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β1 Integrins Expression in Adult Rat Ventricular Myocytes and Its Role in the Regulation of β-Adrenergic Receptor-Stimulated Apoptosis

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Abstract We have shown that the stimulation of β-adrenergic receptors (β-AR) increases apoptosis in adult rat ventricular myocytes (ARVMs). Integrins, a family of αβ-heterodimeric cell surface receptors, are postulated to play a role in ventricular remodeling. Here, we show that norepinephrine (NE) increases β1 integrins expression in ARVMs via the stimulation of α1-AR, not β-AR. Inhibition of ERK1/2 using PD 98059, an inhibitor of ERK1/2 pathway, inhibited α1-AR-stimulated increases in β1 integrins expression. Activation of β1 integrins signaling pathway using laminin (LN) inhibited β-AR-stimulated apoptosis as measured by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL)-staining and flow cytometry. Likewise, ligation of β1 integrins with anti-β1 integrin antibodies prevented β-AR-stimulated apoptosis. Treatment of cells using LN or anti-β1 integrin antibodies activated ERK1/2 pathway. PD 98059 inhibited activation of ERK1/2 by LN, and prevented the anti-apoptotic effects of LN. Thus (1) stimulation of α1-AR regulates β1 integrins expression via the activation of ERK1/2, (2) β1 integrins signaling protects ARVMs from β-AR-stimulated apoptosis, (3) activation of ERK1/2 plays a critical role in the anti-apoptotic effects of β1-integrin signaling. These data suggest that β1 integrin signaling protects ARVMs against β-AR-stimulated apoptosis possibly via the involvement of ERK1/2. J. Cell. Biochem. 89: 381–388, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: integrin; adrenergic receptors; apoptosis; ERK1/2; myocytes

Apoptosis has been shown to occur during heart failure in humans and in animal models of myocardial hypertrophy and failure [Anversa et al., 1996; Gill et al., 2002]. Cardiac myocyte loss due to apoptosis is proposed to be a major factor in the pathogenesis of cardiac disease [Gill et al., 2002]. Levels of norepinephrine (NE) are increased in the hearts of patients with heart failure [Swedberg et al., 1984; Hasking et al., 1988]. We and others have shown that stimulation of β-adrenergic receptor (β-AR) induces apoptosis of cardiac myocytes in vitro and in vivo [Communal et al., 1998, 1999; Shizukuda et al., 1998; Iwai-Kanai et al., 1999; Zaugg et al., 2000].

Integrins, a family of αβ-heterodimeric cell surface receptors, link the extracellular matrix (ECM) proteins and the intracellular cytoskeleton [Hynes, 1992; Giancotti and Ruoslahti, 1999]. Disruption of these linkages has been described in patients with dilated cardiomyopathy [Towbin, 1998] and tachycardia-induced heart failure [Zellner et al., 1991]. Integrins provide a dynamic interaction of environmental cues and intracellular events. Intracellular

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signaling events regulated by integrins include changes in gene expression, intracellular calcium and pH, activation of cytoplasmic kinases including ERK1/2, and organization of actin cytoskeleton leading to changes in cell growth, migration, and differentiation [Giancotti and Ruoslahti, 1999]. Neonatal cardiac myocytes express mainly $\alpha1\beta1$, $\alpha3\beta1$, and $\alpha5\beta1$ integrins, and attach to collagen type I, III, and IV, laminin (LN), and fibronectin [Terracio et al., 1991; Hornberger et al., 2000]. Adult cardiac myocytes express mainly $\alpha3\beta1$ integrins and adhere well to LN and type IV collagen, but poorly to other collagens and fibronectin [Borg et al., 1984; Terracio et al., 1991]. $\beta1$ integrins play a critical role in epidermal growth factor-induced proliferation of fetal cardiac myocytes [Hornberger et al., 2000] and participate in $\alpha1$-adrenergic receptor ($\alpha1$-AR)-induced hypertrophy of neonatal rat cardiac myocytes [Ross et al., 1998]. Recently, using Cre-Lox technology to inactivate the $\beta1$ integrin gene exclusively in ventricular cardiac myocytes, Shai et al. [2002] demonstrated that $\beta1$ integrins play an important role in myocardial fibrosis and cardiac failure.

Loss of attachment to ECM induces apoptosis, also referred as anoikis, in many cells [Meredith et al., 1993; Frisch and Francis, 1994]. $\beta1$ integrins signaling is suggested to provide protection against apoptosis [Giancotti and Ruoslahti, 1999; Mooney et al., 1999]. However, the regulation of $\beta1$ integrins expression, and their role in β-AR-stimulated apoptosis of cardiac myocytes remain largely unknown. In this study, we report that stimulation of $\alpha1$-AR increases $\beta1$ integrins expression, and $\beta1$ integrins signaling protects ARVMs against β-AR-stimulated apoptosis via the activation of ERK1/2.

EXPERIMENTAL PROCEDURES

Myocyte Isolation and Culture

Calcium-tolerant ARVMs were isolated from the hearts of adult male Sprague–Dawley rats (200–220 g) as described [Communal et al., 1998]. Briefly, hearts were perfused retrogradely with nominally Ca^{2+}-free Krebs–Henseleit bicarbonate buffer, minced, and dissociated in the same buffer containing 0.02 mg/ml trypsin and 0.02 mg/ml deoxyribonuclease. The cell mixture was filtered and sedimented through 60 μg/ml bovine serum albumin (Sigma, St. Louis, MO) to separate ventricular myocytes from nonmyocyte cells. The cell pellet was resuspended in ACCT medium consisting of Dulbecco's modified Eagle's medium (DMEM) with 2 mg/ml bovine serum albumin, 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

The ARVMs were then plated in ACCT medium at a density of 30–50 cells/mm^2 on 100-mm culture dishes (Fisher Scientific, Pittsburgh, PA) or glass coverslips (Fisher Scientific) precoated with LN (1 μg/cm^2, Gibco, Carlsbad, CA). After 1 h, the dishes were washed with ACCT to remove the nonadherent cells. Experiments were performed following 16 h of culture.

Cell Treatment

The cells were pretreated with prazosin (PZ; 0.1 μM, Sigma) or propranolol (PRO; 2 μM, Sigma) for 30 min followed by treatment with L-norepinephrine (NE; 10 μM, Sigma) in the presence of ascorbic acid (100 μM) for 24 and 48 h to assess $\beta1$ integrins expression. To study apoptosis, cells were pretreated with PZ for 30 min followed by treatment with NE (10 μM) for 24 h. In some experiments, LN (10 μg/ml, Gibco, Carlsbad, CA), rat anti-$\beta1$ integrin antibodies (10 μg/ml, sodium azide free, Pharmingen, San Diego, CA), or PD 98059 (PD; 10 μM, Calbiochem, La Jolla, CA) were added prior to the addition of NE + PZ.

Western Analysis

Cell lysates were prepared in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, and 0.5% Nonidet P-40) and total proteins (70 μg) were analyzed by western blot using monoclonal anti-$\beta1$ integrin (BD Biosciences, Palo Alto, CA) or phospho-specific ERK1/2 antibodies (New England BioLabs, Beverly, MA) as described [Communal et al., 2000].

TUNEL-Staining

Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed on cells plated on glass coverslips using a Roche Molecular Biochemicals, Indianapolis, IN in situ death detection kit according to the manufacturer's instructions. The percentage of TUNEL-positive myocytes (relative to total myocytes) was determined by counting

200–250 cells in 20 randomly chosen fields per cover slip for each experiment.

Flow Cytometry

Flow cytometry (fluorescence-activated cell sorter analysis) was performed on a FACS Star Plus using Lysis II software (Becton-Dickinson) as described previously [Communal et al., 1998, 1999]. The percentage of apoptotic cells was determined as fraction of cells with hypodiploid DNA content.

Statistical Analysis

All data are expressed as mean ± SE. Comparisons between control and treatments were performed using Student's unpaired *t*-test. Statistical significance of multiple treatments was determined by analysis of variance and a post hoc Tukey's test. Probability (P) values of less than 0.05 were considered to be significant.

RESULTS

NE Increases β1 Integrins Expression in ARVMs

To study if NE regulates β1 integrins expression in cardiac cells, ARVMs were treated with NE (10 μM) and total proteins were analyzed by western blot using anti-β1 integrin antibodies. This analysis showed low basal expression of β1 integrin by ARVMs plated on LN. NE increased β1 integrin expression by 2 ± 0.6 folds ($n = 3$, $P < 0.05$ vs. CTL; Fig. 1A) within 24 h of exposure. This increase in β1 integrins expression was further enhanced following 48 h of NE treatment (Fig. 1B). The increase in β1 integrins expression was inhibited by α1-AR antagonist PZ, but not by β-AR antagonist PRO. A combination of PZ + PRO alone had no effect on β1 integrins expression, however, this combination inhibited NE-mediated increases in β1 integrins expression. A trend towards increased β1 integrins expression was observed when the cells were treated with NE + PRO as compared to NE alone, however, the increase was not found to be statistically significant.

Previously, we have shown that stimulation of α1-AR activates ERK1/2, not p38 kinase and JNKs, in ARVMs [Xiao et al., 2001]. To study if activation of ERK1/2 is involved in α1-AR-stimulated increases in β1 integrins expression, ARVMs were pretreated with PD 98059 (10 μM), a specific inhibitor of ERK1/2 pathway, for 30 min followed by treatment of cells with NE + PRO for 24 h. Analysis of total cell lysate using western blot showed that PD 98059 inhibits α1-AR-mediated increases in β1 integrins expression (Fig. 2).

β1 Integrins Signaling Protects ARVMs Against β-AR-Stimulated Apoptosis

To study if β1 integrins signaling participates in β-AR-stimulated apoptosis in ARVMs, ARVMs were pretreated with LN for 30 min followed by stimulation with NE + PZ for 24 h. LN is a major component of basement membrane and freshly isolated ARVMs adhere

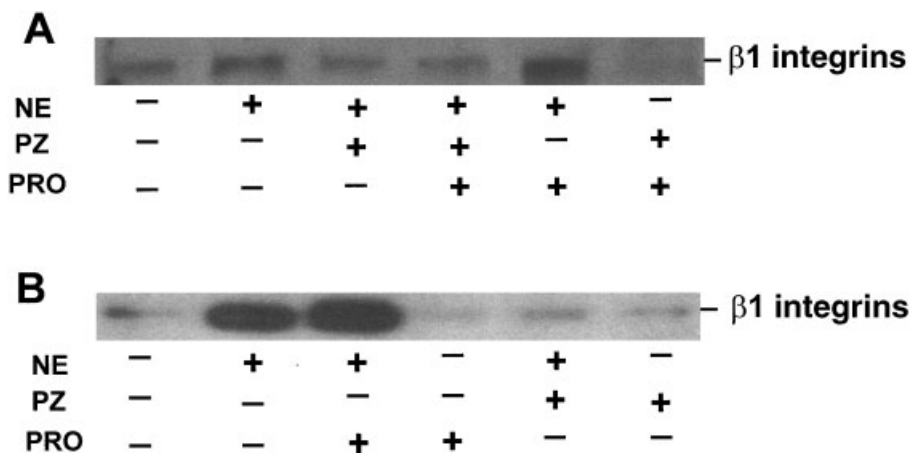

Fig. 1. Expression and regulation of β1 integrins in adult rat ventricular myocytes (ARVMs). ARVMs cultured for 24 h in defined media were pretreated with α1-AR antagonist, prazosin (PZ, 0.1 μM), or β-adrenergic receptor (β-AR) antagonist, propranolol (PRO, 2 μM) for 30 min followed by treatment with norepinephrine (NE, 10 μM) for 24 h (**A**) or 48 h (**B**). Total cell lysates were analyzed by Western blot using anti-β1 integrin antibodies. The experiments were repeated three times with similar results.

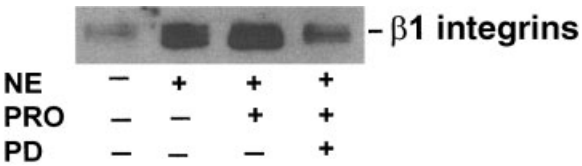

Fig. 2. α1-AR-stimulated increases in β1 integrins expression involves ERK1/2 activation. ARVMs cultured for 24 h in defined media were pretreated with PD 98059 10 μM) for 30 min followed by treatment with NE + PZ for 24 h. Total cell lysates were analyzed by Western blot using anti-β1 integrin antibodies.

efficiently to LN via β1 integrins [Borg et al., 1984]. Measurement of the number of apoptotic cells using TUNEL-staining assay showed that LN alone has no effect on the number of apoptotic cells, however, it inhibits β-AR-stimulated increases in the number of apoptotic cells (Fig. 3A). Flow cytometric analysis of apoptotic cells yielded similar results (Fig. 3B).

To further study the role of β1 integrins in β-AR-stimulated apoptosis, cells were pretreated with anti-β1 integrin antibodies. Integrins clustering with antibodies has been suggested to initiate integrins signaling [Burridge and Chrzanowska-Wodnicka, 1996]. This pretreatment also inhibited β-AR-stimulated increases in the number of apoptotic cells (1.08 ± 0.1, $n = 3$; $P < 0.05$ vs. NE/PZ; $n = 3$, Fig. 3A). Flow cytometric analysis of apoptotic cells yielded similar results (Fig. 3B).

Activation of ERK1/2

Analysis of ERK1/2 activation using phospho-specific antibodies demonstrated that exposure of cells to LN for 30 min increases ERK1/2 phosphorylation (activation) by 4.8 ± 1.6-folds ($P < 0.05$ vs. CTL, $n = 3$). β-AR stimulation weakly activated ERK1/2 (1.9 ± 0.2, $P < 0.05$, $n = 4$) at 15 min. However, the activation of ERK1/2 was significantly higher when cells were pretreated with LN for 15 min followed by β-AR stimulation for 15 min (9.0 ± 1.1 folds, $n = 3$; Fig. 4). PD 98059 inhibited LN− or LN+ NE/PZ-stimulated activation of ERK1/2 (not shown). Activation of ERK1/2 was also observed when cells were stimulated with anti-β1-integrin antibodies for 15 and 30 min (Fig. 5).

Inhibition of ERK1/2 Abolishes the Anti-Apoptotic Effects of Laminin

To study if anti-apoptotic effects of integrins signaling could be mediated via the activation of ERK1/2, ARVMs were pretreated with PD 98059 (10 μM) for 30 min followed by stimulation with LN in the presence of NE + PZ. Pretreatment with PD 98059 prevented the protective effects of LN (Fig. 6). A combination of PD 98059 and LN had no effect on the number of apoptotic cells.

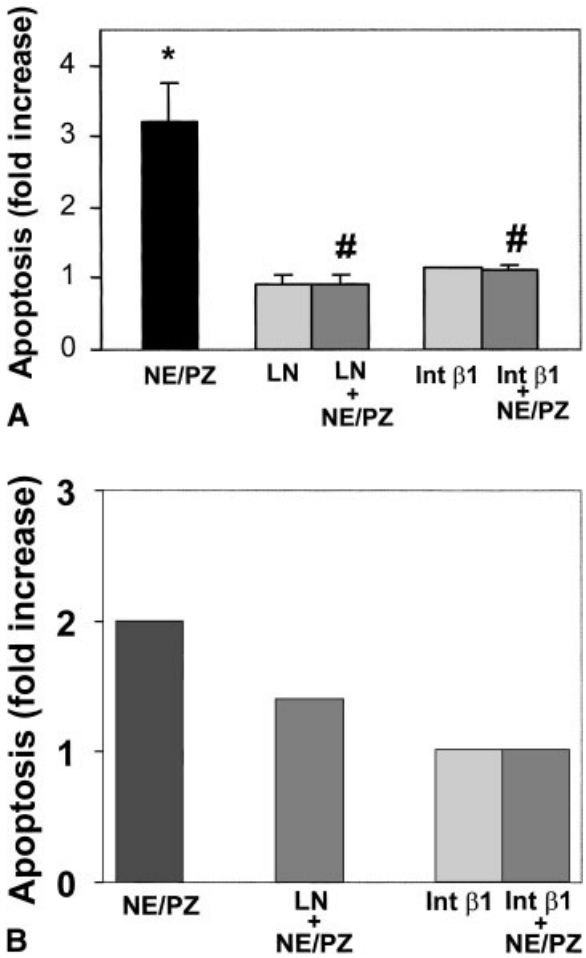

Fig. 3. β1 integrins signaling protects ARVMs against β-AR-stimulated apoptosis. ARVMs plated on cover slips or dishes (P100) were pretreated with laminin (LN; 10 μg/ml) or anti-β1 integrin antibodies (intβ1; 10 μg/ml) for 30 min followed by treatment with NE (10 μM) in the presence of PZ for 24 h. **A**: The number of apoptotic cells on cover slips was quantified using TUNEL-staining assay as described under ''Experimental Procedures.'' *$P < 0.05$ vs. CTL; #$P < 0.05$ vs. NE/PZ; $n = 4$. **B**: The number of apoptotic cells in dishes was quantified using flow cytometry.

DISCUSSION

Stimulation of β-AR induces apoptosis in neonatal and adult cardiac myocytes in vitro [Communal et al., 1998; Iwai-Kanai et al., 1999] and in vivo in rat myocardium following isoproterenol infusion [Shizukuda et al., 1998]. The present study demonstrates that NE increases

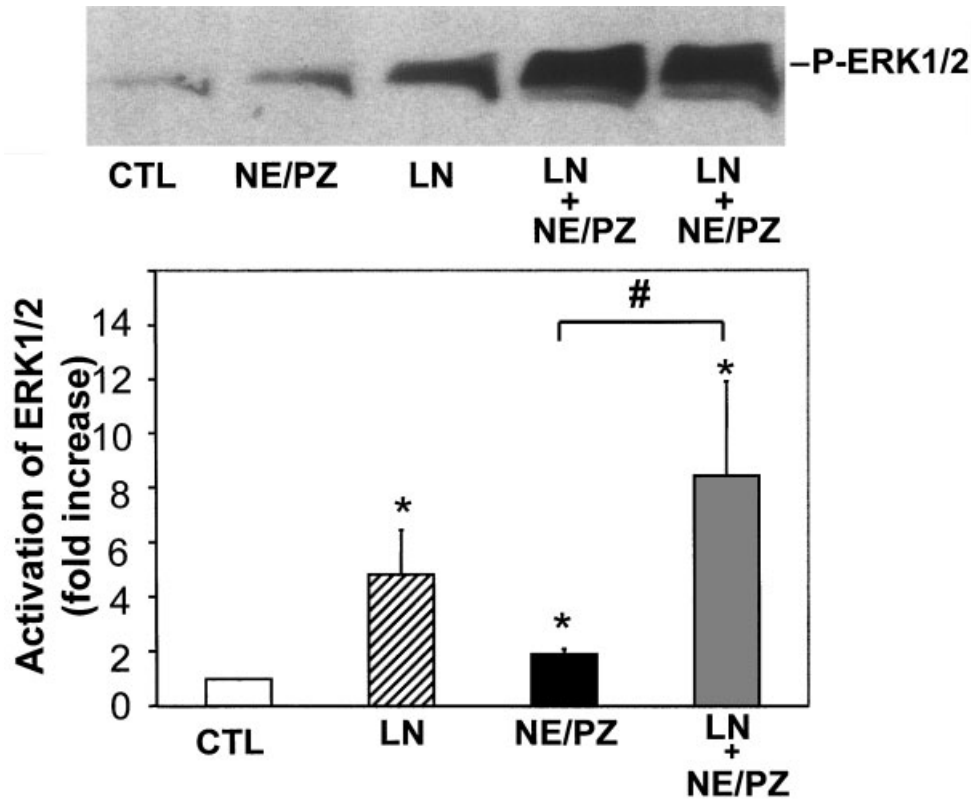

Fig. 4. Treatment of cells with LN activates ERK1/2. ARVMs plated for 24 h in defined media were pretreated with LN for 15 min followed by treatment with NE in the presence of PZ for 15 min. Total cell lysates were analyzed by Western blot using phospho-specific ERK1/2 antibodies. **Upper panel** depicts the activation of ERK1/2, while **lower panel** shows fold changes in activity from three independent experiments. *$P < 0.05$ vs. CTL; #$P < 0.05$ vs. NE/PZ; n = 3.

β1 integrins expression via the stimulation of α1-AR in ARVMs, not β-AR. This increase in β1 intergins expression is mediated via the activation of ERK1/2. Furthermore, the data presented here suggest that the stimulation of β1 integrins signaling protects cardiac myocytes from β-AR-stimulated apoptosis via the activation of ERK1/2 signaling pathway.

Cardiac myocytes express β1 integrins with costameric distribution at the Z line [Terracio et al., 1991]. Here, we demonstrate that NE increases β1 integrin expression in ARVMs via the stimulation of α1-AR, not β-AR. Consistent with these findings, increased expression of β1 integrins, specifically β1D which is expressed exclusively in the heart and skeletal muscle cells, was also observed in neonatal cardiac myocytes following α1-AR stimulation [Pham et al., 2000]. Upregulation of β1 integrin and its heterodimeric alpha (α5) partner has also been observed in the rat heart during cardiac hypertrophy [Terracio et al., 1991]. Previously, we have shown that α1-AR stimulation activates ERK1/2, not p38 kinase and JNKs, in ARVMs, and activation of ERK1/2 plays a critical role in the regulation of α1-AR-induced hypertrophy [Xiao et al., 2001, 2002]. In this study, we show that α1-AR-induced activation of ERK1/2 plays an important role in the regulation of β1 integrins expression. β1 integrins are shown to be involved in α1-AR-induced hypertrophy of neonatal cardiac myocytes [Ross et al., 1998].

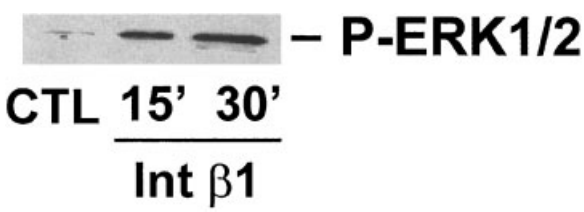

Fig. 5. Anti-β1 integrins antibodies activate ERK1/2. ARVMs plated for 24 h in defined media were treated with anti β1 integrin antibodies for 15 and 30 min. Total cell lysates were analyzed by Western blot using phospho-specific ERK1/2 antibodies.

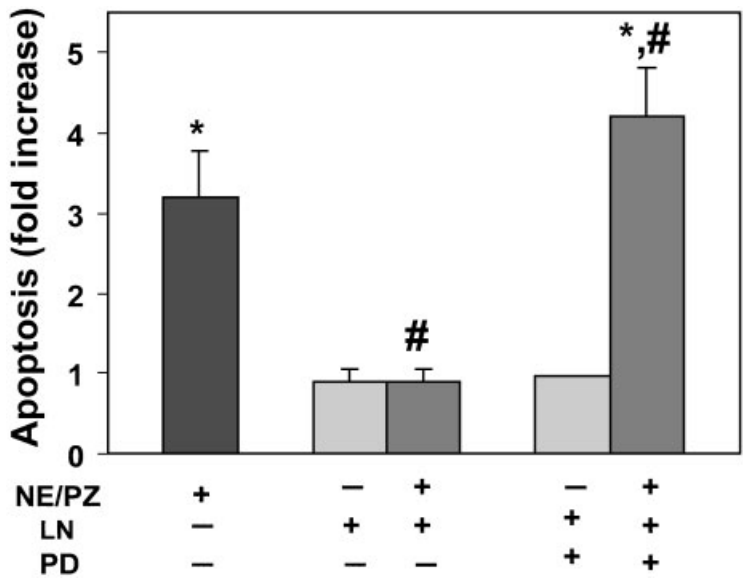

Fig. 6. Inhibition of ERK1/2 inhibits the protective effects of LN in β-AR-stimulated apoptosis. ARVMs plated for 24 h in defined media were pretreated with PD 98059 (10 μM) for 30 min followed by treatment with LN for 30 min. The cells were then stimulated with NE in the presence of PZ for 24 h. The apoptotic cells were quantified using TUNEL-staining as described. *$P < 0.05$ vs. CTL, #$P < 0.05$ vs. NE/PZ; n = 3.

Although not tested here, these observations raise the possibility that α1-AR-stimulated increases in β1 integrins may participate in α1-AR-induced hypertrophy of adult cardiac myocytes.

The novel finding of this study is that stimulation of β1 integrins signaling pathway protects adult cardiac myocytes against β-AR-stimulated apoptosis. Loss of attachment to ECM has been known to cause apoptosis in various cells [Frisch and Francis, 1994; Kuppuswamy, 2002]. Overexpression of focal adhesion kinase is shown to inhibit apoptosis in Madin–Darby canine kidney cells [Chan et al., 1999], while inhibition of integrin-linked kinase induced cell cycle arrest and apoptosis in prostate cancer cells [Persad et al., 2000]. Within the heart, induction of apoptosis occurred during the transition from hypertrophy to early failure and was correlated with the disruption of myocyte β1-integrins anchorage to the adjacent ECM [Ding et al., 2000]. Focal adhesion kinase has recently been shown to protect neonatal cardiac myocytes from undergoing apoptosis [Heidkamp et al., 2002]. Thus, integrins mediated signals may be a general requirement for cell growth and survival, including terminally differentiated cardiac myocytes.

It is interesting to note that ARVMs plated on LN are able to respond to LN and anti-β1 integrin antibodies added to the media. This could be due to the fact that ARVMs are not flat and exhibit β1 integrins expression at the Z-line [Terracio et al., 1991]. Therefore, it is possible that a subpopulation of integrins may not participate in cell adhesion. Soluble ECM proteins are shown to stimulate integrins signaling in other adherent cells. In human umbilical vein endothelial cells, addition of soluble osteopontin inhibits apoptosis of adherent endothelial cells deprived of growth factors [Khan et al., 2002].

Cytoplasmic domain of integrin subunits is known to signal through a number of molecules such as focal adhesion kinase, Akt, small GTPase, Raf, MEK, ERKs, etc. [Giancotti and Ruoslahti, 1999; Ross and Borg, 2001]. In this study, we show that β1 integrins signaling activates ERK1/2 in cardiac myocytes. This finding is supported by the observation that disruption of β1 integrin function in murine myocardium fails to activate p42-ERK in the heart after 7 days of aortic banding [Keller et al., 2001]. Although, we cannot rule out the possibility that activation of ERK1/2 using LN may possibly be due to the presence of mitogens in the LN. However, the rat LN (GibcoBRL) is ≥95% pure by SDS–PAGE, and according to the manufacturer, it is suitable for use in ELISA, production of antiserum, immunoblotting, and

stimulation of neurite growth. Furthermore, treatment of cells using anti-β1 integrin antibodies also activates ERK1/2.

We provide evidence that activation of ERK1/2 plays an anti-apoptotic role in cardiac myocyte apoptosis. These findings are consistent with the data in neonatal cardiac myocytes where ERK1/2 has been shown to play an anti-apoptotic role [Sheng et al., 1997; Iwai-Kanai et al., 1999; Araki et al., 2000]. The ability of cardiotrophin-1 to protect against serum deprivation-stimulated apoptosis was blocked by inhibition of ERK1/2 [Sheng et al., 1997]. Similarly, concurrent stimulation of α1-AR was found to oppose β-AR-stimulated apoptosis in neonatal cardiac myocytes, and this protective effect was blocked by inhibition of ERK1/2 [Araki et al., 2000]. Similar to integrin signaling, both cardiotropin-1 and α1-AR agonist activated ERK1/2 within 30 min. Previously, we have shown that β-AR-stimulated activation of ERK1/2 is not detected until 60 min, and this stimulation of ERK1/2 does not affect β-AR-stimulated apoptosis [Communal et al., 2000]. Possible explanations for these observations may include the time and/or kinetics of activation of ERK1/2. LN activates ERK1/2 prior to the stimulation of cells with β-AR, and the activation of ERK1/2 is greater when cells were stimulated with β-AR in the presence of LN. Previously, using immune-complex kinase assay, we demonstrated that activation of ERK1/2 occurs following 60 min of β-AR stimulation [Communal et al., 2000]. However, in this study we observed ~1.9 fold increase in phosphorylation of ERK1/2 following 15 min of β-AR stimulation. Differences in time of activation of ERK1/2 by β-AR stimulation may include the use of different methods. Western blot analysis of ERK1/2 activity using phospho-specific antibodies is a simple method and does not involve the use of radioactivity, however, it may overestimate the ERK1/2 activity.

In conclusion, the present study provides the first evidence that NE increases β1 integrins expression in adult cardiac myocytes, and β1 integrins signaling plays a protective role against β-AR-stimulated apoptosis. Further studies aimed at dissecting the signaling pathway involved in the activation of integrin-stimulated ERK1/2, and determining the molecular mechanisms by which β1 integrins signaling opposes β-AR-stimulated apoptosis may have important implications for the regulation of myocyte survival.

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