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## **Molecular and cellular characterization of cardiac overload-induced hypertrophy and failure**

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## CHAPTER 2

### Integrin stimulation-induced hypertrophy in neonatal rat cardiomyocytes is NO-dependent

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## Abstract

**Background:** Prolonged myocardial stretch typically leads to hypertrophy of cardiomyocytes. As integrins are cellular receptors of stretch, we hypothesize that integrin stimulation induces cardiomyocyte hypertrophy.

**Methods:** Integrins of neonatal rat cardiomyocytes (NRCMs) were stimulated with a peptide containing the Arg-Gly-Asp (RGD) sequence for 24 h. For comparison,  $\alpha_1$ -adrenergic stimulation by phenylephrine (PE) for 24 h was applied. Saline-treated NRCMs were used as control. The hypertrophic response was quantified by measuring cell surface area (CSA). Phosphorylation of NO-synthase-1 (NOS1) was assessed by immunocytochemistry.

**Results:** CSA was increased by 38% (IQR 31-44%) with RGD and by 68% (IQR 64-84%) with PE vs. control (both  $p < 0.001$ ). NOS-1 phosphorylation was increased by 61% with RGD and by 21% with PE vs. control (both  $p < 0.01$ ). A general NOS inhibitor (L-NAME) inhibited RGD-induced hypertrophy completely, but had no significant effect on PE-induced hypertrophy. Administration of NO-donor to NRCMs co-incubated with RGD + L-NAME partly restored hypertrophy (to 62% of the hypertrophic effect of RGD alone), but had no effect if incubated with PE + L-NAME. Ryanodine and BAPTA-AM inhibited RGD-induced hypertrophy completely, but not that induced by PE.

**Conclusions:** Integrin stimulation of NRCMs by RGD leads to hypertrophy, likely by activation of NOS-1. Abrogation of RGD-induced hypertrophic response upon NOS-inhibition and rescue of this hypertrophic effect by NO-donor suggest that integrin stimulation-induced hypertrophy of NRCMs is NO-dependent.

**Key words:** cardiomyocytes, hypertrophy, integrin,  $\alpha_1$ -adrenergic receptor, phenylephrine, nitric oxide synthase-1

**Abbreviations:** FAK, focal adhesion kinase; nNOS, neuronal nitric oxide synthase; NOS1, neuronal nitric oxide synthase; NO, nitric oxide; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase, also indicated by MAPK/ERK kinase; NRCM, neonatal rat cardiomyocyte; PE, phenylephrine; RGD, -Arg-Gly-Asp- sequence; L-NAME, N $\omega$ -nitro-L-arginine methyl ester; SMTC, S-methyl-L-thiocitrulline; PKC, protein kinase C; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester;

## Introduction

The primary stimulus for cardiac hypertrophy in response to hemodynamic overload is mechanical stretch itself. Upon mechanical stretch *in vitro*, protein synthesis in cardiomyocytes is stimulated associated with expression of “immediate-early” (IE) genes and re-expression of fetal genes [1-6]. As to the mechanosensors involved in stretch-induced protein synthesis of cardiomyocytes, the integrins are likely candidates as they take a central position between the extracellular matrix (ECM) of the tissue on which the stretch is applied and the cellular cytoskeleton through which the cell becomes deformed in response to stretch [7,8]. When ECM proteins interact with integrins, signals are transmitted by integrins to the cell cytoplasm through “outside-in signaling” [7]. Transmission of intracellular signals after integrin ligation is carried by, among others, focal adhesion kinase (FAK), a key cytoplasmic tyrosine kinase [9,10]. FAK associates with several different signaling proteins such as Src-family protein-tyrosine kinases, p130<sup>Cas</sup>, Shc, Grb2, and paxillin [11-14].

Earlier we have shown that short-term integrin stimulation of neonatal rat cardiomyocytes (NRCMs) using a pentapeptide containing the Arg-Gly-Asp (RGD) sequence resulted in elevations of intracellular Ca<sup>2+</sup> and NO concentrations [15]. The latter was considered the result of an increased concentration of the phosphorylated form of NO-synthase-1 (NOS1) [15].

In the present study we compared integrin stimulation-induced cardiomyocyte hypertrophy with a well-established cell model of hypertrophy, PE-induced cardiomyocyte hypertrophy [16], with respect to components of the intracellular signaling cascade [17]. Specifically, NRCMs were exposed to integrin stimulation by RGD or PE for 24 h, followed by assessment of the state of phosphorylation of several candidate signaling molecules, and quantification of the hypertrophic response. The specific roles in the hypertrophic process of NOS1, NO, Ca<sup>2+</sup>, ryanodine receptor, protein kinase C, and ERK1/2 were studied using inhibitors and an NO donor, sodium nitroprusside.

## Materials and Methods

### Cardiomyocyte cultures

Neonatal (2-day-old) rat ventricular cardiomyocytes (NRCMs) were prepared as described before [18]. Briefly, ventricular myocardium was dissociated with collagenase (Worthington, USA) and preplated in plastic dishes (Falcon Primaria, Becton-Dickinson, USA) to allow preferential attachment of non-cardiomyocytes. Then, 1 h later, non-adherent cells were transferred to Petri dishes (ø 35 mm), each containing a glass cover-slip (ø 25 mm) coated with rat-tail collagen (20 µg/mL; Sigma, USA). The culture medium consisted of Ham's F10 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 100 U/mL penicillin and 0.1 g/L streptomycin, all from Invitrogen (the Netherlands). The culture medium was replaced 24 h after seeding by a medium containing Ham's F10 and DMEM (Invitrogen)(1:1, v/v), 5% HS, antibiotics, and

100  $\mu\text{mol/L}$  5-bromo-2'-deoxyuridine (Sigma) to inhibit cardiac fibroblast proliferation. The cultures of NRCMs were grown in a humidified incubator at 37°C and 5% CO<sub>2</sub>. The medium was replaced 48 h after seeding by a medium containing Ham's F10 and DMEM (1:1,  $\frac{1}{v}$ ), 2.5% HS and antibiotics. The experiments had the approval of the Animal Experiments Committee of the Leiden University Medical Center.

### Experimental protocol

Seventy-two hours after seeding the NRCMs, PE (100  $\mu\text{mol/L}$ , Sigma), the pentapeptide Gly-Arg-Gly-Asp-Ser (RGD, 300  $\mu\text{g/mL}$ , Sigma), or PBS (control) were added to the culture medium. Twenty-four hours later, spontaneously beating NRCMs were lysed with ice-cold lysis buffer containing 0.1 mol/L Tris-HCl, 0.1% Tween-20 and 0.5 mmol/L sodium orthovanadate (Sigma), pH 7.5. After freezing and thawing for three times, the cell extracts were sonified two times for 30 s each, at 60% output using the microtip (Branson Ultrasonics, USA). Cell extracts were stored at -20°C before assay.

In separate series of experiments, spontaneously beating NRCMs were incubated with several drugs and agents, added to the culture medium 30 min before adding RGD, PE or PBS for a total duration of 24 h. These drugs and agents were (*i*) the general NOS-inhibitor N $\omega$ -nitro-L-arginine methyl ester (L-NAME, 100  $\mu\text{mol/L}$ , Sigma); (*ii*) the specific NOS1-inhibitor S-methyl-L-thiocitrulline (SMTC, 0.1  $\mu\text{mol/L}$ , Sigma); (*iii*) the MAP kinase inhibitors apigenin (10  $\mu\text{mol/L}$ , Calbiochem, USA) and PD98059 (100  $\mu\text{mol/L}$ , Calbiochem); (*iv*) the protein kinase C inhibitors chelerythrine (10  $\mu\text{mol/L}$ , Sigma) and bisindolylmaleimide (50  $\mu\text{mol/L}$ , Sigma); (*v*) an NO-donor (sodium nitroprusside (SNP), 10  $\mu\text{g/mL}$ , pharmacy of LUMC); (*vi*) an inhibitor of the Ca<sup>2+</sup> release channel of the sarcoplasmic reticulum ryanodine (100  $\mu\text{mol/L}$ , Sigma); (*vii*) the intracellular Ca<sup>2+</sup> chelator BAPTA-AM (50  $\mu\text{mol/L}$ , Invitrogen); (*viii*), and the sarcolemmal L-type Ca<sup>2+</sup> channel blocker verapamil (100  $\mu\text{mol/L}$ , Sigma).

### Protein assay

Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, USA) using bovine serum albumin (2 mg/mL, Pierce) as a standard.

### Western blotting

Ten  $\mu\text{g}$  of protein was loaded per well and run on SDS-PAGE, using NuPage 12% Bis-Tris gels (Invitrogen) and electro-transferred to polyvinylidene difluoride filter (PVDF) membrane (Hybond-P, GE Healthcare, the Netherlands). To reduce non-specific binding, the PVDF membrane was blocked for 1 h at room temperature with blocking buffer (ECL Advance blocking agent, GE Healthcare) in TBST solution composed of 10 mmol/L Tris-HCl, 0.05% Tween-20, 150 mmol/L NaCl, pH 8.0. Thereafter, the PVDF membrane was incubated with primary antibodies diluted 1:50,000 in blocking buffer at room temperature for 60 min, washed four times in TBST solution, and incubated with horseradish peroxidase-conjugated secondary antibodies diluted 1:35,000 in blocking buffer at room temperature for 60 min.

For ANP and GAPDH, the PVDF membrane was incubated with both anti-ANP and anti-GAPDH primary antibodies at the same time. For ERK1-P/ERK1 and ERK2-P/ERK2, the PVDF membrane was incubated with anti-phospho ERK1 and anti-phospho ERK2 antibody followed by incubation with anti-ERK1 or anti-ERK2 antibody, respectively, after stripping the membrane.

Bands were visualized with the enhanced chemiluminescence detection system (ECL Advance western blotting detection kit, GE Healthcare), exposed to X-ray film (GE Healthcare), and analysed using a densitometer (Ultrascan XL, LKB, Sweden).

Primary antibodies used were: rabbit polyclonal anti-ANP antibody (Chemicon), mouse monoclonal anti-GAPDH antibody (Chemicon), and mouse monoclonal anti-phospho ERK1 and anti-phospho ERK2 antibodies (Cell Signaling Technology, USA). Secondary antibodies used were goat anti-mouse IgG conjugated to HRP (Santa Cruz Biotechnology, USA) and goat anti-rabbit IgG conjugated to HRP (Santa Cruz Biotechnology).

### **Immunofluorescence microscopy**

Cultures of NRCMs were processed as described earlier [15]. Primary antibodies used were: mouse monoclonal anti-sarcomeric  $\alpha$ -actinin antibody (Sigma), rabbit anti-phospho (Tyr397) FAK antibody (Upstate, USA), and rabbit anti-phospho NOS1 antibody (Upstate). The primary antibodies were diluted 1:200 in PBS containing 1% FBS. Secondary antibodies were goat anti-rabbit IgG conjugated to FITC (Sigma), goat anti-mouse IgG conjugated to FITC (Sigma), goat anti-rabbit IgG conjugated to Alexa-568 (Molecular Probes, USA) and rabbit anti-mouse IgG conjugated to Alexa-568 (Molecular Probes, USA). The secondary antibodies were diluted 1:150 in PBS containing 1% FBS. Immunofluorescence images were produced using a fluorescence microscope (Eclipse, Nikon, Japan) equipped with a digital camera (DXM1200, Nikon). Images were analysed quantitatively using Image Pro Plus software (Media Cybernetics, USA) providing pixel intensity distributions of blue, green and red fluorescence.

Cell surface areas were measured by morphometric analysis of  $\alpha$ -actinin stained NRCMs using Image Pro Plus software (Media Cybernetics). Surface areas of five cells per field and a total of 60-80 cells per glass cover-slip were determined.

### **Statistics**

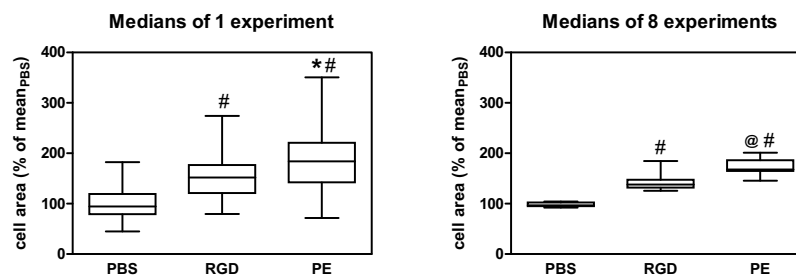
Results are expressed as median with interquartile range (IQR), unless stated otherwise. The significance of differences between means was calculated by Student's t-test and one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test wherever appropriate. Correlations were determined using a parametric test (Pearson). Differences were considered significant at  $p < 0.05$ . SPSS12 for Windows (SPSS Inc., USA) was used for statistical analysis. Experimental data were always compared with data collected in control experiments performed at the same day.

## Results

### Effects of RGD and PE on hypertrophy

Cardiomyocytes incubated with RGD-peptide (300  $\mu\text{g}/\text{mL}$ ) or PE (100  $\mu\text{mol}/\text{L}$ ) for 24 h showed a median increase in cell surface area by 38% (IQR 31-44%;  $p < 0.001$  vs. control) or by 68% (IQR 64-84%;  $p < 0.001$  vs. control) compared with the cells treated with PBS (Fig.1a). The left panel of fig.1a shows the medians, interquartile ranges and ranges of the cell areas in the three groups of one representative experiment, indicating that the large variability of cell surface area data per group represents a true variation in cell surface area within a particular NRCM culture. In the right panel of fig.1a the medians, interquartile ranges and ranges of 8 median values of cell areas obtained from 8 independent experiments are presented. Unlike the large variation of cell areas within a treatment group of one experiment (variation coefficient of  $\approx 30\%$ ), the between-experiment variation in the groups with the same treatment is much lower (variation coefficient of  $\approx 10\%$ ).

After treatment with RGD for 24 h we found an increase in average ( $\pm\text{SD}$ ) ANP/GAPDH ratio by  $49 \pm 9\%$  compared with PBS ( $p < 0.05$ ) (Fig. 1b). After treatment with PE for 24 h we found an increase in average ( $\pm\text{SD}$ ) ANP/GAPDH ratio by  $111 \pm 27\%$  compared with PBS ( $p < 0.05$ ) (Fig. 1b).

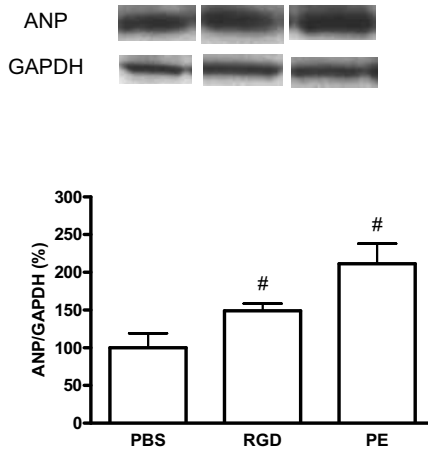


**Figure 1A. LEFT** Box and whisker plots of cell areas of three groups of NRCMs grown in the presence of phenylephrine (PE, 100  $\mu\text{mol}/\text{L}$ ), RGD-containing pentapeptide (RGD, 300  $\mu\text{g}/\text{mL}$ ), or control (PBS) for 24 h. Cell areas are expressed as percentage of the mean value of cell areas of the control culture. The boxes show median, 1<sup>st</sup> and 3<sup>rd</sup> quartiles, and the whiskers show the range. Data shown originate from one representative experiment. Per group  $n=77$ . **RIGHT** Box and whisker plots of median values of cell areas obtained from 8 independent experiments with NRCMs grown in the presence of phenylephrine (PE, 100  $\mu\text{mol}/\text{L}$ ), RGD-containing pentapeptide (RGD, 300  $\mu\text{g}/\text{mL}$ ), or control (PBS) for 24 h.

#  $p < 0.001$  vs. control (PBS), by ANOVA and Bonferroni correction

\*  $p < 0.001$  PE vs. RGD, by ANOVA and Bonferroni correction

@  $p < 0.01$  PE vs. RGD, by ANOVA and Bonferroni correction



**Figure 1B.** ANP/GAPDH protein ratio in NRCMs treated with PBS (control), RGD (300  $\mu\text{g}/\text{mL}$ ), and PE (100  $\mu\text{mol}/\text{L}$ ) for 24 h. Values are mean  $\pm$  SD.  $n=3$  cultures per bar. Representative blotting results are presented (<sup>#</sup>  $p<0.05$  vs. PBS).

### Effects of RGD and PE on activation of FAK, NOS1, ERK1/2 and PKC

After 24 h incubation, RGD had caused an average ( $\pm$ SD) increase in FAK phosphorylation by  $34\pm 27\%$  compared with PBS ( $p<0.05$ ). Incubation with PE for 24 h had caused an average ( $\pm$ SD) increase in FAK phosphorylation by  $111\pm 17\%$  compared with PBS ( $p<0.001$ ) (Table).

NOS1 phosphorylation was increased by  $61\pm 29\%$  (mean $\pm$ SD) by incubation with RGD for 24 h compared to PBS ( $p<0.01$ ), whereas PE had caused an increase in NOS1 phosphorylation by  $21\pm 11\%$  (mean $\pm$ SD;  $p<0.01$ ) (Table).



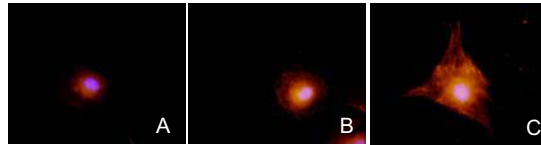
Signaling molecule	Control	+RGD	+PE	<i>P</i> (RGD vs. PE)
FAK-P	100±46	134±27 <sup>#</sup>	211±17 <sup>###</sup>	<0.001
NOS1-P	100±29	161±29 <sup>###</sup>	121±11	<0.01
ERK1-P	100±9	163±18 <sup>##</sup>	74±14	<0.001
ERK2-P	100±5	152±4 <sup>#</sup>	101±40	<0.05

<sup>#</sup> *p*<0.05 vs. Control      <sup>##</sup> *p*<0.01 vs. Control      <sup>###</sup> *p*<0.001 vs. Control

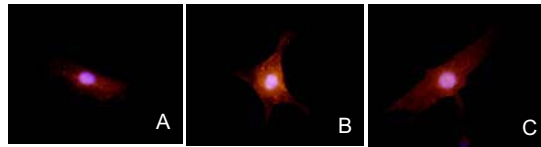
**Table.** Quantification of phosphorylated forms of signaling molecules such as focal adhesion kinase (FAK), nitric oxide synthase-1 (NOS1), and extracellular related kinases-1 and -2 (ERK1 and ERK2, respectively) after 24 h incubation of NRCMs in control medium (Control), medium containing RGD peptide (+RGD, 300 µg/mL), phenylephrine (+PE, 100 µmol/L). Phosphorylated forms of FAK and NOS1 were quantified by immunofluorescence microscopy. Number of independent observations is 5 per group. Phosphorylated forms of ERK1 and ERK2 were quantified by western blot. Number of independent observations is 3 per group.

Figure 2 shows representative immunofluorescent images of NRCMs stained for phosphorylated FAK and NOS1 before and after stimulation with PE (100 µmol/L) or RGD (300 µg/mL).

In NRCMs incubated with RGD for 24 h phosphorylated forms of ERK1 and ERK2 had increased by, on average (±SD), 63±18% and 52±4%, compared with PBS, respectively (both *p*<0.05). Incubation with PE for 24 h had no significant effects on ERK1/2 phosphorylation (Table). However, phosphorylation of ERK1/2 had already peaked during the first hour of incubation with PE, whereas their phosphorylation was still rising at the end of first hour incubation with RGD (data not shown).



**Figure 2a.** Immunofluorescence images of phosphorylated FAK in NRCMs treated with PBS (control, **A**), RGD (300 µg/mL, **B**) and PE (100 µmol/L, **C**).



**Figure 2b.** Immunofluorescence images of phosphorylated NOS1 in NRCMs treated with PBS (control, **A**), RGD (300 µg/mL, **B**) PE (100 µmol/L, **C**). In panels A, B, and C the secondary antibody used was goat anti-rabbit IgG conjugated to Alexa-568 (red). All images were taken at 100x magnification.

Cells pre-treated with MAP kinase inhibitor apigenin in the presence of RGD showed a complete inhibition of hypertrophy ( $p < 0.001$  vs. RGD). Cells pre-treated with MAP kinase inhibitor PD98059 or with the combination of apigenin and PD98059 also showed complete inhibition of RGD-induced hypertrophy ( $p < 0.001$  vs. RGD)(Fig. 3a). Cells pre-treated with apigenin in the presence of PE showed no significant change in CSA compared with cells treated with the “PE only” group. Likewise, cells pre-treated with PD98059 or with the combination of apigenin and PD98059 did not show a significant change in CSA compared with the “PE only” group (Fig. 3a).

Addition of the protein kinase C inhibitors bisindolylmaleimide (50 µmol/L) and chelerythrine (10 µmol/L) reduced the PE-induced hypertrophy by 41% ( $p < 0.001$  vs. “PE only”), whereas the RGD-induced hypertrophy was not significantly influenced by these agents compared to “RGD only” (Fig. 3b).

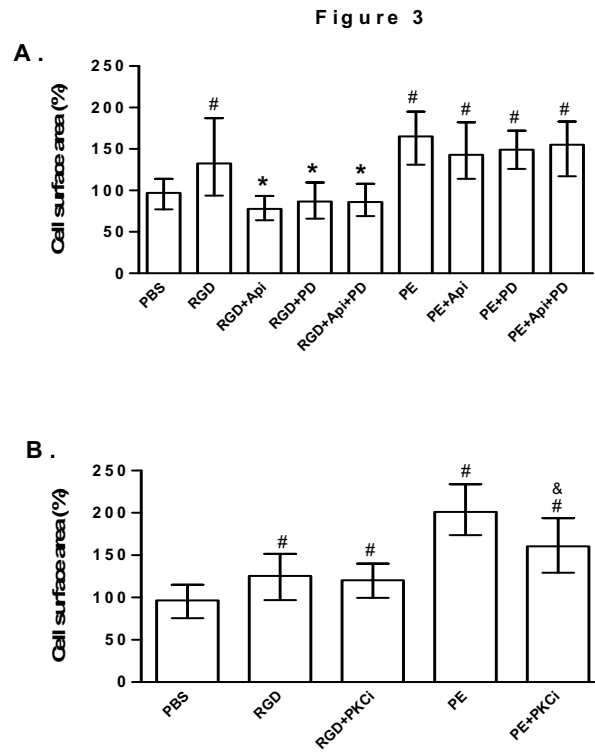


Figure 3

**A.** Effect of RGD (300  $\mu\text{g}/\text{mL}$ ) on cell surface area without and with pre-treatment with the MAP kinase inhibitors apigenin (Api, 10  $\mu\text{mol}/\text{L}$ ), PD98059 (PD, 100  $\mu\text{mol}/\text{L}$ ), and the combination of these two inhibitors and the effect of PE (100  $\mu\text{mol}/\text{L}$ ) on cell surface area without and with pre-treatment of apigenin (10  $\mu\text{mol}/\text{L}$ ), PD98059 (100  $\mu\text{mol}/\text{L}$ ), and the combination of these two inhibitors.

In all series of experiments, the incubations lasted 24 h.

Columns show median values with 1<sup>st</sup> and 3<sup>rd</sup> quartiles. N= 36-83 cells per bar.

#  $p < 0.05$  vs. PBS

\*  $p < 0.05$  vs. "RGD only"

**B.** Effect of RGD (300  $\mu\text{g}/\text{mL}$ ) on cell surface area without and with pre-treatment with protein kinase C inhibitors (PKCi, being 50  $\mu\text{mol}/\text{L}$  bisindolylmaleimide and 10  $\mu\text{mol}/\text{L}$  chelerythrine) and the effect of PE (100  $\mu\text{mol}/\text{L}$ ) on cell surface area without and with pre-treatment with these protein kinase C inhibitors.

In all series of experiments, the incubations lasted 24 h. Columns show median values with 1<sup>st</sup> and 3<sup>rd</sup> quartiles. N= 20-59 cells per bar.

#  $p < 0.05$  vs. PBS

§  $p < 0.05$  vs. "PE only"

**Effect of intracellular NO on RGD- and PE-induced hypertrophy**

NRCMs pre-treated with a general NOS-inhibitor L-NAME (100  $\mu\text{mol/L}$ ) in the presence of RGD showed a complete inhibition of hypertrophy ( $p < 0.001$  vs. RGD) (Fig. 4a). Cells pre-treated with the specific NOS1-inhibitor SMTC (0.1  $\mu\text{mol/L}$ ) in the presence of RGD also showed a complete inhibition of hypertrophy ( $p < 0.001$  vs. RGD)(data not shown).

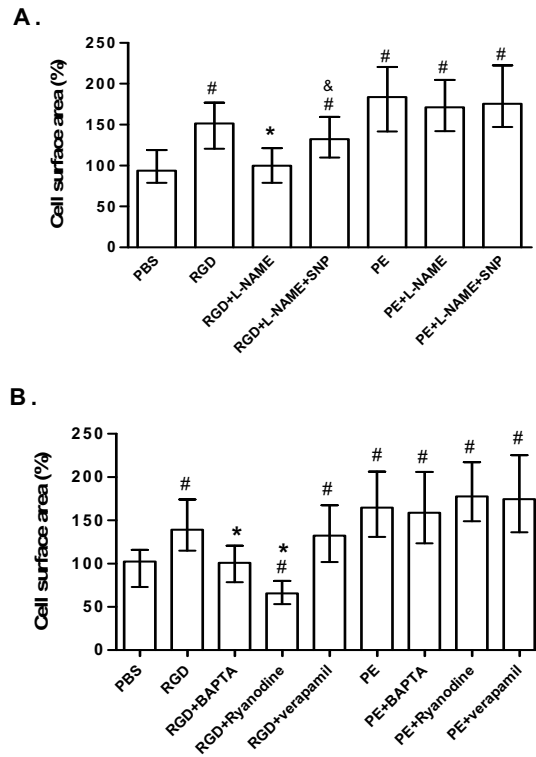
Cells pre-treated with L-NAME (100  $\mu\text{mol/L}$ ) in the presence of PE showed no significant change in CSA compared with the “PE only” group (Fig. 4a). Cells pre-treated with SMTC (0.1  $\mu\text{mol/L}$ ) in the presence of PE showed a 55% inhibition of hypertrophy compared with the “PE only” group ( $p < 0.05$  vs. PE)(data not shown). Addition of SNP (10  $\mu\text{g/mL}$ ) to NRCMs incubated with RGD + L-NAME for 24 h partially restored the hypertrophic capacity of RGD to 132% ( $p < 0.001$  vs. RGD+L-NAME) which is 62% of the full effect by “RGD only”. SNP added to NRCMs incubated with PE + L-NAME had no significant effect (Fig. 4a).

**Effect of intracellular  $\text{Ca}^{2+}$  on RGD- and PE-induced hypertrophy**

Pretreatment of NRCMs by BAPTA (50  $\mu\text{mol/L}$ ) inhibited the hypertrophic effect of RGD completely ( $p < 0.001$  vs “RGD only”). Likewise, blockade of the ryanodine receptor by ryanodine (100  $\mu\text{mol/L}$ ) caused a complete inhibition of RGD-induced hypertrophy. Verapamil (100  $\mu\text{mol/L}$ ) had no significant effect on RGD-induced hypertrophy (Fig. 4b). So,  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum appears to be a prerequisite for the hypertrophic capacity of RGD.

In PE-incubated NRCMs, BAPTA, nor ryanodine, nor verapamil had any effect on the hypertrophic effect of PE (Fig. 4b).

Figure 4



**Figure 4 A.** Effect of RGD (300  $\mu\text{g}/\text{mL}$ ) on cell surface area without and with pre-treatment with L-NAME (100  $\mu\text{mol}/\text{L}$ ) and L-NAME + SNP (10  $\mu\text{g}/\text{mL}$ ) and the effect of PE (100  $\mu\text{mol}/\text{L}$ ) on cell surface area without and with pre-treatment with L-NAME (100  $\mu\text{mol}/\text{L}$ ) and L-NAME + SNP (10  $\mu\text{g}/\text{mL}$ ). Columns show median values with 1<sup>st</sup> and 3<sup>rd</sup> quartiles. N= 72-80 cells per bar.

#  $p < 0.05$  vs. PBS

\*  $p < 0.05$  vs. "RGD only"

&  $p < 0.05$  vs. RGD + L-NAME

**B.** Effect of RGD (300  $\mu\text{g}/\text{mL}$ ) on cell surface area without and with pre-treatment with BAPTA (50  $\mu\text{mol}/\text{L}$ ), ryanodine (100  $\mu\text{mol}/\text{L}$ ) and verapamil (100  $\mu\text{mol}/\text{L}$ ), and the effect of PE (100  $\mu\text{mol}/\text{L}$ ) on cell surface area without and with pre-treatment with BAPTA (50  $\mu\text{mol}/\text{L}$ ), ryanodine (100  $\mu\text{mol}/\text{L}$ ) and verapamil (100  $\mu\text{mol}/\text{L}$ ). In all series of experiments, the incubations lasted 24 h. Columns show median values with 1<sup>st</sup> and 3<sup>rd</sup> quartiles. N= 48-90 cells per bar.

#  $p < 0.05$  vs. PBS

\*  $p < 0.05$  vs. "RGD only"

## Discussion

The present study shows that integrin stimulation with a pentapeptide containing the Arg-Gly-Asp (RGD) sequence for 24 h leads to NRCM hypertrophy *in vitro* via an NO-dependent mechanism. In comparison to the frequently used model of cellular hypertrophy by  $\alpha_1$ -adrenergic stimulation using PE, the intracellular signaling cascades of the two models differed in a number of aspects including dependence upon NO, intracellular  $\text{Ca}^{2+}$ , and protein kinase C.

Cardiac hypertrophy is a fundamental process of adaptation to an increased workload. During development of cardiac hypertrophy, cardiomyocytes undergo specific changes, such as rapid induction of immediate-early genes, quantitative and qualitative changes in gene expression and increased rate of protein synthesis [6]. The primary stimulus for cardiac hypertrophy is mechanical stress and/or an accompanying increase in neurohumoral factors [1-5]. Mechanical stretch of cardiomyocytes *in vitro* produces effects that are generally similar to those observed in the heart exposed to hemodynamic overload [1-5], including activation of MAP kinases [3,19,20].

The cardiomyocyte's mechanosensors are the integrins, being transmembrane proteins that link the extracellular matrix (ECM) to the cellular cytoskeleton at places called focal adhesion sites [21]. The cytoplasmic domain forms links with cytoskeletal proteins and intracellular signaling molecules such as focal adhesion kinase (FAK) [21,22]. Integrins can function as signal transducers that regulate gene expression and cellular growth [7,23]. Integrins also influence the hypertrophic response in cardiomyocytes [24]. Overexpression of  $\beta_1$ -integrin in the cardiomyocyte increases ANP expression and protein synthesis without affecting DNA synthesis [24]. In addition, Kuppuswamy *et al.* have shown an association of  $\beta_3$ -integrin and two non-receptor kinases, FAK and c-Src, with the cytoskeleton in hypertrophic cat hearts [25]. Upon integrin-induced phosphorylation of these kinases, they become activated, may recruit Grb2/Sos and then initiate the Ras/ERK signal transduction pathway [11,26-30]. We found an increase in FAK phosphorylation in both integrin-stimulated as well as PE-stimulated NRCMs after 24 h, although significantly more with PE than with integrin stimulation. Pham *et al.* have shown that  $\alpha_1$ -adrenergic receptor stimulation induced a rapid and sustained phosphorylation of FAK, which appeared to be a prerequisite for a hypertrophic response of  $\alpha_1$ -adrenoceptor agonists, as FAK-mutants had a blunted  $\alpha_1$ -adrenoceptor-induced hypertrophic response [31].

PE, like several other agonists of G-protein coupled receptors, stimulates sarcomeric organization, induces ANP and produces hypertrophy in NRCMs. Moreover, PE stimulates a rapid increase in tyrosine phosphorylation of focal adhesion proteins including FAK, paxillin, and p130Crk-associated substrate and subsequent formation of peripheral focal complexes [32].  $\alpha_1$ -adrenergic receptor-stimulated signaling implies phosphorylation of p90RSK [33]. Eukaryotic elongation factor 2 kinase (eEF2K) is a known p90RSK target. eEF2K phosphorylation was significantly reduced by the protein kinase C inhibitor bisindolylmaleimide [34]. In the nucleus p90RSK phosphorylates transcription factors, including c-Fos, Nur77 and CREB [35,36]. In the present study, inhibition

of protein kinase C reduced PE-induced hypertrophy by 41%, but had no effect on RGD-induced hypertrophy.

Although ERK signaling has been shown to be involved in effective PE-induced cardiomyocyte hypertrophy [37-39], other studies including the present study have shown that the MEK1 inhibitor PD98059 did not block PE-induced cardiomyocyte hypertrophy to a significant extent [40-42]. In the present study, the MAP kinase inhibitors apigenin and PD98059 inhibited hypertrophy considerably in RGD-treated NRCMs, and hardly in PE-treated NRCMs.

The present study has been conducted with NRCMs that upon stimulation of integrins with RGD-peptide revealed increased intracellular concentrations of NO and  $Ca^{2+}$ , in combination with markedly increased concentrations of phosphorylated NOS1 [15]. Accordingly, the NOS inhibitors L-NAME and SMTC prevented the RGD-induced hypertrophy of NRCMs, but had little effect on PE-induced hypertrophy of NRCMs. However, the pro-hypertrophic effect of NO and the anti-hypertrophic effect of NO inhibitors have to be considered in association with integrin stimulation. In the literature we can find studies reporting that NO has pro-hypertrophic effects [43-46] and studies reporting that NO has anti-hypertrophic effects [47-49]. These apparently contradictory observations are explained by taking into account the fact that in the cardiomyocyte NOS3 and NOS1 are localized at sarcolemma and sarcoplasmic reticulum, respectively, leading to NO formation that is confined to particular compartments with different consequences. This is illustrated by the finding that in NOS3-deficient mice, blood pressure is  $\approx 30\%$  higher than in wild-type mice and associated with LV hypertrophy [49], whereas in NOS1-deficient mice blood pressure is similar to that in wild-type mice when awake, and hypotensive under anaesthesia [50,51]. Other factors that may explain why NO may have pro- and anti-hypertrophic effects are (i) the formation of other vasoactive compounds, such as ANP and angiotensin-II, (ii) the formation of superoxide anion due to “uncoupling” of the NOS isoenzyme, thereby causing oxidative stress, (iii) the reaction of NO with superoxide anion to form peroxynitrite, and (iv) direct effects of NO on proteins by means of S-nitrosylation, or indirect effects via stimulation of guanylate cyclase and cGMP formation.

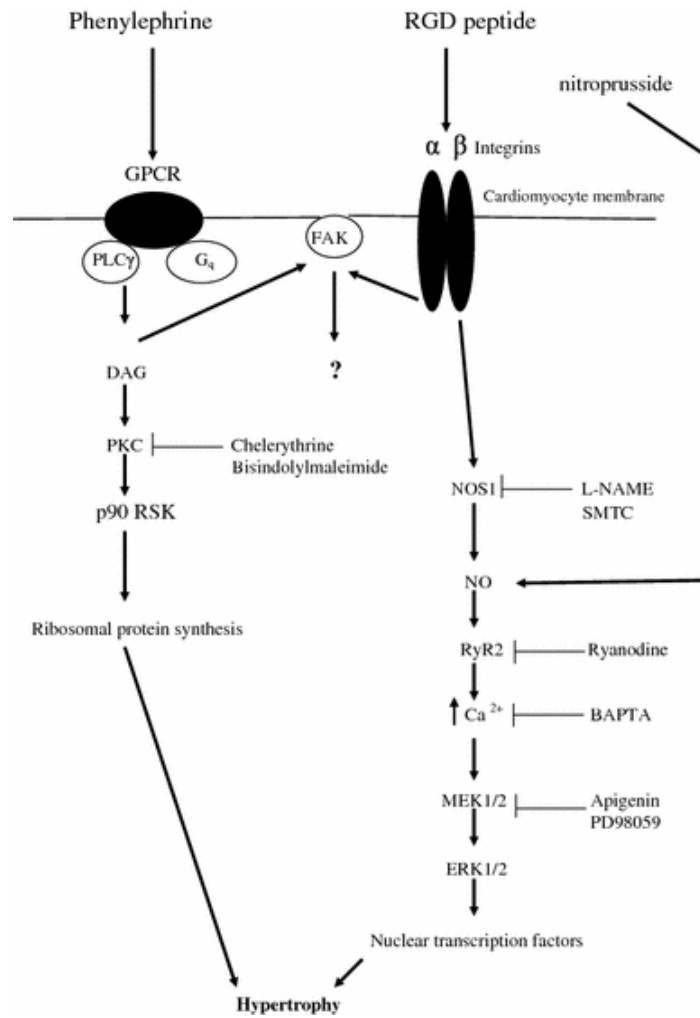
The present study has clearly demonstrated the calcium dependence of the integrin stimulation-induced hypertrophy. This dependence was demonstrated particularly by inhibiting RyR function and by chelating intracellular  $Ca^{2+}$  ions, whereas blockade of  $Ca^{2+}$  influx via L-type  $Ca^{2+}$  channels by verapamil was without any effect.  $Ca^{2+}$  release from the SR appears necessary for integrin stimulation-induced hypertrophy. NO controls RyR2 function probably by NO-induced nitrosylation of RyR2, leading to increased conductance of RyR2 and increased  $Ca^{2+}$  concentration [52-55].

As to the limitations of our study, we note (i) the use of collagen as an ECM substrate to culture the NRCMs; we did not investigate the effects of other ECM components such as fibronectin, laminin and gelatin on NRCM hypertrophy; (ii) the use of neonatal rat cardiomyocytes in vitro; we did not investigate whether adult rat cardiomyocytes were behaving similarly, as in our hands adult rat cardiomyocytes loose vitality within 24 h; (iii) the time window of 24 h which represents acute rather than chronic events.

## Conclusions

The present study demonstrates that although integrin stimulation and  $\alpha_1$ -adrenergic receptor stimulation both lead to hypertrophy of NRCMs *in vitro*, intracellular signaling cascades differ between the two models. In Fig. 5 a hypothetical scheme of both signal transduction pathways is presented, showing that the PE-stimulated signaling cascade is protein kinase C-dependent and that the RGD-stimulated signaling cascade contains both release of  $\text{Ca}^{2+}$  ions from the SR and production of NO. In the latter pathway, we have clearly demonstrated that exogenous NO (donated by nitroprusside) was able to bypass the L-NAME-induced blockade of the signaling pathway. Absence of a hypertrophic response by NOS-inhibition suggests that integrin stimulation-induced hypertrophy is NO-dependent, whereas stimulation by PE appears largely NO-independent.





**Figure 5**

A hypothetical scheme of signaling events leading to hypertrophy induced by  $\alpha_1$ -adrenergic receptor stimulation using phenylephrine and by integrin stimulation using RGD peptide.

Abbreviations: GPCR, G-protein coupled receptor; PLC $\gamma$ , phospholipase- $\gamma$ ; G $_q$ , G-protein q; DAG, diacylglycerol; PKC, protein kinase C; p90RSK, p90 ribosomal S6 kinase; FAK, focal adhesion kinase; NOS1, nitric oxide synthase-1; L-NAME, N $\omega$ -nitro-L-arginine methyl ester; SMTC, S-methyl-L-thiocitrulline; NO, nitric oxide; RyR2, ryanodine receptor-2; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester; MEK1/2, MAP kinase kinase 1/2; ERK1/2, p44 and p42 MAP kinase

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