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Expression of Active Protein Phosphatase 1 Inhibitor-1 Attenuates Chronic Beta-Agonist-Induced Cardiac Apoptosis

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Abstract

Cardiac apoptosis has been considered an important contributing factor to heart failure. Several subcellular mechanisms, including increased protein phosphatase 1 activity, have been suggested to induce apoptosis. Protein phosphatase 1 is regulated by an endogenous inhibitor-1 (I-1) that is activated upon phosphorylation at threonine 35 via protein kinase A. Here, we tested whether cardiac specific overexpression of a constitutively active (T35D, AA 1–65) inhibitor-1 (I-1c), could also affect cardiac apoptosis and heart failure progression induced by prolonged β-adrenergic stimulation. We found that either acute or chronic expression of I-1c reduced isoproterenol (ISO)-induced apoptosis assessed by nuclear condensation, TUNEL staining and DNA fragmentation. The beneficial effects of I-1c were associated with increased expression of the anti-apoptotic protein Bcl-2, decreased expression of the pro-apoptotic protein Bax and reduced levels of active caspases as well as increased activation of ERK. These findings suggest that mitochondrial signaling and ERK activation may be involved in the I-1c cardioprotective effects against apoptosis induced by prolonged β-adrenergic stimulation.

Keywords

Protein Phosphatase 1 Inhibitor-1; Cardiomyocytes; Apoptosis; ERK; Bad

Introduction

For the last decade, apoptosis has been recognized as an important factor contributing to the onset and progression of various cardiac diseases such as myocarditis, acute myocardial infarction and cardiomyopathy [6, 22]. Although the exact mechanism of apoptosis within the heart is not clearly understood, several studies indicate that apoptosis mediates

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cardiomyocyte cell death after myocardial infarction and it is a major determinant of the infarct size upon coronary occlusion, provided that sufficient cellular ATP is present to sustain the apoptotic cascades [22]. Particularly, apoptosis plays a considerable role in the development of heart failure, especially in the transition from the compensatory to the failing phase [2, 25]. Furthermore, several anti-apoptotic strategies have shown promising results in heart failure studies induced by different stressors [20].

Previous studies revealed that an active form of inhibitor-1 (I-1c), a specific and potent inhibitor of PP1, enhanced basal cardiac function by affecting the phosphorylation levels of phospholamban (PLN), and thus the calcium (Ca^{2+}) dynamics in the heart [7, 26]. More importantly, expression of I-1c in mice displayed a dramatic protective effect against the development of heart failure induced by chronic pressure over-load [7, 26]. In addition, adenoviral-mediated expression of I-1c in the setting of pre-existing heart failure restored contractile function and halted the progression of heart failure and fibrosis [7, 26].

Given the remarkable involvement of protein phosphatase 1 (PP1) in the induction of apoptosis [16, 21], it is interesting to speculate that the inhibitor-1 may also protect cardiac cells from death, especially due to apoptosis induced by pathological stress. Indeed, a recently published study from our laboratory demonstrated that inducible expression of the I-1c in mice, improved the heart mechanical recovery as well as cell survival following an ischemic insult through inhibition of apoptosis [24]. One of the underlying mechanisms may involve cardioprotection by the elevated peroxiredoxin II in these hearts [38].

As one of the well-recognized etiologies of heart failure, prolonged β-adrenergic stimulation results in cardiomyocyte apoptosis or death [10, 29]. Moreover, elevated PP1 activity and decreased levels of inhibitor-1 were observed following excessive adrenergic drive in failing hearts [4, 11]. In this study, we found that expression of I-1c decreased chronic ISO-induced cellular apoptosis in acutely infected adult rat cardiomyocytes as well as in transgenic hearts. This protective effect may be associated with altered expression/activity of caspase 3, caspase 8, Bcl-2 and ERK, all being considered active players in the apoptotic cascades.

Materials and methods

Myocyte isolation and cell culture

Left ventricular myocytes were isolated from adult male Sprague-Dawley rats (~ 250 g; Harlan Laboratory, Indianapolis, IN), as previously described [8, 13]. The animals were handled according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Cincinnati. Following cell isolation, cardiomyocytes were plated on laminin-coated culture dishes for 1 to 2 hours and allowed to recover at 37°C, in a humidified 5% CO_2 incubator. Plated myocytes were then infected with adenovirus expressing the truncated, active form of I-1 (I-1c) at a multiplicity of infection of 500 for 2 h. Following infection, cells were maintained in culture for 24 hours before the addition of ISO (10μmol/L, Sigma, St. Louis, MO). All dishes were supplemented with ascorbic acid (0.1 mmol/L, Sigma, St. Louis, MO) to prevent oxidation of ISO.

Animal preparation and osmotic minipump infusion

Generation of mice with cardiac-specific expression of truncated T35D-inhibitor-1 has been described previously [7, 26]. Adult wild type (WT) and transgenic mice (TG), inbred on a FVB/N background, were studied at 8 to 10 weeks of age. All procedures were in accordance with the Institutional Guidelines for Animal Research. Osmotic minipumps (model 2002; Alzet) containing saline solution or ISO (50 μg/g/day), were implanted into 10-week-old male mice over a period of 14 days, as described previously [14]. Following 14 days of chronic ISO treatment, the pumps were excised and the hearts were immediately

collected for subsequent experiments. In some experiments, the pumps were excised after 12 days of ISO treatment and cardiac contractile parameters were assessed by catheterization after 2 days [23]. The heart rate, and the first derivative of left ventricular (LV) pressure development (+dP/dt) and decline (−dP/dt) were monitored.

Heart homogenates and immunoblotting

Hearts were snap frozen in liquid nitrogen and homogenized in Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with phenylmethylsulphonyl fluoride 1mM (PMSF) and complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Equal amounts of protein samples from individual heart homogenates were analyzed by SDS-PAGE, as previously described [28]. Immunoblotting analysis was performed using primary antibodies at 1:1000 dilution, corresponding to the protein under analysis: Bcl-2 and Bax (from Invitrogen, Carlsbad, CA), caspase-3 (Asp175), caspase-8 and phosphorylated/total p38, ERK, JNK, Akt, Bad (Cell Signaling, Danvers, MA) and α-actin (Sigma, St. Louis, MO). The secondary antibody conjugated with horseradish peroxidase was used at a 1:5000 dilution. The protein bands were visualized using the SuperSignal West Pico chemiluminescence substrate kit (Pierce, Rockford, IL) or the ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Signals were quantified using ImageQuant 5.2 analysis software (GE Healthcare, Little Chalfont, UK). The densitometric values corresponding to TG samples were normalized to control WT samples. The data were expressed as fold change relative to control values. Actin was used as an internal standard. The findings were similar to those obtained with calsequestrin as an internal standard.

Detection of apoptosis using terminal dUTP nick end-labeling (TUNEL) assay

Mouse hearts were collected and the atrial tissue was removed. The ventricles were fixed in 10% buffered formalin and then embedded in paraffin according to standard procedures. The TUNEL assay was performed on 3 sections each of 3 μm thick, using the ApopTag Plus Peroxidase In situ Apoptosis Detection Kit (Chemicon, Burlington, MA), according to the manufacturer's instructions. The kit detects the DNA strand breaks generated upon DNA fragmentation by enzymatically labeling the 3'-OH ends in the apoptotic bodies with chemically labeled nucleotides. TUNEL-positive nuclei were counted on each mid-ventricular section from 10 randomly chosen fields per section. The TUNEL-positive nuclei were expressed as a percentage of the total counted nuclei.

Detection of apoptosis by measuring nuclear DNA fragmentation and DNA condensation

Following ISO treatment, mouse hearts were collected and homogenized in cell lysis buffer (RIPA) containing 1 μmol/L dithiothreitol (DTT) and 50 μmol/L PMSF. The heart homogenates were centrifuged at 13 000*g* for 10 minutes and apoptosis was assessed in the supernatants using the Cell Death Detection ELISA^{PLUS} assay kit (Roche, Indianapolis, IN). The kit employs a photometric enzyme immunoassay for quantitative *in vitro* determination of the cytoplasmic histone-associated DNA fragments. The measurements corresponding to the individual samples were normalized to a positive control provided in the kit, consisting of a DNA-histone complex and expressed as percentages in relation to the positive control. Alternatively, we used Hoechst 33342 a fluorescent dye that stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells. Data were expressed as the number of pyknotic nuclei counted in cells infected with a virus carrying I-1c in the presence or absence of ISO treatment, normalized to the number of pyknotic nuclei in cells infected with an empty virus expressing only a green fluorescent protein (GFP), in the absence of ISO treatment.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined with ANOVA, followed by Duncan multiple range comparison test using Super ANOVA (Abacus Concepts Inc). Differences were considered statistically significant at a value of $P<0.05$.

Results

The active inhibitor-1 attenuates cardiac apoptosis induced by chronic isoproterenol treatment

The truncated, active I-1c has been shown to prevent cardiac cell apoptosis under ischemia/reperfusion conditions [24]. To examine whether this form of inhibitor-1 affects apoptosis induced by chronic β-agonist treatment *in vivo*, we treated mice expressing I-1c and their wild type littermates with ISO by mini-pump infusion over a period of 14 days (50 μg/g/day). The TUNEL-staining assay revealed a lower proportion of TUNEL-positive nuclei in the myocardium of TG mice after ISO treatment, compared to WT (Figure 1a and 1b). With an ELISA-based method measuring DNA fragmentation, we further confirmed that compared with saline treatment, chronic ISO treatment induced a dramatic increase in apoptosis by 69% in WT mice compared with TG mice where apoptosis increased by only 25%. In saline control groups, there were no differences in cellular apoptosis between WT and TG hearts (Figure 1b, 1c).

To further confirm this finding in a system that lacks compensatory mechanisms which may occur in the transgenic hearts, we infected isolated rat adult cardiomyocytes with an adenovirus carrying the I-1c gene linked to a green fluorescent protein (GFP) gene that serves as an expression marker. As control, we used cardiomyocytes infected with an adenovirus expressing GFP only. Twenty-four hours following infection, the cells were incubated with 10μmol/L ISO for an additional twenty-four hours. We then evaluated the nuclear condensation in cardiomyocytes as an index of apoptosis, using the vital dye Hoechst 33342 (Figure 2a). Consistent with previous reports [10 13], chronic ISO treatment increased apoptosis, demonstrated by a 2.1-fold increase in the number of pyknotic nuclei in control or GFP infected cells (Figure 2b). Although ISO treatment also increased apoptosis in I-1c infected cells, this effect was attenuated compared to control. Overall, these data indicate that I-1c protects against cellular apoptosis induced by chronic β-adrenergic stimulation.

The active inhibitor-1 attenuates the expression levels of pro-apoptotic proteins

To elucidate the mechanisms underlying the anti-apoptotic effect of I-1c, we assessed the expression levels of key players involved in either the intrinsic or the extrinsic apoptotic cascades. Chronic ISO treatment of WT animals decreased the expression levels of the antiapoptotic protein Bcl-2, while it increased the pro-apoptotic protein Bax (Figure 3a, 3b). However, these changes were diminished in the I-1c hearts. Interestingly, the ratio of Bcl-2 to Bax was two fold higher in TG (0.76 ± 0.12) than in WT mice hearts (0.38± 0.02; Figure 3b). Surprisingly, in the absence of ISO treatment, TG mice exhibited a higher Bcl-2/Bax ratio (by 43%) compared with WT mice although this did not lead to any differences in apoptosis, as assessed in figures 1 and 3. We further determined the levels of caspase 8, commonly recognized as an extrinsic pro-apoptotic molecule. In saline treated groups, the expression levels of cleaved caspase 8 were similar between WT and TG animals. ISO treatment, however, resulted in an increase (by 43%) in the protein levels of cleaved caspase 8 in WT animals, while this increase was attenuated in TG mice (Figure 3a, 3c). A similar pattern of alteration was also observed for cleaved caspase 3, an active element involved in both extrinsic and intrinsic apoptotic pathways [32] (Figure 3a, 3d). Altogether, these observations suggest that I-1c may maintain a high Bcl-2/Bax ratio that would prevent the

release of mitochondrial apoptotic signals and preserve cell survival under chronic ISO treatment.

The active inhibitor-1 attenuates apoptosis by modulating the phosphorylation state of the pro-apoptotic protein Bad

Given that phosphorylated Bad, one of the Bcl-2 family members, has been reported to be a target of PP1 [1] and that dephosphorylated Bad has a pro-apoptotic character [3], we tested the phosphorylation levels of Bad at three sites, ser112, ser136 and ser155. Interestingly, chronic ISO treatment caused varied alterations in the different phospho-sites of Bad. It elicited an increase in phosphorylation at ser112 by 2.48-fold, a decrease at ser136 by 63% and no changes at ser155 (Figure 4b, 4c, 4d). Also, phosphorylation levels at the ser112 and ser155 sites were dramatically increased in the active inhibitor-1 animals at the basal state (2.7-fold and 2.6-fold, respectively). Importantly, the phosphorylation level at ser155 in the transgenic mice remained higher than WT under chronic ISO treatment (by 59%, Figure 4d).

The active I-1c affects phosphorylation levels of ERK

Given the significant role of MAP kinase pathways in cardiac apoptosis [2, 5], we tested the phosphorylation levels of p38, ERK and JNK (Figure 5a). The level of phosphorylated p38 was increased similarly in WT and active inhibitor-1 animals following ISO treatment (Figure 5b). The ERK phosphorylation level was decreased by 55% with ISO treatment in WT mice. However, under ISO treatment, the level of ERK phosphorylation was higher (by 53%, Figure 5c) in TG compared with WT mice. The levels of phosphorylated JNK (Figure 5d) or Akt (data not shown) were not altered by either chronic ISO treatment or expression of inhibitor-1. These data indicate that in I-1c mice under chronic stress, the ERK signaling pathway appears to be activated. Altogether the data suggest that attenuation of apoptosis observed experimentally in I-1c mice under chronic stress, might be mediated not only through the activation of the mitochondrial-related pro-survival mechanisms mentioned above, but also through activation of the ERK survival signaling cascade.

Discussion

Recently, cell death/apoptosis has been recognized as an important player in heart failure development and especially the transition from compensated to failing status. A key characteristic of human and experimental heart failure is elevated activity of PP1. Importantly, inhibition of PP1 by a truncated and constitutively active (T35D) inhibitor-1 enhanced basal cardiac function, attenuated heart failure induced by chronic over-load and protected against ischemic injury [7, 26]. In this study, we report that this active form of inhibitor-1 may also attenuate apoptosis induced by chronic β-adrenergic stimulation in the heart. Prolonged activation of the sympathetic nervous system and constantly elevated catecholamine levels contribute to the progression of heart failure [19]. Apoptosis induced by chronic β-adrenergic stimulation may be one of the underlying mechanisms in this process [10, 29]. Furthermore, reduced levels of inhibitor-1 and elevated activity of PP1 have been observed following longterm β-adrenergic stimulation [4, 11]. In this study, we observed that expression of the active inhibitor-1 attenuated cardiac apoptosis associated with prolonged β-stimulation. The underlying mechanisms may involve altered expression and phosphorylation levels of proteins that belong to the Bcl-2 family and/or MAP kinase pathways. Indeed, the MAP kinase pathways have been shown to play an important role in cardiac apoptosis [2]. Furthermore, accumulating evidence indicates that various protein kinases [2, 18] and phosphatases [33] may regulate apoptosis by targeting cytosolic and/or mitochondrial proteins. Specifically, phosphorylation of ERK or JAK was shown to induce cardioprotection [2, 15], whereas phosphorylation of JNK or p38 had a predominantly pro-apoptotic effect [2, 18]. Thus, the observed increases in phosphorylation of ERK, which has

been reported to be under regulation by PP1 through MEK [36], may contribute to the protective effects under prolonged ISO-stimulation of transgenic hearts.

Interestingly, we observed for the first time that chronic ISO exposure caused opposed changes in the phosphorylation levels of different sites in Bad. In its phosphorylated form, Bad promotes cell survival whereas in its dephosphorylated form, Bad associates with Bcl-X_L at the mitochondrial membrane, preventing its antiapoptotic activity and promoting cell death. It has been reported that Bad phosphorylation at ser155 triggers the dissociation of Bad from Bcl-X_L [21, 31], which in turn leads to cell survival. Although the available data suggest that PP1 is involved in Bad dephosphorylation at ser112 and ser136 [21, 22], there is no evidence suggesting that PP1 is also involved in the dephosphorylation of ser155. Our data indicate that phosphorylation of ser155 was increased under basal conditions in I-1c hearts and higher phosphorylation levels were also observed upon chronic exposure to ISO (Figure 4d). This may partially contribute to the cardioprotective effects mediated by the active inhibitor-1. However, a limitation of this study is that there was no evidence presented that the findings associated with chronic ISO stimulation could be prevented by pre-incubation with a beta-blocker.

In addition to the possibility of directly regulating the phosphorylation levels of the signaling proteins, the active inhibitor-1 may elicit its effects on signaling transduction by other mechanisms including Ca^{2+}-dependent cascades. It is well known that the active inhibitor-1 can regulate the sarcoplasmic reticulum function and thus Ca $^{2+}$-homeostasis, mainly by affecting the phosphorylation level of phospholamban [7, 26]. Indeed, accumulating evidence suggests that the SR may be involved in the apoptotic processes by interacting with the mitochondria [9, 17]. In particular, phospholamban has been implicated in myocardial cell apoptosis through a Ca^{2+}-dependent pathway [34, 35]. Based on the critical role of inhibitor-1 in regulating the phosphorylation level of phospholamban and SR function, it is interesting to propose that at least part of the beneficial effects of this molecule may be attributed to its effects on improved Ca^{2+} homeostasis. Interestingly, these I-1 beneficial effects may appear contradictory to recent findings in an I-1 knockout and an I-1c inducible mouse model. It was reported that under conditions of increased adrenergic drive, the I-1 deficient mouse shows cardioprotection [12], while the inducible I-1c model, generated on an I-1 deficient background, exhibits a decline in fractional shortening accompanied by an increase in left ventricular dilation, hypertrophy and increased interstitial fibrosis [37]. However, we did not observe cardiac deterioration following β-adrenergic stress. Actually, not only apoptosis was halted by I-1c overexpression but contractility remained enhanced in I-1c hearts (assessed by catheterization) (+dP/dt: 7052±489 and 9216±375; −dP/dt: 7075±421 and 8902±555 in WT and TG mice, respectively). Furthermore, the end-diastolic pressure (EDP) was much lower (4.0±0.5) in I-1c than WT hearts (7.1±1.4). This apparent discrepancy between our findings and those reported earlier [12, 37] may be due to differences in the mouse genetic background (FVB/N vs. C57Bl/6J), overexpression of I-1c in 13 the presence (present study) or absence [37] of endogenous I-1 and environmental factors in mouse husbandry.

As indicated above, changes in Ca^{2+}-cycling play a vital role in cellular apoptosis [27]. The active inhibitor-1 may prevent cell death by its ability to improve the Ca^{2+} balance in cells under stress. Interestingly, based on the universal effects of Ca^{2+} on multiple cellular pathways [27], this mechanism may allow this form of inhibitor-1 to offer cell protection under different pathways. For example, endoplasmic reticulum (ER) stress is a proposed detrimental mechanism for acute ischemia/reperfusion induced cell injury [30]. Our recent data showed that apoptosis induced by ischemia/reperfusion was decreased by inducible expression of active inhibitor-1 through attenuated ER stress [24]. Meanwhile, as revealed in this study, increased phosphorylation of signaling proteins such as Bad and ERK may be the

major mechanisms for the protective effects in the transgenic hearts, responding to prolonged β-adrenergic activation. Thus, the active inhibitor-1 may prevent cell death through pathways associated with improved Ca^{2+} homeostasis and phosphorylation of key signaling players in the heart.

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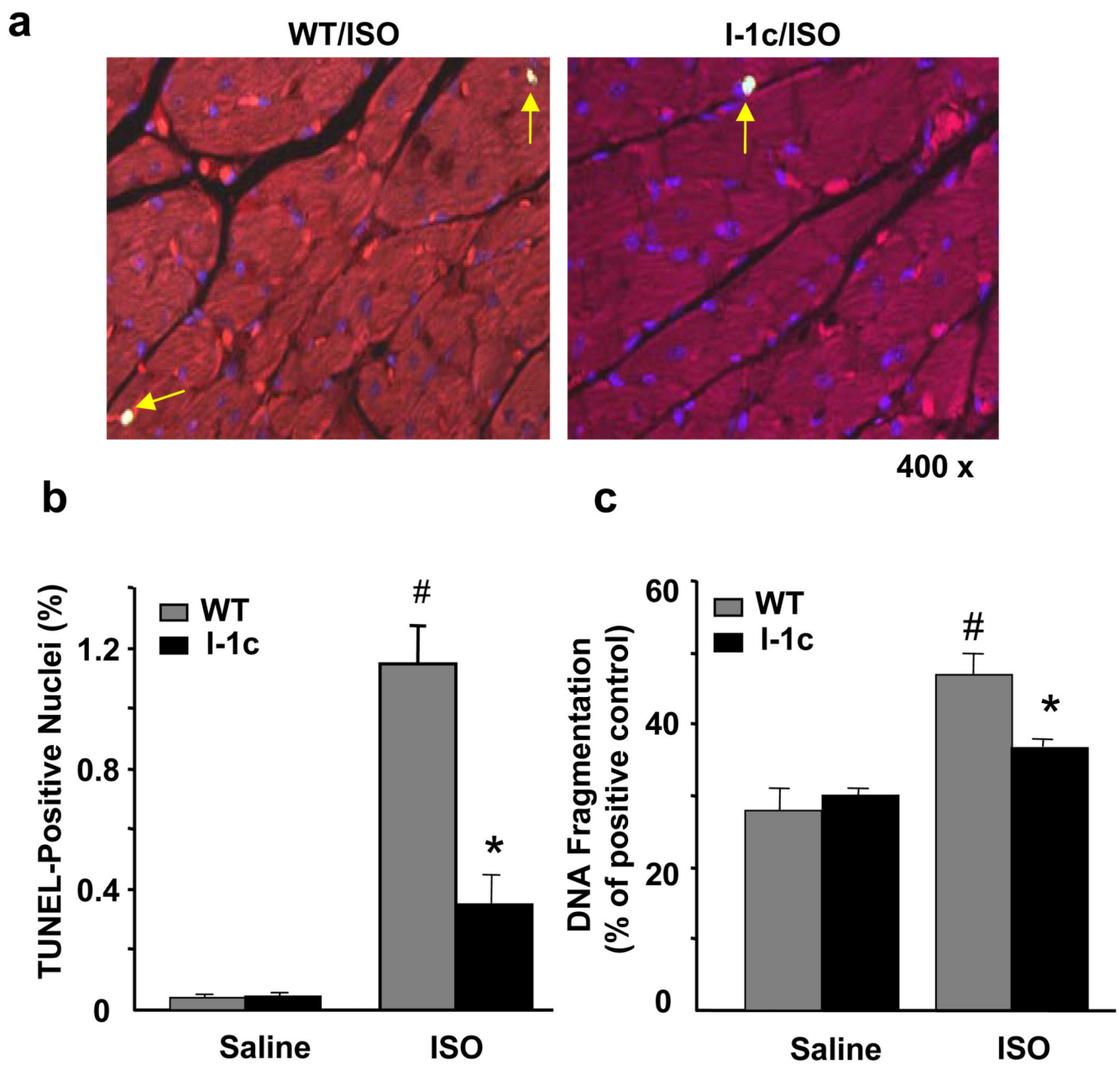

Fig.1.

Active inhibitor-1 (I-1c) expression protects hearts from apoptosis induced by chronic isoproterenol treatment. **a)** Triple-staining with anti-α-sarcomeric actin antibody, DAPI, and TUNEL to determine apoptosis in ISO–treated wild type or active inhibitor-1 cardiomyocytes (arrows indicate TUNEL-positive nuclei); **b)** Counts of TUNEL-positive nuclei (n=5); **c)** DNA fragmentation assay measured with a cell death detection ELISA kit in ISO-treated WT and I-1 hearts (n≥3); **P*<0.05 compared with ISO-treated WT hearts

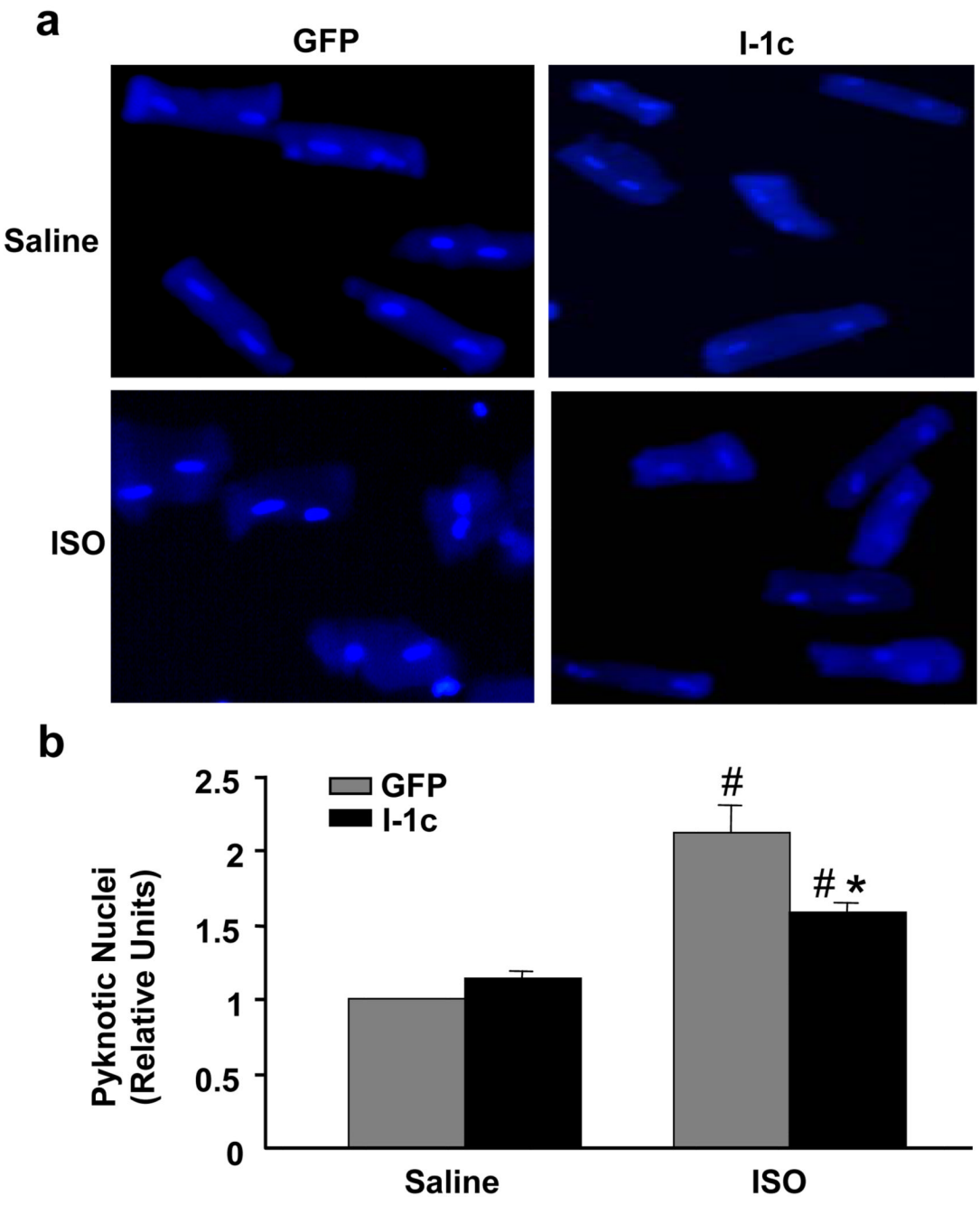

Fig.2.

Active I-1c expression protects adult rat cardiomyocytes from apoptosis induced by prolonged isoproterenol treatment. **a)** Adenoviral-infected cells were stained with Hoechst 33342 after exposure to ISO for 24 hours and fluorescence was examined (magnification 300×); **b)** Basal and ISO-induced pyknotic nuclei under Hoechst staining were counted and expressed as the percentage of total nuclei (n=3); **P*<0.05 compared with ISO-treated WT hearts; # *P*<0.05 compared with saline-treated hearts

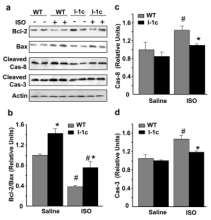

Fig.3.

Active I-1c expression diminishes the changes in the protein levels of apoptosis-regulatory molecules induced by chronic isoproterenol treatment. **a)** Representative blots; **b)** Ratio of Bcl-2/Bax. **c)** Cleaved caspase 8; **d)** Cleaved caspase 3. n≥3, **P*<0.05 compared with ISO-treated WT hearts; # *P*<0.05 compared with saline-treated hearts

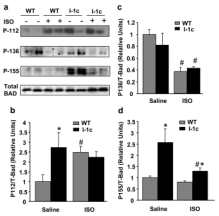

Fig.4.

Active I-1c expression alters the phosphorylation levels of Bad. **a)** Representative blots; **b)** Ratio of phosphorylated ser112/total Bad; **c)** Ratio of phosphorylated ser136/total Bad; **d)** Ratio of phosphorylated ser155/total Bad. n≥3;**P*<0.05 compared with ISO-treated WT hearts; # *P*<0.05 compared with saline-treated hearts

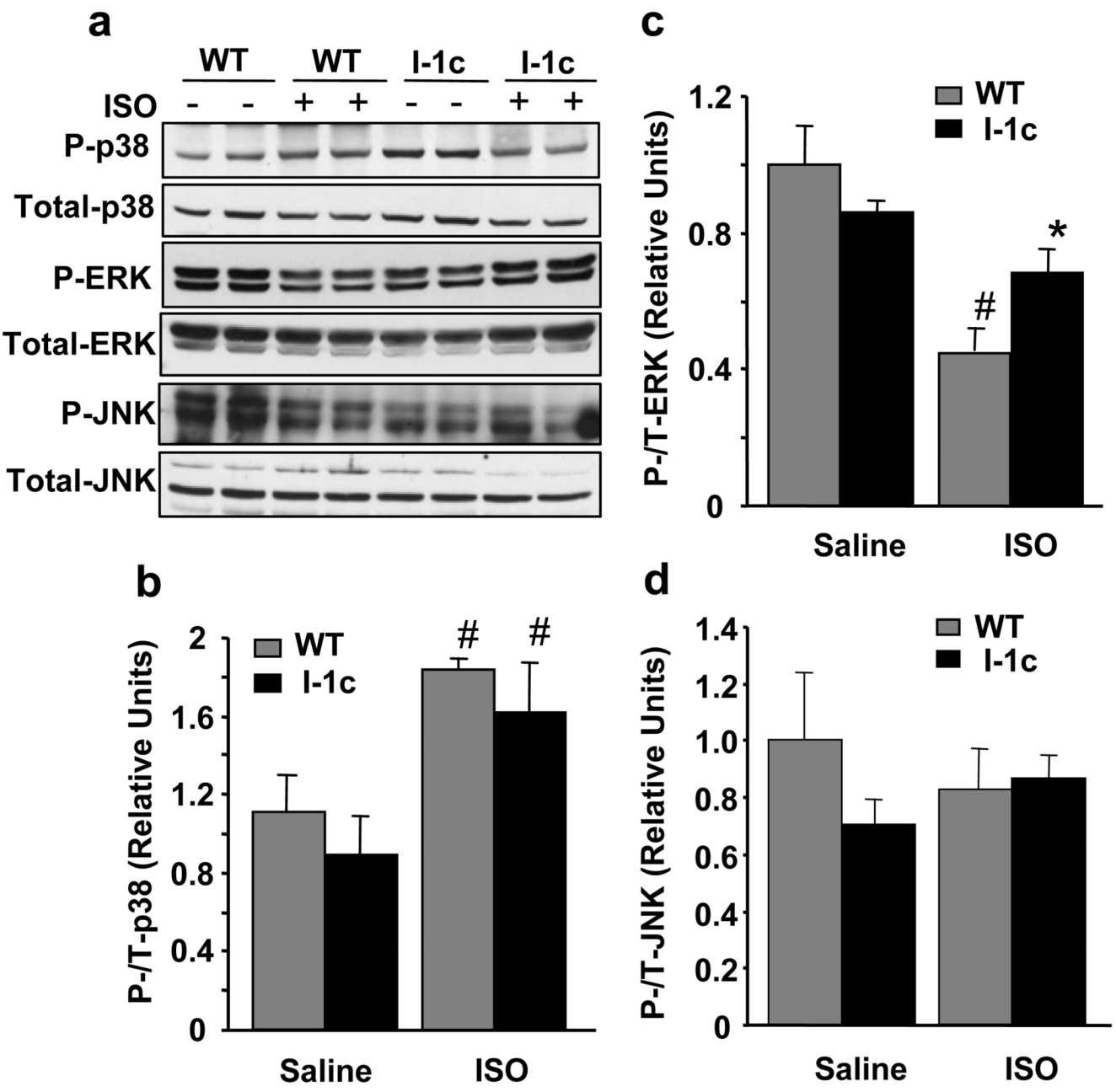

Fig.5.

Active I-1c expression diminishes the changes in the levels of phosphorylated ERK induced by chronic isoproterenol treatment. **a)** Representative blots; **b)** Ratio of phosphorylated /total p38; **c)** Ratio of phosphorylated /total ERK; d) Ratio of phosphorylated /total JNK. n≥3; **P*<0.05 compared with ISO-treated WT hearts; # *P*<0.05 compared with saline-treated hearts