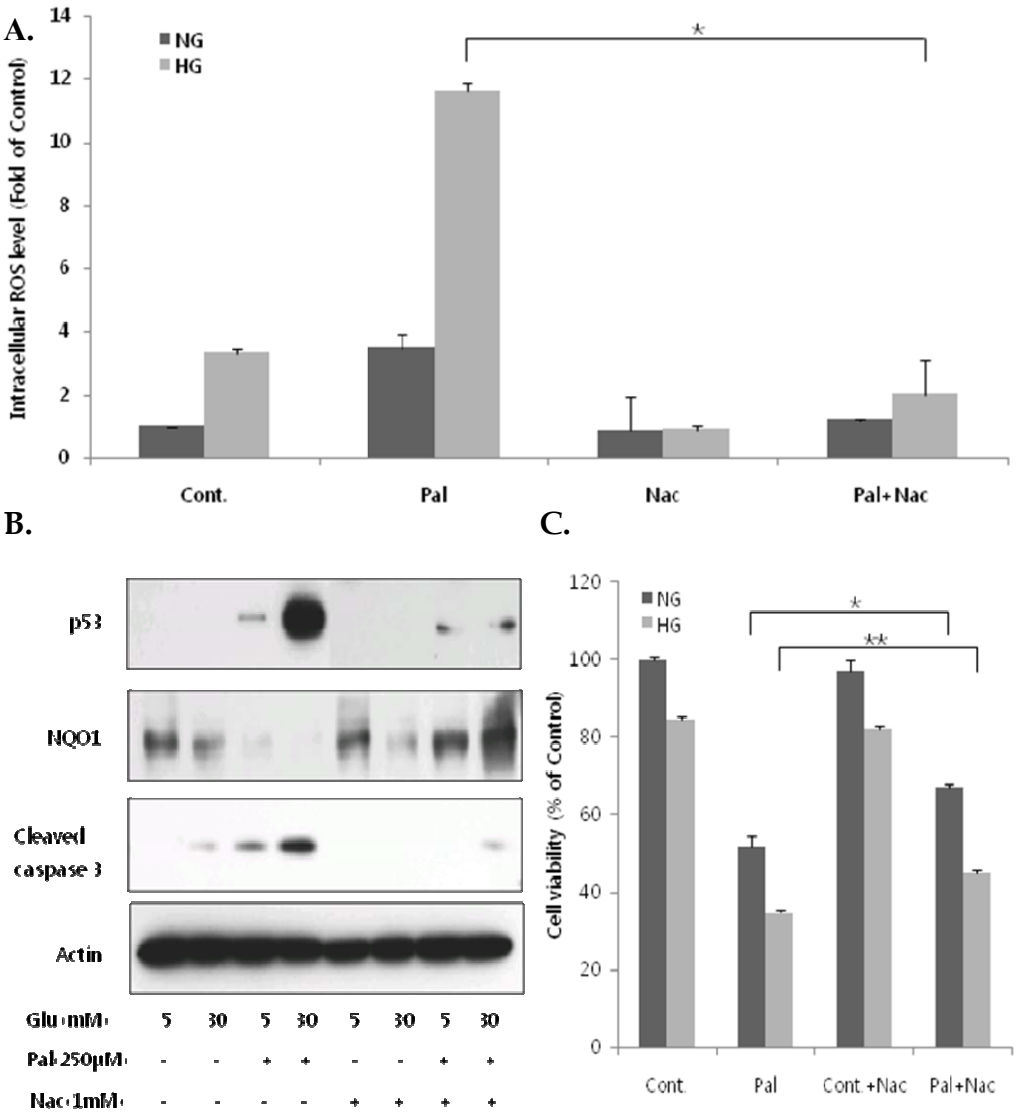

Fig 7. NAC decreases glucolipotoxicity-induced p53 expression and normalizes NQO1 levels. H9c2 cells were preincubated with 1 mM NAC for 1 h and then incubated with normal (5 mM) or high (30 mM) glucose alone or together with 250 μM palmitate for 18 h, and then assayed by western blotting and MTT assay. For ROS experiments, cells were incubated as described, except that the final incubation was 3 h instead of 18 h on the same conditions above. (**A**) NAC significantly attenuated elevated ROS levels induced by high glucose and palmitate. (**B**) NAC significantly inhibited glucolipotoxicity-induced increases in p53 expression and

cleaved caspase-3 levels, and increased NQO1 expression. (**C**) NAC increased the viability of cells incubated with low or high glucose in the presence of palmitate (***$P < 0.001$, *$P < 0.05$). The data are presented as means ± SEs of three independent experiments.

IV. DISCUSSION

Chronic hyperglycemia and hyperlipidemia in patients with diabetes are known to generate ROS, leading to apoptosis of many cell types, including pancreatic β-cells[17] and cardiomocytes[18]. In the present study, we focused on glucolipotoxicity-induced apoptosis in cardiomyocyte and the underlying apoptotic pathways. Our results demonstrated that glucolipotoxicity-induced apoptosis of H9c2 cells was mediated by stimulation of a pro-apoptotic pathway involving both increased generation of ROS and upregulation of p53 and caspase-3, as well as by suppression of an anti-apoptotic pathway involving competitive binding of p53 to ARE-containing promoter regions required for Nrf2-mediated induction of *NQO1*. Three aspects of our results deserve highlighting.

First, our finding that glucotoxicity or lipotoxicity alone are partially capable of inducing apoptosis in H9c2 cardiomyoblast cells (Figs. 1, 2, 3 and 7) is consistent with some previous reports however, there is some controversy about whether glucotoxicity alone is capable of inducing apoptosis, especially in cardiomyocytes[1, 18-19]. On the other hand, palmitate-induced lipotoxicity has been found to induce apoptosis in cardiomyocytes in all reports[1, 19-23]. One of these studies reported that glucotoxicity and lipotoxicity synergized in inducing apoposis[19], but another did not[1]. In other systems, notably pancreatic β-cells, interacting glucotoxicity and lipotoxicity pathways also synergize to induce apoptosis[24]. In the current study, we found that high

glucose combined with palmitic acid consistently caused apoptosis (Figs. 1, 3, 5 and 7), and clarifying the concept of synergistic glucolipotoxicity in H9c2 cardiomyoblasts. Our observations that high glucose in the presence of palmitic acid induced apoptotic cell death and increased intracellular ROS levels in H9c2 cells, taken together with the finding that NAC reduced ROS levels and attenuated apoptosis (Fig. 7A and C), suggest that glucolipotoxicity-induced apoptosis was triggered by intracellular ROS generation. By extension, our results imply that there is a threshold of intracellular ROS level which is a starting point of apoptosis.

Second, in concordance with previous reports, we found that glucolipotoxicity induced intracellular ROS generation, resulting in increased expression of p53 and higher levels of cleaved caspase-3 in H9c2 cells. The tumor suppresser protein, p53, is a transcription factor that is well known to promote either cell-cycle arrest or apoptosis[25-26]. In addition, a number of studies have shown that elevated levels of ROS are capable of inducing p53 expression by producing DNA damage[20-21]. Interestingly, p53 expression was not affected by glucotoxicity or lipotoxicity alone, but was markedly increased by glucolipotoxicity (Figs. 3 and 5), suggesting that high levels of ROS may be required to induce p53 expression. The finding that treatment with NAC concomitantly abrogated both p53 expression and intracellular ROS levels hints at an important pro-apoptotic pathway linking p53 and ROS in this cardiomyoblast cell line. Evidence that this represents a reciprocal pathway

(i.e., p53 affects on ROS) is provided by the observation that inhibition of p53 activity by PFT-α markedly lowered intracellular levels of ROS (Fig. 6). This mechanism is consistent with previous reports that p53 transcriptionally activates a specific subset of genes encoding ROS-generating enzymes[27-28] and/or inhibits antioxidant enzymes such as NQO1. These reports and our results together suggest the existence of a positive feedback loop in which glucolipotoxicity-induced intracellular ROS generation stimulates p53 expression, which in turn induces an accumulation of ROS. Collectively, the effects of NAC and PFT-α on ROS levels, p53 expression, and cleaved caspase-3 levels described here (Fig. 6 and 7) indicate that p53-dependent mechanisms may play an important role in the development of apoptosis in cardiomyocyte on the glucolipotoxicity condition.

Third, we investigated the role of inhibition of the Nrf2-NQO1 signaling pathway, which is an important defense mechanism against oxidative stress[29], in glucolipotoxicity-induced, p53-mediated apoptosis in rat cardiomyoblasts. Once dissociated from its negative regulator Keap1, Nrf2 activates antioxidant genes, such as those for NQO1, glutathione S-transferase and r-glutamylcysteine synthase, by binding to ARE regions in the promoters of the corresponding genes[13]. In this study, Nrf2-dependent activation of the antioxidant gene, *NQO1*, was essentially abolished by upregulation of p53 (Figs. 3, 5 and 7). It is known that Nrf2 and Keap1 expression are not affected by p53 overexpression or by endogenously induced p53[13]. Instead,

our ChiP assays confirm that p53 binds to ARE regions of the *NQO1* promoter (Fig. 4), as previously reported[13]. Thus, despite upregulation of Nrf2 by glucolipotoxicity, p53 apparently represses Nrf2 transcriptional activity by displacing NRf2 bound to the *NQO1* promoter, resulting in the near-abrogation of NQO1 expression, diminished antioxidant activity, and further ROS-/p53-dependent apoptosis. However, additional p53- and ROS-independent apoptotic pathways also contribute to the observed cardiomyocyte cell death because NAC and PFT-α did not fully abolish glucolipotoxicity-induced apoptosis.

In summary, high glucose (30 mM) in the presence of palmitic acid (250 μM) induced apoptotic cell death in cardiomyocytes. This glucolipotoxicity-induced apoptosis was p53-dependent and was mediated through a dual mechanism: ROS-dependent stimulation of pro-apoptotic signaling through the caspase cascade, and inhibition of the anti-apoptotic Nrf2-NQO1 signaling pathway**.**

V. CONCLUSION

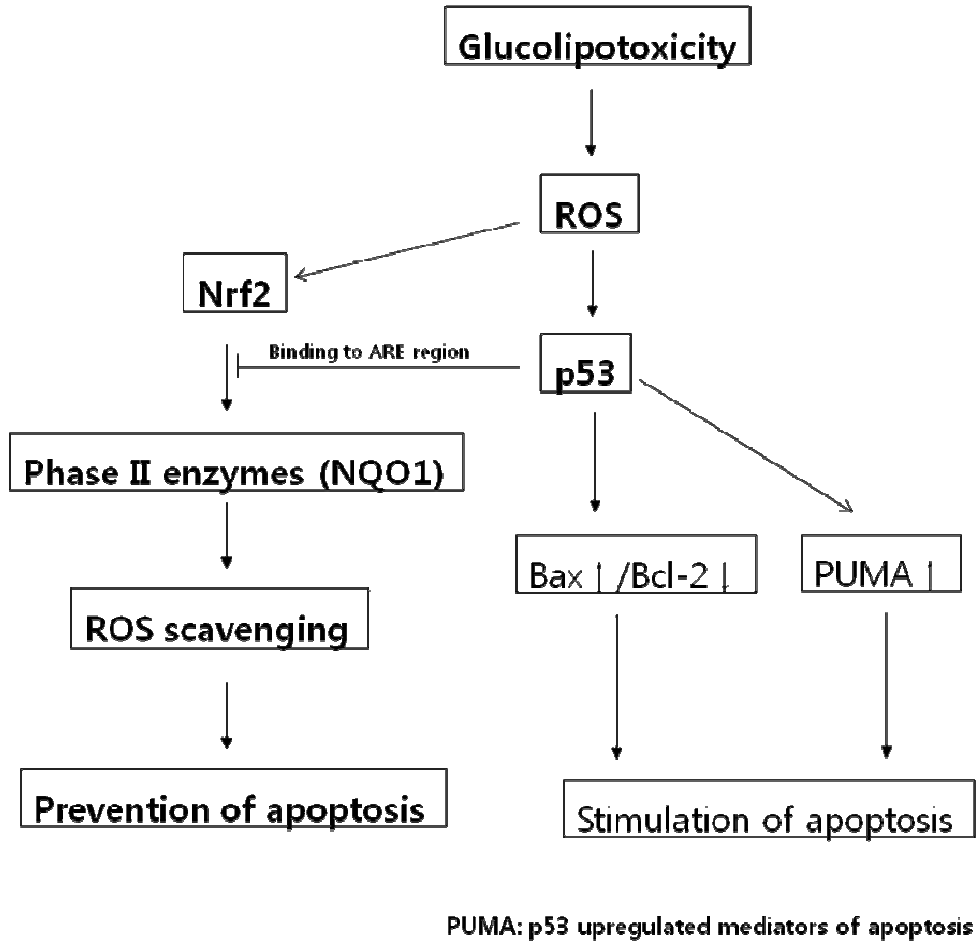

Fig 8. Model of p53-dependent apoptosis in H9c2 cells via inhibition of the anti-apoptotic Nrf2-NQO1 pathway. The glucolipotoxicity caused by combined exposure to high glucose (30 mM) and palmitic acid (250 μM) generates ROS, which increase p53 and Nrf2 levels. Induction of Nrf2, a positive transcriptional regulator of ROS-scavenging phase II enzymes, leads to a reduction in ROS levels and thereby prevents apoptosis. However, p53 interrupts Nrf2 transcriptional activity by binding to ARE regions. Hence, ROS accumulate and then lead to stimulation of apoptosis by p53.

To identify the apoptosis pathway by palmitic acid and high concentrated glucose induced glucolipotoxicity, we performed several experiments, and we found p53 involved pathway.

We obtain the following results.

1. ROS generation by palmitate and high concentrated glucose increases p53 expression, and Nrf2 was interrupted transcriptional activity by p53, therefore NQO1 which is a representative of phase II enzymes was decreased.

2. We could reduce apoptosis of H9c2 cells by treatment with pifithrin-α as a p53 inhibitor on glucolipotoxicity conditions.

3. We demonstrated that p53 was increased by ROS. When ROS was scavenged by N-acetyl-L-cystein (NAC), p53 was decreased and cell viability was increased.

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<ABSTRACT (IN KOREAN)>

종양 억제 p53 경로를 통한 rat 심근배아세포의 당지방독성에 의한 세포자살

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고혈당은 자유지방산과의 상승작용으로 활성산소(ROS)를 생성하고 세포자살을 일으킨다. 이번 실험에서 우리는 palmitic acid(C16:0)와 높은 당의 당지방독성 효과를 rat의 심근세포주인 H9c2 를 이용하여 실험하였고 종양 억제 p53 과 관련된 세포자살 경로에 초점을 맞추었다. H9c2 세포에 5mM normal glucose, 30mM high glucose와 250 μM palmitic acid를 각각 처리하거나 섞어서 처리하였다. 세포의 생존과 자살은 각각 MTT assay와 western blot을 이용한 cleaved caspase 3 를 통해 확인하였고 ROS의 생성 여부는 H_2DCFDA로 염색 후 FACS analysis로 확인하였다.

30mM high glucose와 250μM의 palmitat를 함께 처리 했을 때에만 p53 과 cleaved caspase 3 의 발현이 증가하는 것을 확인 할 수 있었고 5mM glucose condition과 비교 했을 때 ROS 생성이 2.5 배 증가하는 것을 확인 할 수 있었다. p53 억제제인 pifithrin-α를 처리 한 경우 당지방독성에 의해 증가된 ROS와 p53 발현이 모두 감소하는 것을 확인 할 수 있었다. 250μM palmitic acid가 처리된 경우, Nrf2 의 발현이 증가되는 반면 NQO1 의 발현은 감소하는 것을 확인하였고 pifithrin-α를 처리 하였을 때 NQO1 의 발현이 다시 증가하는 것을 볼 수 있었다. 염색체 면역 침강법을 이용하여 p53 이 NQO1 의 ARE 지역과 결합하는 것을 확인하였다. Rat 심근세포주에서 당지방독성에 의해 ROS가 증가하고 증가된 ROS에 의해 p53 의 발현이 증가하였다. 증가된 p53 은 Nrf2 의 작용을 방해함으로써 NQO1 의 발현을 막아 다시 ROS를 생성하게 되면서 결과적으로 세포자살 억제 경로를 막게 된다. 이번 실험에서 우리는 증가된 p53 이 원래 자신의 세포자살 활성 능력과 함께 Nrf2 의 세포자살 억제경로를 방해하는 작용도 같이 함을 보였다.

핵심 단어: p53, NQO1, 당지방독성, ARE region, 활성산소종 (ROS), palmitate, 고혈당, 세포자살