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SHORT COMMUNICATION

Activator protein-1 and caspase 8 mediate p38α MAPK-dependent cardiomyocyte apoptosis induced by palmitic acid

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Abstract

Lipoapoptosis of cardiomyocytes may underlie diabetic cardiomyopathy. Numerous forms of cardiomyopathies share a common end-pathway in which apoptotic loss of cardiomyocytes is mediated by p38α mitogen activated protein kinase (MAPK). Although we have previously shown that palmitic acid (PA), a saturated fatty acid (SFA) elevated in plasma of type 2 diabetes mellitus and morbid obesity, induces apoptosis in cardiomyocytes via p38α MAPK-dependent signaling, the downstream cascade events that cause cell death remain unknown. The objective of this study was to investigate mechanisms involved in palmitic acid-induced cardiomyocyte apoptosis. Human adult ventricular cardiomyocyte line (AC16 cells) exposed to high physiological levels of PA for 16 h showed enhanced transcription and phosphorylation of c-fos and c-jun subunits of AP-1 and transcription of caspase 8. When AC16 cells were transfected with small interfering RNA specific against p38α MAPK (si-p38α) for 24 or 48 h, the amplified phosphorylation of c-fos was dose-dependently attenuated, and procaspase 8 was dose-dependently reduced. With translational knockdown of c-fos, PA-induced apoptosis was diminished. Inhibition of caspase 8 for 24 h reduced apoptosis in PA-treated cardiomyocytes. These findings provide evidence for induction of apoptosis in cardiomyocytes exposed to high SFA by a novel pathway requiring activation of c-fos/AP-1 and caspase 8. These results demonstrate how elevated plasma SFA may lead to continual and cumulative loss of cardiomyocytes and potentially contribute to the development of diabetic cardiomyopathy.

Keywords P38α · AP-1 · Caspase 8 · Apoptosis · Diabetic cardiomyopathy

Introduction

According to the Center for Disease Control, the number of diabetics increased from 5.6 million to a staggering 20.9 million in the U.S. between 1980 and 2010. When patients with diabetes develop heart failure, mortality rate is ten times higher than among diabetics who remained heart failure-free [1]. Despite its importance, our understanding of how diabetic cardiomyopathy develops remains unclear [2]. Perturbations in myocardial substrate and energy metabolism has been proposed as a potential mechanism. Patients with type 2 diabetes mellitus (T2DM) develop markedly elevated plasma saturated fatty acid (SFA) levels, [3] which may promote cardiomyocyte apoptosis, [4] and lead to the development of cardiomyopathy [5].

Work in our laboratory showed that high physiological concentrations of SFA trigger the p38 mitogen activated protein kinase (MAPK) pathway and amplify the inflammatory response to low level lipopolysaccaride (LPS) exposure [6]. p38α MAPK signaling has also been implicated as being critical to programmed cell death of cardiomyocytes in many cardiomyopathies including the hypertrophic, doxorubicin-induced, and amyloidogenic forms [2, 7–11]. We recently demonstrated that p38α MAPK mediates cardiomyocyte apoptosis induced by the most abundant SFA in plasma,

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palmitic acid (PA) [4]. However, the mechanisms that lead to activation of p38α MAPK, cellular dysfunction and subsequent apoptosis in cardiomyocytes remain unclear.

p38 MAPK associates with and phosphorylates the c-fos subunit of AP-1 transcription factor in response to UV light and boosts gene expression [12]. Activator protein 1 (AP-1) is a dimeric protein complex, formed by association between members of the Jun and the Fos subfamilies of proteins, [12, 13] and is a reasonable putative candidate for mediation of the above PA effect on cardiomyocyte cell death.

Activation of p38 MAPK has been linked to increasing caspase 8 activity and apoptosis in response to stressors such as manganese and sphingosine [14, 15]. AP-1 binds to a c-jun/AP-1 site in the promoter region for caspase 8 and increases its transcription [16]. Procaspase 8 is activated by cleavage by a caspase 8 homologue, cellular Flice Inhibitory Protein (cFLIP), which exists in two forms, long and short. Either can form heterodimers with caspase 8 but, only the long form, $cFLIP_L$, activates caspase 8, whereas the short form, $cFLIP_S$ which lacks the catalytic domain, blocks recruitment and activation of procaspase 8 [17, 18]. Activated p38α MAPK has been shown to block phosphorylation of $cFLIP_S$, but not $cFLIP_L$, thereby promoting caspase 8 activation [17].

In order to probe the p38α MAPK-dependent pathway that leads to PA-mediated cardiomyocyte apoptosis, we knocked down or inhibited putative p38α MAPK substrates, AP-1, specifically c-fos and c-jun subunits, and caspase 8 to determine whether they served critical and indispensable roles in mediating programmed cell death.

Methods

AC16 cells, derived from adult human ventricular cardiomyocytes, were exposed to PA, after treatment with small interfering RNA (siRNA) or specific pharmacological inhibitors, or combinations of these reagents. In the initial experiments, cells were treated with PA for 16 h. For the siRNA knockdown and inhibitor experiments, cells were transfected with siRNA for 24 or 48 h by electroporation or the inhibitor for 24 h, washed in phosphate-buffered saline, and resuspended in fresh media with PA for 16 h. At 24 h, and 48 h post-transfection, as well as at 16 h post-PA treatment, fluorescent microscopy demonstrated high transfection efficiencies (Suppl. Fig. S1). Analysis of gene and protein expression, and mode of cell death was performed using standard laboratory methods or commercially available kits. A detailed "Methods" section is available in the Online Supplement.

Results

PA increased expression and phosphorylation of AP-1 transcription factor family members, c-fos and c-jun

We previously showed that PA-induced apoptosis in cardiomyocytes is attenuated by translational reduction of p38α MAPK [4]. To extend these experiments, we determined the role of PA in activating its substrate, c-fos, and a potential partner, c-jun, the subunits of which the AP-1 transcription factor is composed. Exposure to high PA levels led to dose-dependent increases in c-fos and c-jun mRNA levels (Fig. 1a). Similarly, Western blot analysis showed similar increases in total c-fos and c-jun protein levels in response to the PA treatment. Furthermore, phosphorylated (p-) c-jun and c-fos also increased dose-dependently with PA treatment (Fig. 1b, c). The increase in phosphorylation of c-fos markedly outpaced the increase in total c-fos protein level, and the magnitude of the increase in p-c-fos appeared greater than the increase in p-c-jun (Fig. 1b, c). Confocal microscopy imaging was consistent with dose-dependent increases in c-fos (Fig. 1d, Supplemental Fig. S2), p-c-fos (Fig. 1E, Supplemental Fig. S3), c-jun (Fig. 1F, Supplemental Fig. S4), and p-c-jun (Fig. 1G, Supplemental Fig. S5).

Phosphorylation of c-fos was regulated by p38α MAPK dose-dependently even though total c-fos protein concentration was not significantly altered

To investigate the role of p38α MAPK in mediating the signaling changes that occur in response to PA in cardiomyocytes, AC16 cells were transfected with siRNA specific against p38α (si-p38α), before being exposed to high PA levels, which markedly reduced p38α, without affecting p38β (Supplemental Fig. S6) [4]. Total c-fos levels was not significantly changed with si-p38α knockdown, whereas p-c-fos concentrations declined dose-dependently (Fig. 2a, b). At the highest dose of si-p38α, 120 pmol, the PA-induced increase in p-c-fos was entirely abrogated.

JNK1 MAPK binds to and phosphorylates c-jun, in an analogous role to that of p38α MAPK to c-fos [19]. The expression and phosphorylation of c-jun are also increased under conditions of stress, [20–22] including exposure to PA. However, it is not known whether p38α MAPK plays a direct role in regulating c-jun. We tested whether translational knockdown of p38α MAPK would alter c-jun expression and p-c-jun levels. Interestingly, the reduction in p38α MAPK led to a biphasic p-c-jun response; at a low dose of p38α siRNA, p-c-jun

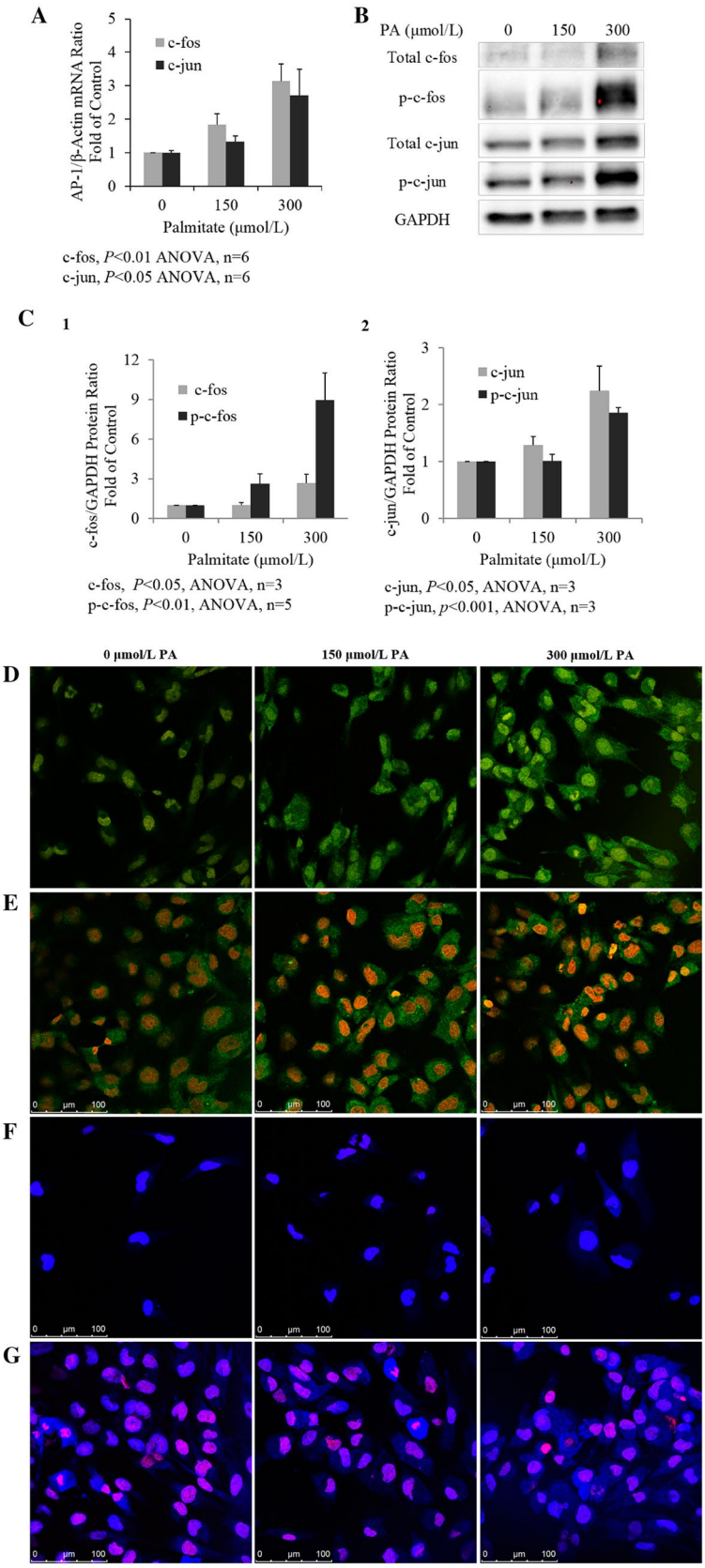


Fig. 1 Palmitic acid up-regulated AP-1 transcription factors, c-fos and c-jun, by increasing transcription, translation, and phosphorylation in a cardiomyocyte cell line. When AC16 cells were treated with 0, 150, or 300 μmol/L PA for 16 h, transcriptions of c-fos and c-jun increased dose-dependently (**a**), as did total and phosphorylated protein concentrations (**c1-2**). A representative Western blot image is shown **b**. PA-treated cardiomyocytes were stained with DAPI for nuclear staining (red), or with Alexa 546 dye (green) for c-fos (**d**) and p-c-fos (**e**), and Alexa 488 dye for c-jun (**f**) and p-c-jun (**g**). qPCR (n = 6 experiments) and Western blot data (n = 3–5 experiments), are presented as mean ± SEM. Statistical significance compared with control was determined by one-way ANOVA

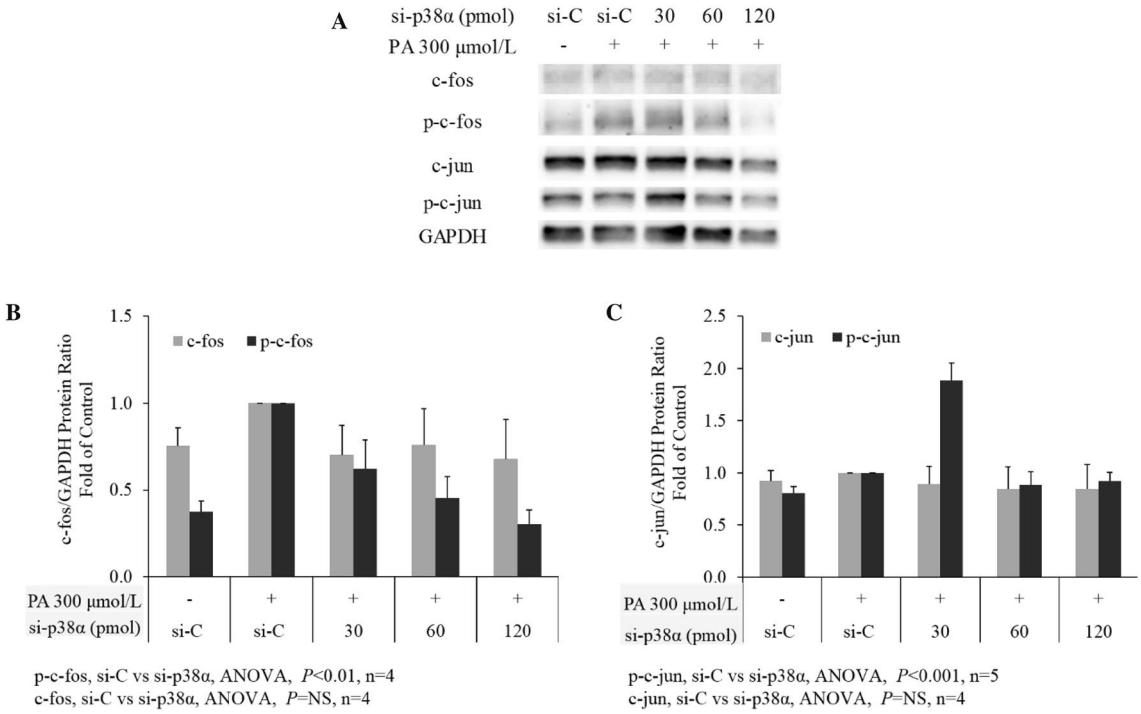


Fig. 2 p38α MAPK specific siRNA significantly reduced p-c-fos without affecting total c-fos levels. Scrambled sequence siRNA (30 pmol si-C) + 300 μmol/L PA was the negative control for experimental groups treated with increasing doses of si-p38α siRNA. Therefore, data from treated groups were calculated as fold change from this control. Translational reduction of p38α MAPK in PA-treated cells produced significant, dose-dependent decreases in p-c-fos without significantly changing total c-fos concentrations (**a, b**). Low dose si-p38α MAPK knockdown (30 pmol) significantly increased p-c-jun, whereas higher doses showed no changes from control. Total c-jun level was unchanged by si-p38α MAPK knockdown (**a, c**)

was increased but this stimulation was not seen at higher si-p38α doses. Similar to total c-fos, total c-jun concentration was unchanged by si-p38α knockdown (Fig. 2a–c).

PA increases caspase 8 transcription but procaspase 8 protein concentration increase was not significant

As the caspase 8 promoter is known to possess an AP-1 binding sequence, [16] we examined whether the PA-induced increases in protein concentrations, and phosphorylations of AP-1 subunits, c-fos and c-jun, resulted in increase in caspase 8 transcription. As expected, high PA treatment induced dose-dependent increase in caspase 8 mRNA expression (Fig. 3a). However, although concentrations of both full length procaspase 8 and cleaved 43 kD increased, these were not significant (Fig. 3b, c). Consistent with this, confocal staining of procaspase 8 did not show a clear change with PA treatment (Fig. 3d). Confocal images also showed that procaspase 8 was restricted to the cytosolic compartment (Fig. 3d, Supplemental Fig. S7).

Translational reduction of p38α MAPK decreased procaspase 8 levels dose-dependently

Prior studies showed that p38 MAPK activation increased caspase 8 activity by releasing it from the inhibition by c-FLIP$_S$ [17, 18]. We sought to determine whether PA exposure also depended on the p38 MAPK regulation of caspase 8. We tested the effect of si-p38α knockdown on procaspase 8 protein concentration. Procaspase 8 level was dose-dependently reduced by translational decrease of p38α MAPK (Fig. 3e, f) suggesting that p38α MAPK activity was necessary to maintain procaspase 8 concentration.

Translational knockdown of c-fos/AP-1 attenuates PA-induced increase in apoptosis of cardiomyocytes

After we established that p38α MAPK regulated phosphorylation of c-fos in PA-treated cardiomyocytes, we examined whether this step was indispensable to PA-induced apoptosis. We blocked c-fos expression by siRNA knockdown, thereby also reducing p-c-fos level, and assessed its effect on Annexin V/PI staining by flow cytometry. PA-induced

apoptosis was significantly reduced by the c-fos specific siRNA knockdown for 24 h or 48 h (Fig. 4a). The result for c-fos knockdown for 24 h was similar to 48 h (Supplemental Fig. S8), therefore data were combined for analysis.

Caspase 8 inhibition attenuated PA-induced increase in apoptosis

Prior studies had suggested that p38α MAPK could increase caspase 8 activity by relieving its inhibition by $cFLIP_S$. Therefore, we tested whether specific, irreversible inhibition of caspase 8 with z-IETD-fmk would block PA-induced cardiomyocyte apoptosis. Indeed, PA-induced cardiomyocyte apoptosis was significantly attenuated by z-IETD-fmk, in magnitude similar to p38α or c-fos knockdown (Fig. 4b).

Translational reduction of p38β enhanced PA-induced apoptosis

Several studies had suggested that p38β MAPK isoform possessed an anti-apoptotic function, in contrast to p38α MAPK [17, 23, 24]. We tested this hypothesis by translational reduction of p38β MAPK. We found that si-p38β knockdown significantly enhanced apoptosis in PA-treated cardiomyocytes (Fig. 4a).

Discussion

Many laboratories have shown that p38 MAPK-dependent signaling mediates apoptosis in response to diverse insults, and may lead to dilated cardiomyopathy [8, 9, 11, 25, 26]. We recently reported that cultured human cardiomyocytes become apoptotic at a higher rate when exposed to high concentrations of PA and that phosphorylated p38α MAPK mediates this.

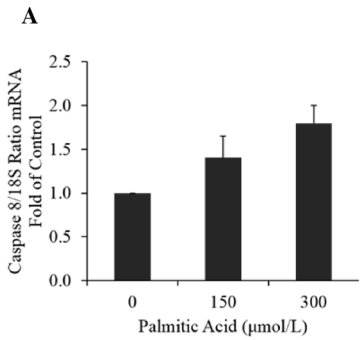
In the present study we demonstrated that PA up-regulated c-fos and c-jun, two members of the AP-1 family, and increased transcription of caspase 8, a protease that initiates the apoptotic program. Furthermore, we showed for the first time, to our knowledge, that suppression of p38α MAPK expression dose-dependently blunted the PA-induced increase in c-fos phosphorylation, but not that of c-jun, and dose-dependently reduced procaspase 8 levels. These findings suggest that phosphorylated c-fos and caspase 8 mediate PA-induced apoptosis in human cardiomyocytes.

Conversely, we also showed that p38β MAPK knockdown amplified PA-induced apoptosis beyond the level seen with PA treatment alone. Consistent with this, prior studies had shown that p38β MAPK lacked p38α MAPK's ability to phosphorylate and recruit $c\text{-}FLIP_S$ [17], and protected against tumor necrosis factor-alpha(TNFα)-induced apoptosis [24]. When it was overexpressed, hydrogen peroxide (H_2O_2)-induced apoptosis was attenuated [23]. Our data confirms the anti-apoptotic role of p38β MAPK in PA-treated cardiomyocytes.

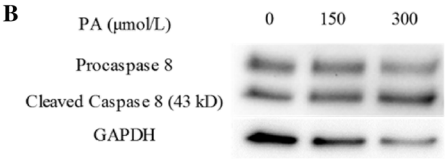
Unlike Jun proteins, Fos proteins do not bind to DNA directly nor form homodimers, so can only exert its effect as part of a heterodimeric AP-1 which binds to DNA via the partner Jun protein [13]. In our experiments, the concentration of c-fos was not changed by suppressing p38α MAPK with siRNA whereas the phosphorylated form, p-c-fos was reduced, which suggests that phosphorylation is a likely mechanism by which p38α MAPK activates c-fos to a proapoptotic function. Previous studies have shown that c-fos possesses many phosphorylation sites and that the effect of phosphorylation varied and was dependent on the specific site [27–29]. For example, the half-life of c-fos was extended to 2 h from the basal 30–45 min when phosphorylated at its extreme C terminus [29]. Given the marked reduction in p-c-fos with the p38α MAPK knockdown, it seems likely that stabilization of p-c-fos, and prolongation of half-life, is taking place via phosphorylation of the extreme C-terminus site. Phosphorylation of the C-terminal transactivating domain, which renders c-fos more active, would also be consistent with out findings. Additionally, given the reduction in p-c-fos and the lack of change in c-fos protein, the ratio of p-c-fos/fos must be decreased with p38α MAPK knockdown. Given these changes, formation of heterodimers that include p-c-fos, that are known to be active [13] is likely reduced. Therefore, the attenuation of apoptosis seen with the p38α MAPK knockdown appears to be mediated by a reduction in AP-1 via reduced p-c-fos as one of its heterodimeric partners.

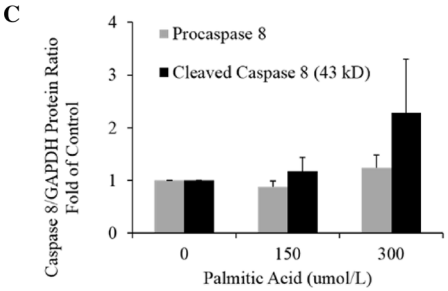
With p38α MAPK knockdown, there was a dose-dependent drop in procaspase 8 protein concentration, suggesting that activated p38α MAPK is required to maintain procaspase 8 levels. Caspase 8 promoter is known to contain a c-jun/AP-1-responsive element mediating elevated transcription [16]. The increase in caspase 8 transcription seen in our study may have been due to the increased binding of the c-fos/c-jun heterodimer to the AP-1-responsive element in the caspase 8 promoter. The data from the present study demonstrate that the increased caspase 8 mRNA expression and presumably increased translation rate, was not matched by increased concentration of the procaspase 8 protein. This suggests that, with p38α MAPK knockdown, the cleavage and activation, or degradation of procaspase 8 in PA-treated cardiomyocytes was even greater in magnitude than the synthesis rate which is likely suppressed. In addition, caspase 8 activity is regulated by other pathways triggered by SFA but not mediated by p38α MAPK so changes seen this study may be due to these [30, 31].

In summary, we demonstrated that c-fos, an AP-1 subunit, and caspase 8 are critical and indispensable factors in PA-induced apoptosis in a human cardiomyocyte cell line.

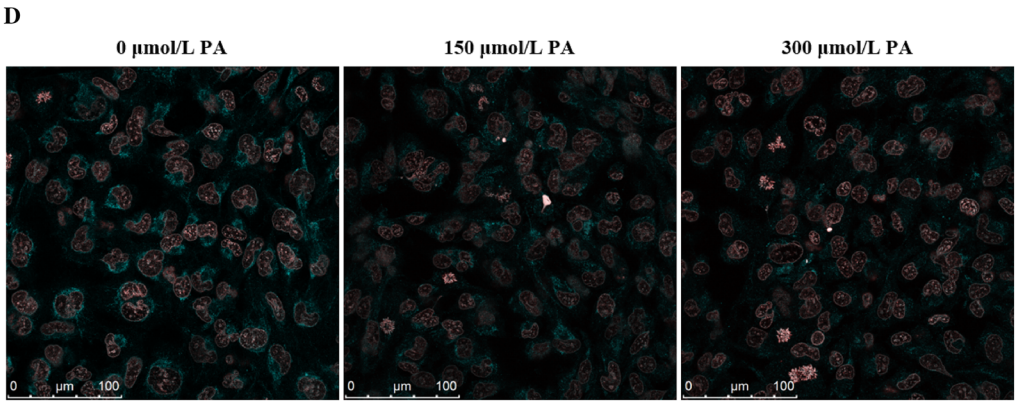


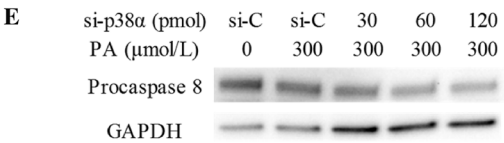
P<0.05 ANOVA, n=5

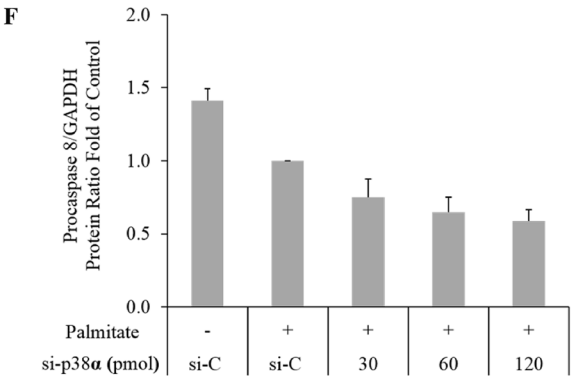




Procaspase 8, *P*=NS, ANOVA, n=5
Cleaved Caspase 8 (43 kD), *P*=NS, ANOVA, n=4







Procaspase 8, si-C vs si-p38α, ANOVA, p<0.05, n=5

◂**Fig. 3** Palmitic acid increased caspase 8 transcription but the increases in full length and cleaved caspase 8 protein concentrations were not significant (**a–c**). Representative images from confocal microscopy of procaspase 8 staining in PA-treated AC16 cells appeared to be consistent with this result (**d**). The images are overlays of nuclei stained with DAPI (red) and procaspase 8 or cleaved caspase 8 stained with Alexa 546 dye (green). siRNA knockdown of p38α MAPK in PA-treated cells produced significant, dose-dependent decreases in procaspase 8 levels (**e, f**). Data from treated groups were calculated as fold change from the si-C + 300 µM PA control group. Representative Western blots (**b, e**), and densitometry quantifications (**c, f**) are shown. Relative caspase 8 mRNA levels were determined by qPCR and relative protein levels by Western blot analysis and presented as mean ± SEM. Statistical significance compared with control was determined by one-way ANOVA

PA induces phosphorylation of p38α MAPK [4] which, in turn, phosphorylates c-fos, and thus may stabilize and prolong its half-life and activity. Phosphorylated c-fos, as a partner in heterodimeric AP-1, likely increases caspase 8 mRNA expression, potentially mediating the maintenance of its concentration by p38α MAPK. Activated p38α MAPK also likely increases caspase 8 activity directly by relieving its inhibition by $cFLIP_S$. We propose that the two parallel pathways converge on caspase 8 and mediate p38α MAPK-dependent PA-induced apoptosis in human cardiomyocytes to which p38β MAPK plays an antagonistic, anti-apoptotic role (Fig. 4).

This study suggests that p38α MAPK, c-fos/AP-1, and caspase 8 may be prime targets for silencing or inhibition to prevent death of cardiomyocytes due to high SFA concentration to prevent or treat diabetic cardiomyopathy.

It also suggests directions for future studies. Upstream of p38 MAPK, preliminary data from our laboratory (not shown) confirms recent reports that PA exposure [32] or high fat diet [33] elevates TXNIP expression and leads to lipotoxic injury. Ischemia/reperfusion injury has been shown to upregulate thioredoxin interacting protein (TXNIP) but inhibition of p38 MAPK abrogated it [34]. A potential mechanism for these events was suggested by a recent study in which direct binding of TXNIP and p38 MAPK was demonstrated [35]. Further upstream, TXNIP expression was increased in diabetic smokers by relief of its repression by microRNA (miRNA)-17 by a long noncoding (lnc) RNA, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) [36]. SFA may also provoke innate immune response, via activation of Toll-like receptor (TLR) 2 and TLR4 pathways, leading to cell death and organ injury [31, 37, 38]. Direct binding of PA with TLR4 accessory protein [39], with downstream involvement of miRNA-194 [40], resulting in inflammation and myocardial injury have been reported.

Collectively, the findings above hint at a complex regulatory cascade controlling the effect of high plasma saturated fatty acid levels on programmed cell death of cardiomyocytes potentially involving innate immunity, lncRNA, miRNA, TXNIP and p38α-dependent signaling pathway. These offer a multitude of potential targets for further study.

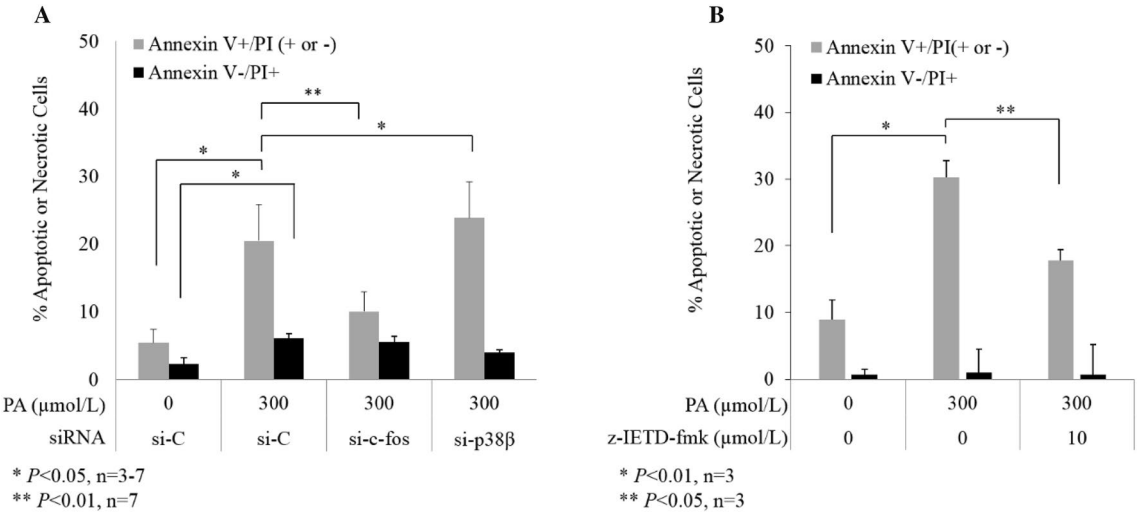


Fig. 4 siRNA knockdown of c-fos or inhibition of caspase 8 attenuated PA-induced apoptosis in a cardiomyocyte cell line. Exposure to high PA concentration for 16 h significantly increased apoptosis in AC16 cells, consistent with previous results. When c-fos translation was knocked down for 24 h or 48 h (**a**) then exposed to PA, percentage of apoptotic cells was significantly reduced, whereas si-p38β knockdown elevated it further. PA treatment increased necrosis but neither si-c-fos or si-p38β knockdown prevented it. Inhibition of caspase 8 with z-IETD-fmk reduced apoptosis to a similar degree to the c-fos knockdown, without significant change in necrosis. PA treatment significantly increased apoptosis in both the c-fos knockdown and caspase 8 inhibition experiments as expected (**a** and **b**)

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Compliance with ethical standards

Disclosures We have no disclosures to make.

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