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# p38α mitogen-activated kinase mediates cardiomyocyte apoptosis induced by palmitate

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ABSTRACT

*Rationale:* The mechanisms underlying lipotoxic/diabetic cardiomyopathy remain poorly understood. Saturated fatty acid (SFA) levels, elevated in obesity and type 2 diabetes, induce apoptosis in many cell types including cardiomyocytes. Signaling pathways, including the p38α mitogen-activated kinase (MAPK)-dependent pathway, have been implicated in apoptosis due to a diverse range of insults.
*Objective:* We tested the hypothesis that SFA-induced cardiomyocyte apoptosis is dependent on p38α activation.
*Methods and results:* Human adult ventricular cardiomyocytes (AC16 cells) were exposed to high physiological levels of palmitate (PA), a SFA. The apoptotic response was measured using annexin-V by flow cytometry, and the p38α-dependent pathway was evaluated using a p38 inhibitor PD169316, and by p38α small interfering RNA (siRNA) knockdown. PA exposure for 16 h dose-dependently increased apoptosis in AC16 cardiomyocytes (control: 2.6 ± 0.6%, 150 μM PA: 3.5 ± 0.9%, 300 μM PA: 11.5 ± 1.6%, $n = 4$, $p < 0.01$). PA did not change total p38α protein levels, but increased p38α phosphorylation dose-dependently ($n = 5$, $p < 0.01$). PD169316 tended to reduce PA-induced apoptosis ($n = 4$, $p = 0.05$). Specific p38α siRNA markedly reduced the expression of p38α but not p38β ($n = 3$, $p < 0.0001$), and dose-dependently attenuated PA-induced apoptosis (control siRNA: 7.7 ± 1.0%, 300 μM PA: 34.4 ± 5.0%, 300 μM PA + 30 pmol siRNA: 23.7 ± 4.4%, 300 μM PA + 60 pmol siRNA: 19.7 ± 2.6%, 300 μM PA + 120 pmol siRNA: 17.3 ± 2.8%, $n = 4$, $p < 0.0001$).
*Conclusions:* These results demonstrate that PA induces p38α activation, and reducing p38α expression attenuates PA-induced cardiomyocyte apoptosis. Our results support a potential mechanism by which high plasma SFA levels through p38α activation may lead to the development of lipotoxic/diabetic cardiomyopathy.

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## 1. Introduction

Elevated plasma free fatty acid levels seen in Type 2 diabetes and morbid obesity [1], have been postulated as a possible mechanism for the development of diabetic/lipotoxic cardiomyopathy [2,3]. High plasma saturated fatty acid (SFA) levels lead to metabolic abnormalities such as increased ceramide levels and oxidative stress, and have been shown to induce apoptosis in many cell types [4–7]. In other established, important cardiomyopathies such as hypertrophic, doxorubicin-induced, and amyloidogenic, the p38α mitogen-activated protein kinase (MAPK)-dependent pathway has been implicated in apoptotic loss of cardiomyocytes leading to the development of cardiac dysfunction. In contrast, only one study that has directly linked SFA-induced apoptosis to p38 MAPK's, and it suggested an indirect antiapoptotic role for p38β [8]. These somewhat contradictory data prompted consideration of further studies.

The p38 MAPK family is involved in cellular responses including inflammation and apoptosis that occur subsequent to trauma and chemical extracellular stress [9]. Of the four known p38 MAPK isoforms, α, β, γ, and δ, p38α facilitated apoptosis in most models [10–12], whereas p38β was antiapoptotic in others [8,13]. Initially, p38α and p38β were thought to be the predominant isoforms in the adult human myocardium with very little if any expression of p38γ or p38δ [14–16]. However, p38γ was discovered to be expressed at significant levels in the heart as well [17]. Further studies have led to the current understanding that all four isoforms

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**Non-standard abbreviations and acronyms**

| | |
|---|---|
| MAPK | Mitogen activated protein kinase |
| SFA | Saturated fatty acid |
| PA | Palmitate |
| siRNA | Small interfering RNA |
| c-FLIP$_s$ | Cellular Fas-associated death domain-like interleukin-1β converting enzymes (FLICE) inhibitory enzyme short |
| AP-1 | Activator protein 1 |
| C/EBP | CCAAT/enhancer binding protein |
| qPCR | Quantitative polymerase chain reaction |
| DISC | Death-inducing signal complex |

are expressed in cardiomyocytes with p38α and p38γ predominating, and p38β and p38δ present at lower levels [18].

Studies from several laboratories indicated that p38 MAPK isoforms are involved in the development of cardiomyopathies. For example, p38 MAPK activation and myocardial apoptosis were seen in rats administered doxorubicin, both of which were blocked by a p38 MAPK inhibitor [19]. When cardiac hypertrophy was induced by pressure overload via aortic banding, myocardial apoptosis increased, but to a lesser degree in the dominant-negative p38α MAPK mice than in the wild-type mice [10]. Additionally, amyloidogenic light chain proteins induce cardiomyocyte apoptosis via a non-canonical p38α activation [11]. Collectively, these results point toward p38α MAPK activation as a common endpoint integrating signaling from a diverse set of stressors driving apoptosis in cardiomyocytes.

Nonetheless, it remains to be clarified whether elevated plasma concentrations of SFA might play a causative role in inducing diabetic/lipotoxic cardiomyopathy and whether p38 MAPK signaling may provide the mechanistic link. In the present study, we tested the hypothesis that (1) PA induces cardiomyocyte apoptosis, and (2) the p38α-dependent pathway mediates this effect.

## 2. Material and methods

### 2.1. Cell culture

AC16 cell line was obtained through the generosity of Dr. Mercy Davidson at Columbia University. These cells are immortalized human adult ventricular cardiomyocytes that express key cardiomyocyte-specific transcription factors, contractile and structural proteins [20]. AC16 cell line has been used in multiple published studies of cardiomyocyte biology [21–24].

AC16 cells (passage 4–15) were cultured in growth media [F12/DMEM (1:1) supplemented with 12.5% fetal bovine serum, 1% penicillin/streptomycin, and 1% fungizone] under standard cell culture conditions (37 °C, humidified, 95% air/5% CO2), and split every 2–3 days to maintain confluence below 95%.

Cell culture media and supplements were obtained from Invitrogen (Gaithersburg, MD). PD169316, a p38 inhibitor, was obtained from EMD Millipore (Billerica, MA). Quantitative Real-Time PCR (qPCR) and Western blot reagents were from Bio-Rad (Hercules, CA). Flow cytometry antibodies and reagents were from eBioscience (San Diego, CA). Specific antibodies to p-p38, total p38, total p38α, total p38β, and GAPDH were from Cell Signaling Technology (Danvers, MA). All other reagents not otherwise specified were from Sigma–Aldrich (St. Louis, MO).

### 2.2. Experimental design

The day before experiments were conducted, AC16 cells were split into six well plates and studied at 70–80% confluence. For the initial experiments investigating the effects of PA, cells were treated with 0, 150, and 300 μM of PA for 4, 16, or 24 h. In experiments with PD169316, cells were pretreated with the inhibitor 2 h prior to exposure to PA for 16 h. We chose the 150–300 μM range of PA used in the present study as it is within the physiological range seen in diabetes and morbid obesity, and therefore, may be clinically relevant.

### 2.3. siRNA transfection

For the p38α translational knockdown experiments, double-stranded siRNA specific for p38α and non-targeting control siRNA were obtained from Dharmacon (Lafayette, CO). Sequences (5′-G GUCUCUGGAGGAAUUCAAtt-3′, 3′-ttCCAGAGACCUCCUUAAGUU-5′) were obtained from Tourian et al. [13]. AC16 cells were transfected using the Amaxa Nucleofector II and Cell Line Nucleofector Kit L (Lonza, Walkersville, MD). Following manufacturer's protocols, approximately 125,000 AC16 cells per group were incubated with the nucleofector solution, and mixed with siRNA to p38α or negative control siRNA (Dharmacon, Lafayette, CO). Cells were incubated in a humidified incubator at 37 °C/5% CO2 for 48 h before being treatment with PA for 16 h.

### 2.4. qPCR

RNA from AC16 cells was isolated using the Bio-Rad Aurum Total RNA Mini Kit, following manufacturer's instructions. One μg of cDNA was synthesized following manufacturer's protocol by reverse transcription using a Bio-Rad iScript cDNA Synthesis Kit. Quantitative real time PCR was performed with a Bio-Rad iQ5 thermal cycler, 2× Syber Green reagent, and primers designed for each specific gene from IDT (Coralville, IA). $C_t$ values were obtained using the standard dilution method.

### 2.5. Western blotting

AC16 cells extracts were prepared by incubation with RIPA buffer (Thermo Scientific, Rockford, IL), supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail 2. The protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific). The extracted proteins were boiled in protein buffer for 5 min, separated by 10% SDS–PAGE, and transferred to a PVDF membrane. The membrane was incubated at room temperature for 1 h in TBS-T (Tris-buffered saline containing 0.1% Tween 20) containing a blocking buffer. The membrane was then incubated overnight at 4 °C with the primary antibodies, washed with TBS-T buffer three times, and then incubated with a horseradish peroxide-conjugated secondary antibody for 1 h at room temperature. The membrane was developed with exposure to the ECL reagent (Bio-Rad Laboratories, Hercules, CA) for 5 min.

## 3. Results

### 3.1. PA induces dose-dependent apoptosis in adult human cardiomyocytes (AC16 cells)

PA induced a significant dose-dependent increase in apoptosis in AC16 cells after 16 h treatment (1.3-fold and 4.4-fold higher than control at 150 μM and 300 μM PA, respectively), at 24 h (2.6-fold

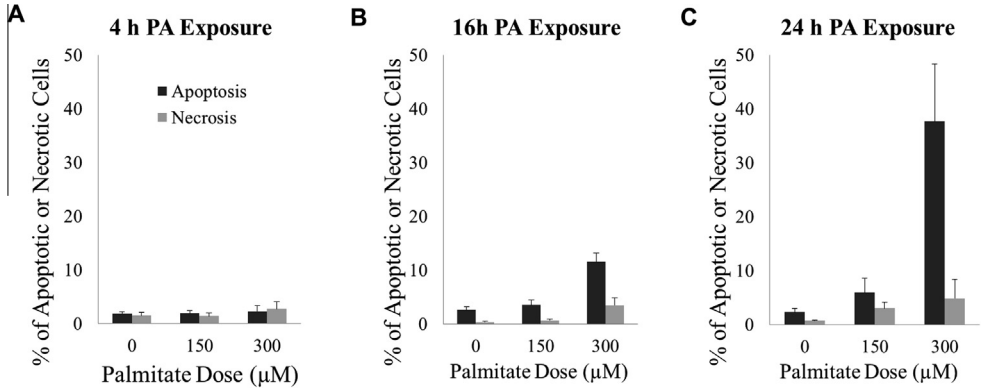


**Fig. 1.** Palmitate induced apoptosis in cells derived from human adult ventricular cardiomyocytes. AC16 cells were treated with 0 μM, 150 μM, or 300 μM PA for 4, 16 h, or 24 h, then apoptosis and necrosis determined by flow cytometry after staining with annex-V and propidium iodide (A–C). Data are presented as mean ± standard error of the mean (SEM) from $n = 3–5$ experiments, performed in duplicate. Statistical significance compared with control was determined by one-way ANOVA: 4 h, $p$ = NS, 16 h, $p < 0.001$, 24 h, $p < 0.05$.

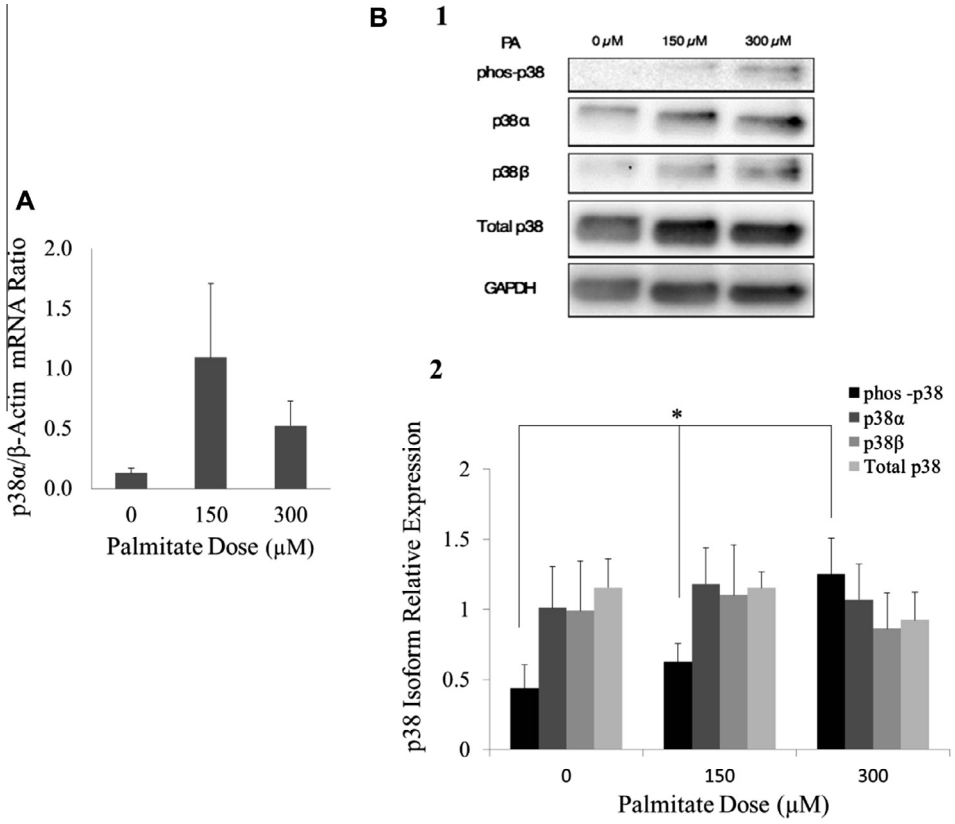


**Fig. 2.** Palmitate up-regulates p38 signaling by dose-dependently increasing p38 activation without changing the mRNA transcription level or the total p38 protein expression. (A) Relative mRNA transcription of p38α was widely variable and not significantly changed by PA. (B) Relative protein levels of phosphorylated p38, total p38α, total p38β, and total p38α+β were determined by Western blot analysis. Representative Western blots (1), and densitometry quantification (2) from 5 experiments are shown. *Overall ANOVA $p < 0.01$.

and 16.6-fold higher than control at 150 μM and 300 μM PA, respectively) (Fig. 1A–C). A comparison among experiments suggested a time-dependent increase in PA-induced apoptosis. As the 16 h treatment allowed optimal discrimination of the pro-apoptotic effect of different PA doses, had good reproducibility, and caused less toxicity than the 24 h exposure, subsequent experiments were performed at this duration of PA exposure. Although necrosis also rose at 16 h (control: 0.3 ± 0.2%, 150 μM PA: 0.7 ± 0.3%, 300 μM PA: 3.5 ± 1.7%, $n = 4$, $p < 0.05$), it was far lower than apoptosis.

### 3.2. PA induces dose-dependent activation of p38α MAPK

Following 16 h exposure to PA, there was not a significant change in p38α mRNA expression (Fig. 2A). Consistent with this finding, p38α protein levels were also not changed (Fig. 2B). Additionally, p38β and total p38 protein levels remained unchanged as well. However, phosphorylated p38 protein (phos-p38), dose-dependently increased (43% and 187% higher than control at 150 μM and 300 μM PA, respectively).

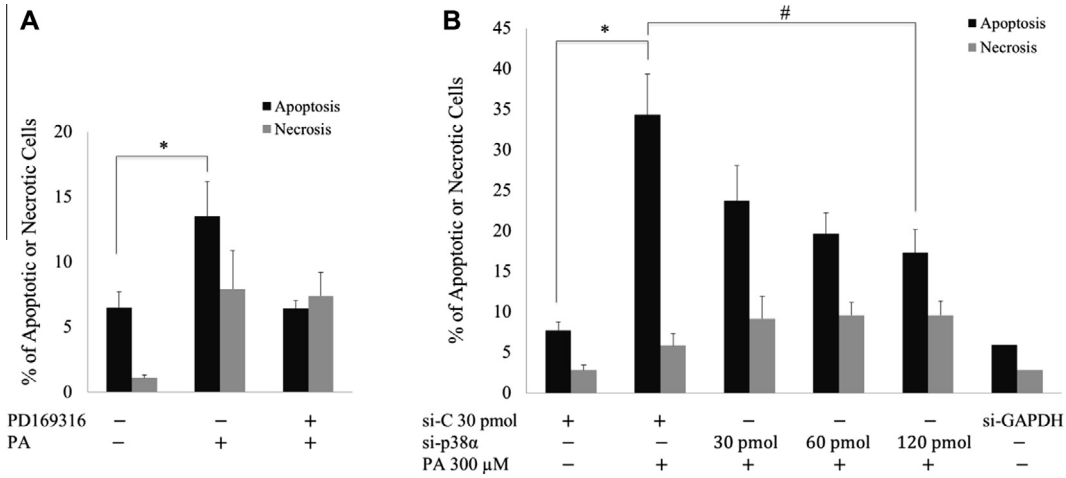


**Fig. 3.** p38α MAPK mediates PA-induced apoptosis in AC16 cells. (A) PD169316, a nonspecific inhibitor of both p38α and p38β tended to reduce PA-induced apoptosis but did not reach significance although PA increased apoptosis in AC16 cells as expected ($n = 4$, $^{*}p < 0.05$). Apoptosis and necrosis were determined as described previously. (B) However, more specific translational knockdown of p38α by siRNA significantly attenuated PA-induced apoptosis in AC16 cells ($n = 4$, $^{*}p \leqslant 0.01$ vs Control, $^{\#}p < 0.0001$ overall ANOVA for si-C + PA vs si-p38α + PA groups). Cells were transfected with siRNA, either with control siRNA or increasing doses, 30, 60, and 120 pmol of p38α siRNA. GAPDH siRNA served as positive control. After 48 h, cells were treated with either with PA vehicle or 300 μM PA for an additional 16 h.

### 3.3. Inhibition of p38 and PA-induced cardiomyocyte apoptosis

To determine whether p38 MAPK-dependent signaling mediated PA-induced apoptosis in cardiomyocytes, we treated AC16 cells with PD169316, a nonspecific inhibitor of both p38α and p38β. It reduced PA-induced apoptosis but this decline did not reach significance (Fig. 3A). This result was likely due to the nonspecific inhibition of both isoforms [13].

### 3.4. p38α knockdown attenuated PA-induced cardiomyocyte apoptosis

Given the unclear result of the pharmacological and nonspecific inhibition of p38 activation, we next examined the effects of the p38α-specific knockdown on PA-induced cardiomyocyte apoptosis. As we hypothesized, blocking p38α translation and thereby its activation with a specific siRNA significantly reduced PA-induced apoptosis. This inhibition of PA-induced apoptosis in AC16 cells by prior transfection with the p38α siRNA was dose-dependent (Fig. 3B). Necrosis was not significantly affected. In comparison and as expected, PA treatment of AC16 cardiomyocytes transfected with si-Control induced a large apoptotic response without a significant increase in necrosis.

### 3.5. Effects of PA and translational knockdown of p38α on the expression of p38 isoforms

Underlying the effect of p38α siRNA on PA-induced apoptosis was an extensive reduction in p38α levels and thereby in its activation. PA treatment for 16 h following a 48 h transfection with the negative control siRNA produced a significant, 76% increase in phos-p38 in the si-C+300 μM PA group (Fig. 4). When p38α siRNA was used, large dose dependent reductions were seen (66%, 76%, and 85% decreases compared to the si-C + 300 μM PA group, $n = 3$, ANOVA $p < 0.0001$). Although quantitative immunoblot assays for phos-p38α and phos-p38β were attempted with antibodies specific to each, no clear interpretation was possible due to massive nonspecific staining (data not shown).

In contrast to the significant changes in p38α, phos-p38, and total p38 protein levels, si-p38α transfection did not lead to a change in total p38β level. Comparisons of the si-Control + PA 300 μM PA group to the si-Control + PA Vehicle group confirmed that PA treatment did not result in changes in total p38α, total p38β, nor total p38 protein levels in AC16 cells.

## 4. Discussion

Our lab had previously shown that palmitate induced p38 activation in monocytes, and that this was an essential step in amplification of the innate immune inflammatory response to lipopolysaccharide [25]. Previous studies had shown that p38 MAPK is involved in the induction of apoptosis in response to diverse insults, and may underlie a variety of well-established cardiomyopathies [10–12,19,26]. Although PA induces apoptosis in many cell types [4–7], to our knowledge, no study has shown that SFA-induced apoptosis is causally linked to p38α MAPK activity in cardiomyocytes. The present study demonstrates for the first time that the apoptosis of human cardiomyocytes induced by exposure to elevated concentrations of PA is dependent on p38α activation.

Our initial experiments demonstrated that exposure of human cardiomyocytes to high PA levels led to dose-dependent programmed cell death, consistent with prior studies [27–29], and further, that p38α phosphorylation was also dose-dependently increased (Fig. 2B) in agreement with earlier work [30].

We then determined whether the PA-induced apoptosis was dependent on the p38α activation by using a p38 inhibitor, PD169316. It tended to reduce PA-induced cardiomyocyte apoptosis but did not reach significance. PD169316 is a nonspecific inhibitor of both p38α and p38β isoforms [13]. However, p38α facilitates apoptosis whereas p38β has antiapoptotic effects [11–13]. Furthermore, the comparative potency of PD169316 against p38α or p38β is not known. It seems plausible that the opposing effects of PD169316 on the two p38 isoforms may have contributed to the ambiguous effect of PD169316 on PA-induced apoptosis in cardiomyocytes in the present study.

Therefore, we conducted further experiments with a more specific tool, siRNA specific to p38α, to assess its role in PA-induced cardiomyocyte apoptosis. Western blot assays confirmed that we achieved a marked translational knockdown of p38α expression with the p38α siRNA. The parallel reduction in PA-induced apoptosis provided strong evidence that p38α activation is an essential step in the signaling cascade from the insult of high PA exposure to eventual apoptosis of cardiomyocytes.

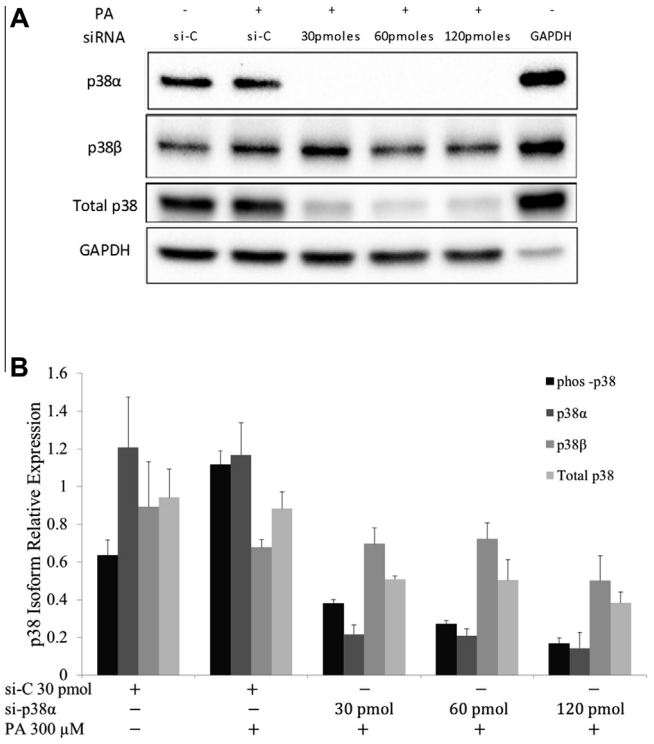


**Fig. 4.** p38α specific siRNA produced marked reduction in phos-p38, p38α, and total p38 protein levels without affecting p38β. (A and B) PA treatment for 16 h following a 48 h transfection with the negative control siRNA produced a significant increase in phos-p38 in the si-C + 300 μM PA group ($n = 3$, $p < 0.05$) without effects on total p38α, total p38β, or total p38 levels. With p38α siRNA transfection, large dose dependent reductions were seen in phos-p38 ($p < 0.0001$), p38α ($p < 0.0003$), and total p38 ($p < 0.001$).

The molecular mechanism that leads from p38α activation to apoptosis is not clearly known. However, p38α has been shown to activate caspase 8 independent of Fas-associated death domain protein in response to manganese and also, interestingly, to sphingosine, a metabolic product of PA [31,32]. Furthermore, among the MAPK-dependent transcription factors, AP-1 and C/EBP have been linked to up-regulated caspase 8 activity. AP-1 increases caspase 8 expression [33], and C/EBP associates directly with caspase 8 complex protein and disrupts procaspase 8 cleavage and activation [34]. In Jurkat cells, Fas treatment activates p38α which inhibits the phosphorylation and association of c-FLIP$_s$ in the death-inducing signal complex (DISC), a well-established proapoptotic pathway for extracellular death stimuli. As c-FLIP$_s$ inhibits recruitment of procaspase 8 to the DISC and its cleavage into the active form, activated p38α promotes caspase 8 cleavage and activation and apoptosis [13]. These observations point to other potential avenues for further studies.

In conclusion, the present study demonstrates that in cardiomyocytes exposed to high yet physiological palmitate levels, dose-dependent increase in p38α activation occurs without a change in total p38α protein expression. When the level of p38α activation is translationally reduced, PA-induced apoptosis is significantly reduced, consistent with an essential role for p38α-dependent pathway in this process.

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## Disclosures

None.

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