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UNEXPECTED ANTIHYPERTROPHIC RESPONSES TO LOW-LEVEL STIMULATION OF PROTEASE-ACTIVATED RECEPTORS IN ADULT RAT CARDIOMYOCYTES

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Abstract

Activators of protease-activated receptors PAR-1 and PAR-2 such as thrombin and synthetic hexapeptides promote hypertrophy of isolated neonatal cardiomyocytes at pathological concentrations. Since PAR-activating proteases often show dual actions at low vs. high concentrations, the potential hypertrophic effects of low-level PAR activation were examined. In H9c2 cardiomyoblasts, mRNA expression of the hypertrophic marker atrial natriuretic peptide (ANP) was significantly increased only by higher concentrations of thrombin, trypsin or the synthetic PAR-2 agonist SLIGRL. The dual PAR-1/PAR-2 agonist SFLLRN did not influence basal ANP mRNA expression in H9c2 cells. Low concentration of thrombin or trypsin (up to 0.1 U/ml) or of the synthetic ligands SFLLRN and SLIGRL (1 μ M) however all suppressed ANP mRNA expression stimulated by Ang II. The PAR-1 selective ligand TFLLRN exerted a comparable effect as SFLLRN. In adult rat cardiomyocytes, protein synthesis determined by [3 H]phenylalanine incorporation was not increased by various PAR agonists at concentrations 10-fold lower than conventionally used to study PAR function *in vitro* (10 μ M for SFLLRN or SLIGRL, 0.1 U/ml for thrombin or trypsin). The positive control endothelin-1 (ET-1, 60 nM) however significantly increased protein synthesis in adult rat cardiomyocytes. Addition of low concentrations of PAR agonists to cardiomyocytes treated with ET-1 or Ang II suppressed [3 H]phenylalanine incorporation induced by the hypertrophic stimuli. The inhibitory effect of SFLLRN effect was partially reversed by the PAR-1 antagonist RWJ56110. These findings suggest that physiological concentrations of PAR activators may suppress hypertrophy, in contrast to the pro-hypertrophic effects evident at high concentrations. PAR-1 and PAR-2 may dynamically control cardiomyocyte growth, with the net effect critically dependent upon local agonist concentrations. The precise significance of proposed concept of bimodal PAR function in cardiomyocytes remains to be defined, particularly *in vivo* where hemodynamic and other regulatory factors may counteract or mask the direct cellular actions described here.

Introduction

Interest is emerging in the role of coagulant and inflammatory cell-derived proteases in myocardial ischemia/reperfusion injury, cardiac remodeling and hypertrophy (Antoniak *et al.*, 2011; Pawlinski *et al.*, 2007; Strande *et al.*, 2007). Cardiac hypertrophy is an essential adaptive response of the mature myocardium to sustain cardiac output when wall tension increases, but prolonged or excessive hypertrophy increases the risk for sudden death and progression to heart failure. Hypertrophic cardiomyocyte growth is characterised by increased protein synthesis and cell size, a fetal gene program and an altered sarcomere structure (Ritchie *et al.*, 2009). Classical triggers include myocyte stretch and neurohumoral factors such as endothelin-1 (ET-1) and angiotensin II (Ang II).

The coagulant protease thrombin activates similar cellular signaling pathways as ET-1 and Ang II, and has been reported to induce pro-hypertrophic signalling and morphometric changes in isolated neonatal cardiomyocytes (Glembotski *et al.*, 1993). Cellular actions of thrombin are mediated via protease-activated receptors (PAR), which are activated through proteolytic cleavage of the NH₂-terminal domain. This unmasks a tethered ligand sequence that autoactivates the receptor. Synthetic peptides mimicking this endogenous ligand sequence allow for specific PAR activation independently of receptor cleavage (Coughlin, 2000; Macfarlane *et al.*, 2001). Via PAR, clotting factors such as thrombin and activated factor X (FXa), as well as various tryptic or neutrophil-derived enzymes, can modulate cellular phenotype and function (Coughlin, 2000).

Cardiomyocytes express three of the four known PAR subtypes (Pawlinski *et al.*, 2007; Sabri *et al.*, 2003; Sabri *et al.*, 2000): PAR-1 is the prototypical receptor for thrombin and FXa, PAR-2 responds primarily to trypsin, tryptase and FXa but not to thrombin, and PAR-4 is a low-affinity receptor activated by high concentrations of thrombin and by neutrophil cathepsin G (Coughlin, 2000; Macfarlane *et al.*, 2001). PAR-3 not detected in rodent myocytes. (Sabri *et al.*, 2000) Like thrombin, synthetic PAR-1 and PAR-2 activating peptides are pro-hypertrophic agonists in isolated neonatal cardiomyocytes (Glembotski *et al.*, 1993; Pawlinski *et al.*, 2007; Sabri *et al.*, 2000). The hypertrophic response to PAR-4 activation is only weak compared to PAR-1, possibly since it activates alternative signalling pathways. (Sabri *et al.*, 2000)

Whether PAR-1 and PAR-2 also promote hypertrophic growth of adult cells has not been described, but cardiomyocyte-specific PAR-1 overexpression induces eccentric hypertrophy *in vivo*, suggesting that PAR also control hypertrophic growth in mature myocardium (Pawlinski *et al.*, 2007). In this model, hypertrophy is prevented by genetic deletion of tissue factor (TF) (Pawlinski *et al.*, 2007), raising the notion that normal myocardium continually generates low levels of thrombin and FXa that are “sensed” by overexpressed PAR-1, leading to hypertrophic signalling.

The concentrations of thrombin and synthetic activating peptides that induce hypertrophy of cardiomyocyte cultures are relatively high, generally 1 U/ml and ≥100 μmol/L respectively. These amounts are conventionally used to study cellular PAR function, and in the case of coagulant proteases approximate the levels generated during the clotting of human plasma (Rosenkranz *et al.*, 2011). As such, they are however representative of pathological PAR activation, which raises the question: how does constitutive, low-level PAR activation influence growth signalling in normal

myocardium, i.e. in the absence of artificial receptor over-activity or the high levels of coagulant proteases generated during ischemia/reperfusion injury? Depending on concentration, thrombin can exert dual actions on cellular growth, survival, migration and inflammatory signaling (Bae *et al.*, 2009; Murray *et al.*, 2006; Striggow *et al.*, 2000). Similarly trypsin, a PAR-2 activator, evokes vascular contractile responses at low concentrations, while higher levels induce relaxation (Assem *et al.*, 2004). The influence of non-pathological PAR activation on cardiomyocyte growth has not previously been determined. Our aim was therefore to examine how low level PAR stimulation impacts on protein synthesis, an early marker of hypertrophy.

Materials and methods

Materials

Angiotensin II (Ang II), human/porcine endothelin-1 (ET-1), thrombin and trypsin were purchased from Sigma (St. Louis, MO). The synthetic PAR activating peptides SFLLRN (dual PAR-1/PAR-2 activator), SLIGRL (PAR-2 selective activator) and TFLLRN were synthesised by Auspep (Parkville, Australia) or the BMFZ (Düsseldorf, Germany). RWJ56110 was generously provided by Dr Thomas Mercolino, Director External Relations, Johnson & Johnson Pharmaceutical Research and Development NJ. All reagents for cell culture were of tissue culture grade and all other materials were of analytical grade or higher and purchased from Sigma except where indicated.

Cell culture

H9c2 rat cardiomyoblasts were obtained from Sigma and utilized at passage 5-7 after 24h serum-deprivation. Cardiomyocytes were isolated from male Sprague-Dawley rats (200–280 g) as described (Ritchie *et al.*, 1998; Rosenkranz *et al.*, 2003) and resuspended in serum-free bicarbonate-buffered medium 199 (M199; with Earle's salts and 25 mM HEPES, JRH Biosciences; Lenexa, KS) supplemented with 0.2% albumin (bovine fraction V), 2 mM L-carnitine, 5 mM creatine, 5 mM taurine, 25 µg/ml gentamicin (Life Technologies; New York, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin (CSL Biosciences; Parkville, Australia). Cells were plated onto laminin (10 µg/ml, Collaborative Biomedical Products; Bedford, MA)-coated six-well plates (Falcon/Becton Dickinson; Lincoln Park, NJ) at a density of $\geq 8 \times 10^4$ cells per 35-mm well and equilibrated overnight at 37°C (5% CO₂ in air). Serum-free medium was refreshed prior to study. This technique yields <7% nonmyocyte contamination (Rosenkranz *et al.*, 2000). H9c2 cardiomyoblasts were cultured in Dulbeccos modified Eagles Medium containing 10% fetal calf serum (both GibcoBRL, Rockville, MD, USA) as described (Ritchie *et al.*, 2012). Cells were allowed to reach 90% confluence and were synchronized by serum-deprivation for 24h prior to use.

Cellular markers of myocyte hypertrophy

For measurement of ANP mRNA expression, H9c2 cardiomyoblasts were stimulated for 24h with study drugs as indicated. Total RNA was extracted with TriReagent® (Sigma-Adrich, München, Germany) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) as instructed by the manufacturers. Target gene expression was determined by real-time PCR relative to 18S ribosomal RNA, utilising QuantiTect Primer Assays (Qiagen, Hilden, Germany) and Quantace SensimixPlus SYBR-Green reagent (Bioline, Luckenwalde, Germany) in the ABI 7300 cycler (Applied Biosystems) as instructed by the manufacturers.[26]. Measurement of de novo protein synthesis was determined by [³H]phenylalanine incorporation as a conventional marker of cellular hypertrophy (Ritchie *et al.*, 1998; Rosenkranz *et al.*, 2003). Adult rat cardiomyocytes were incubated for 2h at 37°C in serum-free M199 containing 2 µCi/ml L-[2,3,4,5-³H]phenylalanine (NEN Lifesciences; Boston, MA) ± study drugs. These included endothelin (ET-1, 60 nM), Ang II (1 µM), thrombin or trypsin (both 0.001-0.100 U/ml), PAR-1 and PAR-2 synthetic ligands (both 1-30µM) and/or the PAR-1 antagonist RWJ56110 (10 µM). Following incubation, DNA and protein was precipitated with trichloroacetic acid and resuspended in 0.3 M sodium hydroxide (Ritchie *et al.*, 1998; Rosenkranz *et al.*, 2003) and [³H]phenylalanine incorporation was determined by liquid scintillation counting and normalised to DNA content as determined fluorimetrically using PicoGreen reagent (Molecular Probes; Eugene, OR).

Statistical analysis

Average results from 3 replicates per treatment were expressed as % of paired control and presented as mean±standard error from n number of individual myocyte preparations. Comparative statistical analyses utilised one-way analysis of variance (ANOVA) with Dunnett's post-hoc multiple comparisons procedure. Values of P < 0.05 were accepted as significant.

Results

Thrombin and synthetic PAR-1 and PAR-2 activators have been shown to elicit pro-hypertrophic responses at high concentrations (Glembotski *et al.*, 1993; Sabri *et al.*, 2000). Given that PAR activating proteases often show dual actions at low vs. high concentrations, the potential hypertrophic effects of low-level PAR activation were examined. In H9c2 cardiomyoblasts, thrombin concentration-dependently (over 0.01-10 U/ml) increased ANP mRNA expression, a cellular marker of hypertrophy, but this effect was very modest and reached statistical significance only at the highest concentration used (Fig. 1a). Unexpectedly, in Ang II (1 µM)-stimulated cells, ANP mRNA expression was *suppressed* by the lower concentrations of thrombin, while higher concentrations did not significantly influence Ang II-induced ANP mRNA levels (Fig. 1b). Basal ANP mRNA was also increased by higher but not lower concentrations of trypsin (Fig. 2a). The synthetic PAR-1/PAR-2 ligand SFLLRN was without effect at all concentrations studied (Fig. 2b), while the PAR-2 ligand SLIGRL exhibited hypertrophic actions at 3 µM and above (Fig. 2c). Trypsin, at the highest concentration that did not itself increase ANP mRNA expression (0.1 U/ml), significantly blunted

Ang II-stimulated hypertrophy in H9c2 cells, as did 1 μ M of the synthetic ligands SFLLRN, TFLLRN or SLIGRL (Fig. 2d).

[3 H]Phenylalanine incorporation, a measure of protein synthesis, was determined as a further marker of hypertrophy in adult rat cardiomyocytes. Unlike the positive control ET-1 (60 nM) which increased protein synthesis by approximately 30%, low concentrations (i.e. 10-fold lower than conventionally used to study PAR function *in vitro*) of PAR agonists (0.1 U/ml for thrombin or trypsin, 10 μ M for SFLLRN or SLIGRL) were without effect (Fig. 3). In cardiomyocytes co-stimulated with either ET-1 (Fig. 4a) or Ang II (Fig. 4b), however, low concentrations of thrombin or trypsin (0.001-0.1 U/ml) suppressed protein synthesis. The dual PAR-1/PAR-2 activator SFLLRN and the PAR-2 activator SLIGRL similarly inhibited [3 H]phenylalanine incorporation induced by ET-1 (Fig. 4c). The antihypertrophic effect of 10 μ M SFLLRN was partially reversed by the PAR-1 antagonist RWJ56110 (10 μ M, Fig. 4d).

Discussion

Cardiomyocyte hypertrophy is the direct cellular response to biomechanical stress, allowing the myocardium to sustain cardiac output when wall tension increases. Hypertrophic growth is characterised by increased protein synthesis and cell size, rather than proliferation, and is accompanied by a fetal gene program and altered sarcomere structure. While early cardiomyocyte growth represents an essential adaptive response to sustain cardiac output, prolonged or excessive pathological hypertrophy significantly increases the risk for sudden death and progression to heart failure (Frey *et al.*, 2003; Ritchie *et al.*, 2009; Tiyyagura *et al.*, 2006). Classical triggers of cardiomyocyte hypertrophy include myocyte stretch and neurohumoral factors such as ET-1 and Ang II (Yamazaki *et al.*, 1998). Pro-hypertrophic responses have also been reported with high levels of PAR agonist, ie. 100-300 μ M of activating peptides or up to 1 U/ml of thrombin (Glembotski *et al.*, 1993; Pawlinski *et al.*, 2007; Sabri *et al.*, 2000), which approximates the amount generated during clotting of human plasma (Rosenkranz *et al.*, 2011). This effect can likely be attributed to PAR-1, with only minor contribution by PAR-4, which is only weakly hypertrophic (Glembotski *et al.*, 1993; Pawlinski *et al.*, 2007; Sabri *et al.*, 2000; Sabri *et al.*, 2002).

The present study provides the first evidence that physiological (i.e. at low concentrations) level activation of PAR-1 and PAR-2 counteracts hypertrophic responses to two different stimuli in adult cardiomyocytes. This is contrary to the reported role of both receptors in events leading to post-ischemic cardiac remodelling (Glembotski *et al.*, 1993; Pawlinski *et al.*, 2007; Sabri *et al.*, 2000; Sabri *et al.*, 2002), specifically cardiac fibroblast proliferation and *stimulation* of cardiomyocyte hypertrophy. In H9c2 cardiomyoblasts, thrombin showed a modest trend to increase ANP mRNA expression in a concentration-dependent manner, but this hypertrophic effect was surprisingly weak and reached statistical significance only at the highest concentration (10 U/ml), unlike the strong ANP response reported previously in neonatal rat cardiomyocytes at concentrations above 0.001 U/ml. (Glembotski *et al.*, 1993; Pawlinski *et al.*, 2007; Sabri *et al.*, 2000; Sabri *et al.*, 2002). In the presence of the hypertrophic stimulus Ang II, thrombin by contrast attenuated the stimulated level of ANP expression in H9c2 cardiomyoblasts when applied at concentrations $\leq$ 0.1 U/ml.

Thrombin at 1-10 U/ml did not significantly impact on the Ang II response. Similarly, in adult rat cardiomyocytes, low concentrations of thrombin did not influence protein synthesis when applied alone, but strongly suppressed [³H]phenylalanine incorporation in cardiomyocytes stimulated with ET-1 or Ang II.

SFLLRN, the dual PAR-1/PAR-2 agonist, did not itself appear to exert pro-hypertrophic effects in the two models examined in this study, but like thrombin strongly inhibited hypertrophic responses to Ang II and ET-1 at low concentrations. In H9c2 cells, the inhibitory effect of SFLLRN was mimicked by the PAR-1 selective ligand TFLLRN, while in adult rat cardiomyocytes, the antihypertrophic SFLLRN effect could be partially blocked with the selective PAR-1 antagonist (RWJ56110). These observations confirm that PAR-1 contributes to the SFLLRN response while the residual inhibition can be attributed to PAR-2 activation (Lerner *et al.*, 1996). Accordingly, low concentration of the PAR-2 agonists trypsin or SLIGRL counteracted hypertrophic responses to ET-1 or Ang II in both H9c2 and adult rat cardiomyocytes. Unlike the PAR-1 activators however, at higher concentrations both trypsin and SLIGRL showed strong hypertrophic responses when applied alone, at least in the H9c2 cell-line. Potentially PAR-2 is more effectively coupled to pro-hypertrophic signalling than PAR-1, while antihypertrophic responses appear comparable.

The apparent contradiction between some of the effects shown here compared with those reported in other studies may arise in part from fundamental differences between the immature cells used in the earlier studies, and the adult cardiomyocytes described here. MAPK activation for example is an essential signal for hypertrophic growth in neonatal but not adult cardiomyocytes; similarly the mechano-sensing component of the neonatal response to stretch appears to be absent in the adult phenotype (Clark *et al.*, 1993; Schluter *et al.*, 1999; Takahashi *et al.*, 1992), while calcium handling and hence excitation–contraction coupling, a trigger for cardiomyocyte growth, (Roderick *et al.*, 2007) are not fully developed in immature cells (Janowski *et al.*, 2006; Maltsev *et al.*, 1999; Tibbits *et al.*, 2002). However, given that cardiomyocyte-specific PAR-1 overexpression induces hypertrophy *in vivo*, (Pawlinski *et al.*, 2007) it is likely that PAR-mediated control of hypertrophic growth will occur in both mature and immature phenotypes. The major difference between the previous studies and our current findings is that we utilised at least 10-fold lower concentrations of PAR agonists. Numerous groups have reported a concentration-dependent, bimodal response to PAR activation in a variety of cell types, where low concentrations (<0.5 U/ml) are generally protective, while higher levels evoke pathological effects. Thus low concentrations of proteases such as thrombin or trypsin (<0.5 U/ml) increase proliferation, migration and growth factor release from endothelial cells, enhance smooth muscle contractility, protect against ischemic neuronal degeneration, and exert anti-inflammatory actions, while higher concentrations (>0.5 U/ml) have the opposite effects. (Assem *et al.*, 2004; Bae *et al.*, 2009; Borrelli *et al.*, 2001; Jiang *et al.*, 2002; Murray *et al.*, 2006; Striggow *et al.*, 2000; Zain *et al.*, 2000) Our findings now demonstrate that cardiomyocytes also possess a dual responsiveness to PAR activation, with high concentrations of agonist likely to promote or at least support hypertrophy, while low concentration appear to counteract hypertrophic signalling.

Potentially, PAR-1 and PAR-2 represent unique regulatory switches controlling cardiomyocyte growth, with the net effect critically dependent upon local agonist concentrations. We propose that in normal myocardium, a continuous low level of activation may serve to counteract hypertrophic signals and thereby maintain

physiological protein turnover rates. Thrombin and FXa are likely candidates responsible for this endogenous “brake” mechanism. Tissue factor, prothrombin and prothrombinase are expressed in the myocardium and could conceivably allow for local generation of clotting factors, as has been described in other myocyte-rich extrahepatic tissues (Levy *et al.*, 2000; McBane *et al.*, 1997). The inhibition of hypertrophy in PAR-1 overexpressing mice with concomitant genetic deletion of tissue factor (TF) (Pawlinski *et al.*, 2007), implies that normal myocardium continually generates low levels of thrombin and FXa that are “sensed” by overexpressed PAR-1, leading to hypertrophic signalling. In myocardium damaged during ischemia/reperfusion, large amounts of blood-borne prothrombin may be activated by TF-expressing cardiomyocytes (Erlich *et al.*, 2000), conceivably increasing local levels to 1 U/ml or more. In this situation, PAR-1 and PAR-2 activation is augmented and the receptors undergo a functional switch to instead promote hypertrophic changes in cardiomyocytes. Interestingly, the hypertrophy induced by PAR activators is reported to be morphologically distinct from that elicited by adrenergic stimuli, and reflects the changes induced by the mechanical stimulus of volume overload rather than pressure overload, as seen with phenylephrine (Pawlinski *et al.*, 2007).

Thus PAR-1 and PAR-2 appear to play a unique and distinct role in the dynamic control of cardiomyocyte growth, with low level activation counteracting hypertrophic signals but at higher levels leading to increased hypertrophy. The reported upregulation of PAR-1 in failing hearts (Moshal *et al.*, 2005) is consistent with an ongoing contribution to disease progression. The precise significance of the proposed concept of bimodal PAR function in cardiomyocytes remains to be defined, particularly *in vivo* where hemodynamic and other regulatory factors may counteract or mask the direct cellular actions described here.

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Figure legends

Fig. 1. a In H9c2 cardiomyoblasts, thrombin modestly increased basal gene expression of the hypertrophic marker ANP, shown as mean $\pm$ SEM of n=3-4 individual experiments, expressed as fold change vs. control. *P<0.05 vs control. **b** Ang II (1 μ M)-stimulated ANP mRNA expression was counteracted by thrombin at low but not high concentrations. Data show mean $\pm$ SEM of n=3 individual experiments, expressed as fold change of Ang II alone. *P<0.05 vs. Ang II.

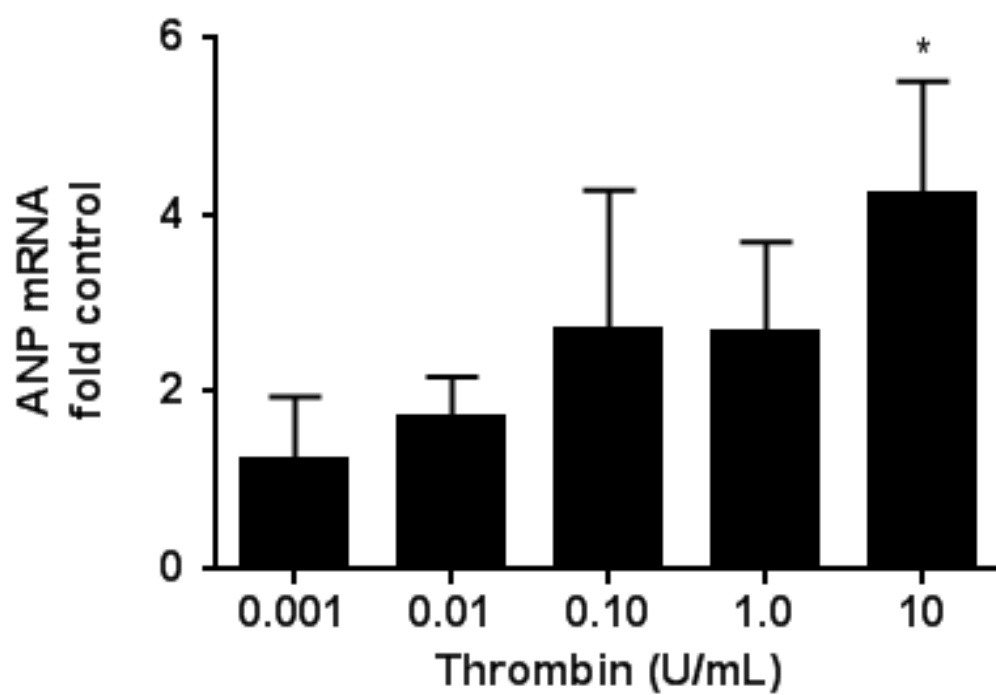
Fig. 2. a Concentration-dependent effects of trypsin, **b** SFLLRN or **c** SLIGRL on gene expression of the hypertrophic marker ANP in H9c2 cardiomyoblasts, shown as mean $\pm$ SEM of n=3 individual experiments, expressed as fold change vs. control. *P<0.05 vs control. **d** Inhibition of Ang II (1 μ M)-stimulated ANP mRNA expression by a single low concentration of trypsin (0.1 U/ml) or the synthetic PAR activators SFLLRN, TFLLRN or SLIGRL (all 1 μ M). Data show mean $\pm$ SEM of n=3 individual experiments, expressed as fold change of Ang II alone. *P<0.05 vs. Ang II.

Fig. 3. [3 H]phenylalanine incorporation, a measure of *de novo* protein synthesis, in adult rat cardiac myocytes stimulated with thrombin, trypsin (both 0.1 U/ml, n=3-4), SFLLRN or SLIGRL (both 10 μ M, n=6). The hypertrophic agonist ET-1 (60 nM) served as positive control (n=6). Data show mean $\pm$ SEM, expressed as fold change of control. *P<0.05 vs. control.

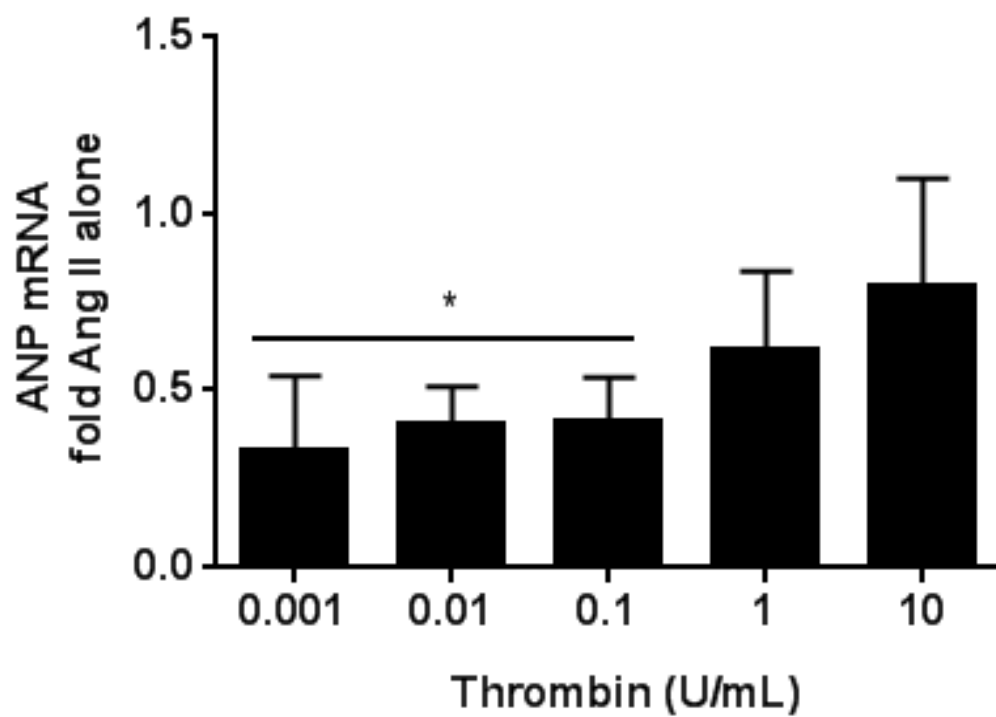
Fig. 4. a Concentration-dependent effects of thrombin and trypsin on [3 H]phenylalanine incorporation in adult rat cardiac myocytes co-stimulated with ET-1 (60 nM, all n=5) or **b** Ang II (1 μ M, all n=3). dual PAR-1/PAR-2 agonist SFLLRN and the PAR-2 selective

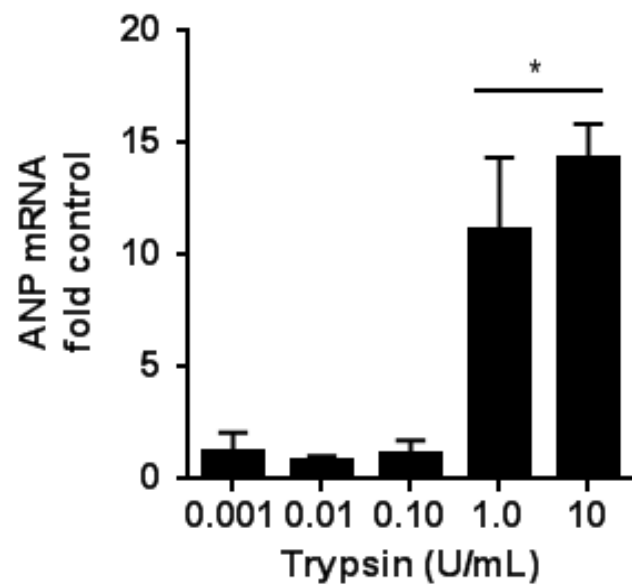
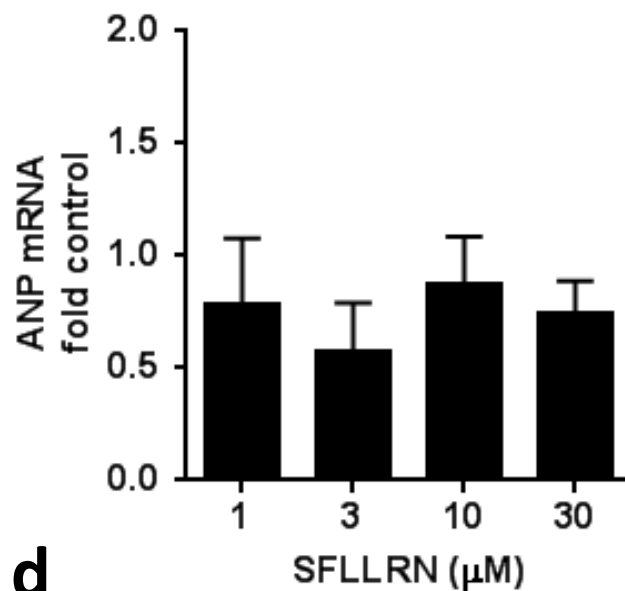
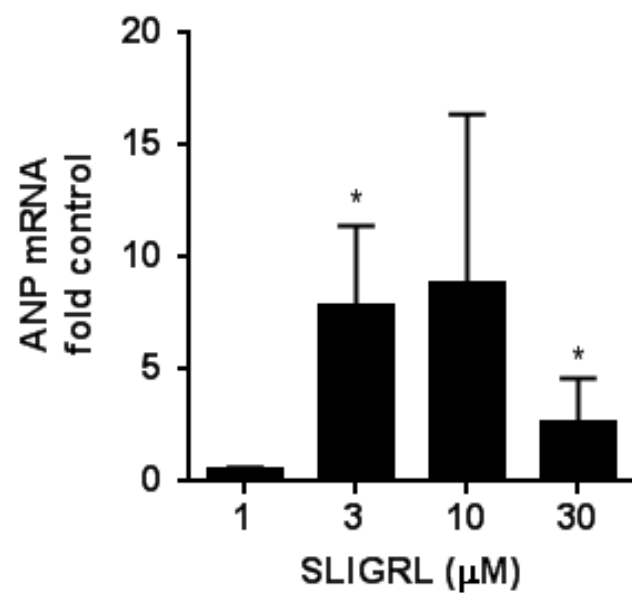
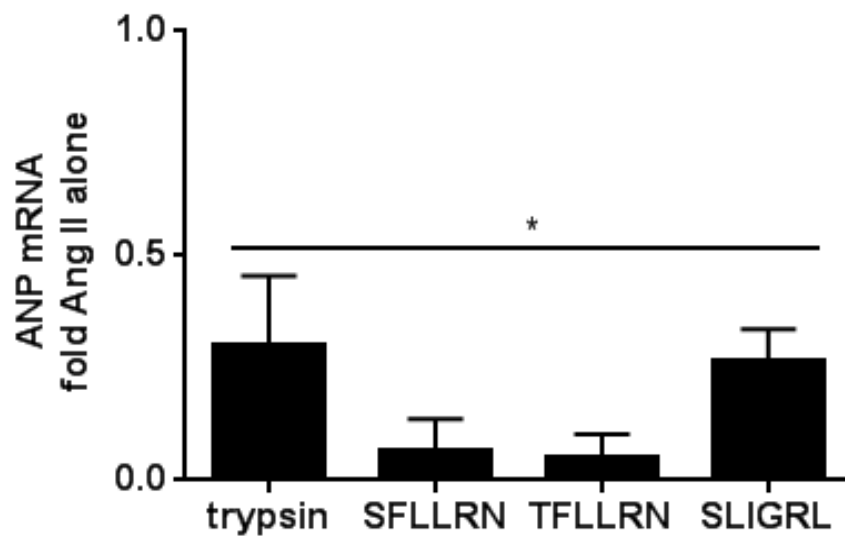
agonist SLIGRL (all n=6). **c** Concentration-dependent effects of SFLLRN and SLIGRL on ET-1-stimulated [³H]phenylalanine incorporation in adult rat cardiac myocytes (all n=6). Data show mean±SEM, expressed as % of ET-1 or Ang II alone, *P<0.05 vs. control, #P<0.05 vs. ET-1 or Ang II. **d** The PAR-1 antagonist RWJ56110 partially counteracts the antihypertrophic action of SFLLRN (both 10 µM, n=4) in ET-1 stimulated adult rat cardiomyocytes. Data show mean±SEM, normalised to DNA content and % of control. *P<0.05 vs. control, #P<0.05 vs. ET-1 or Ang II,

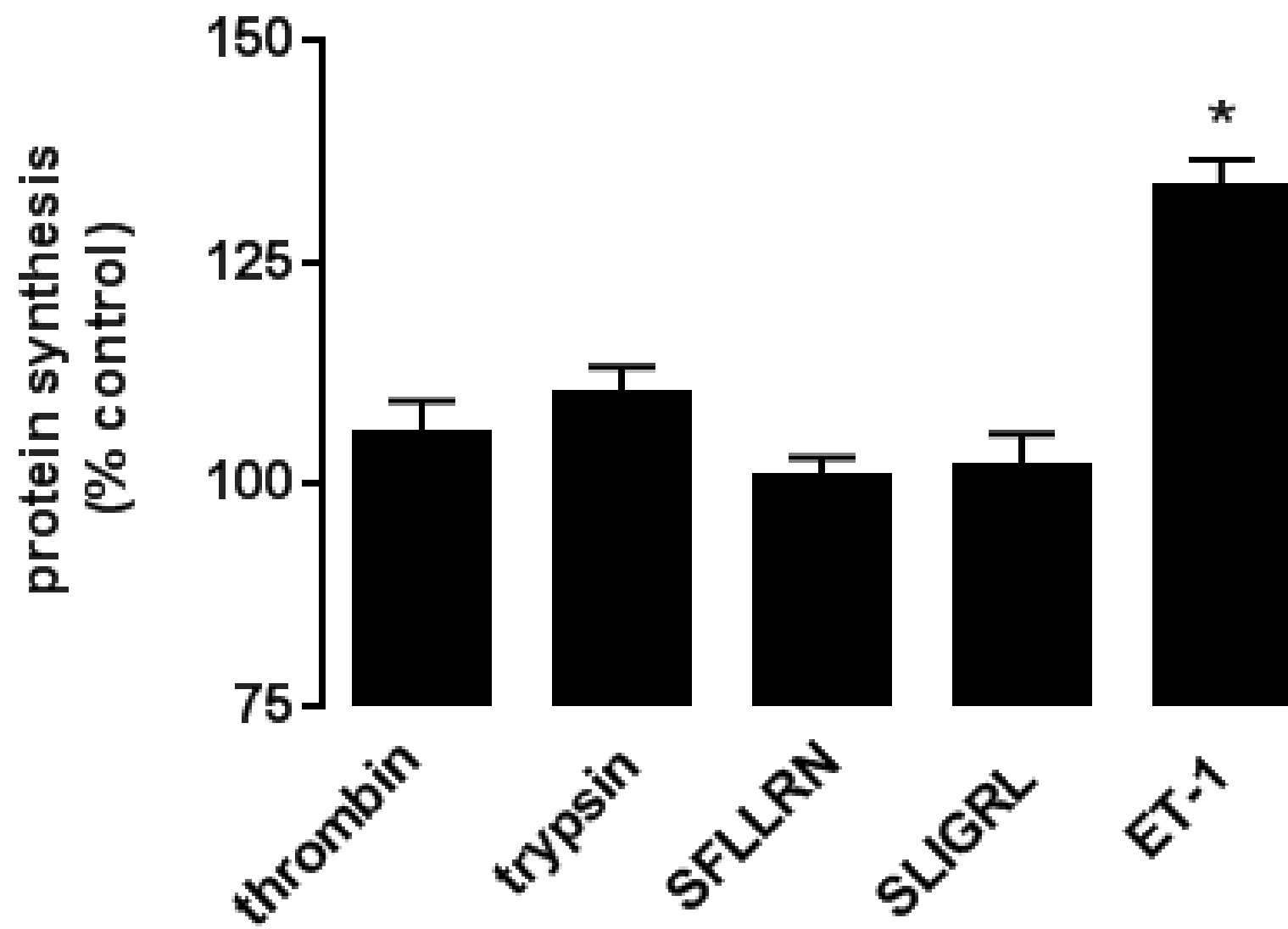
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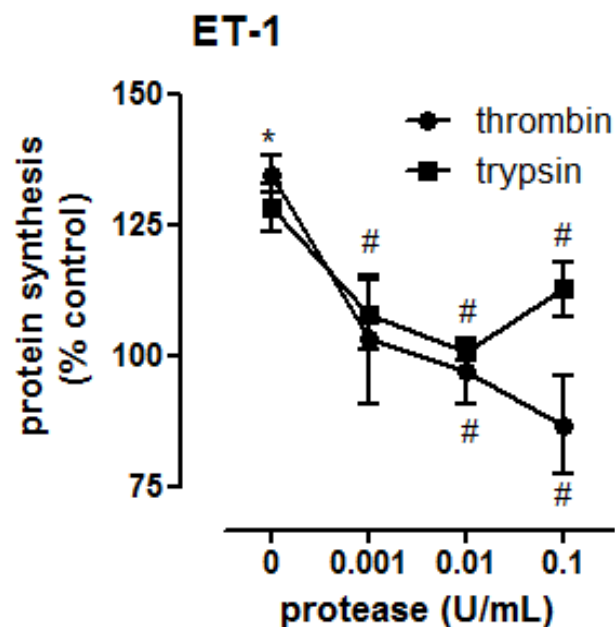
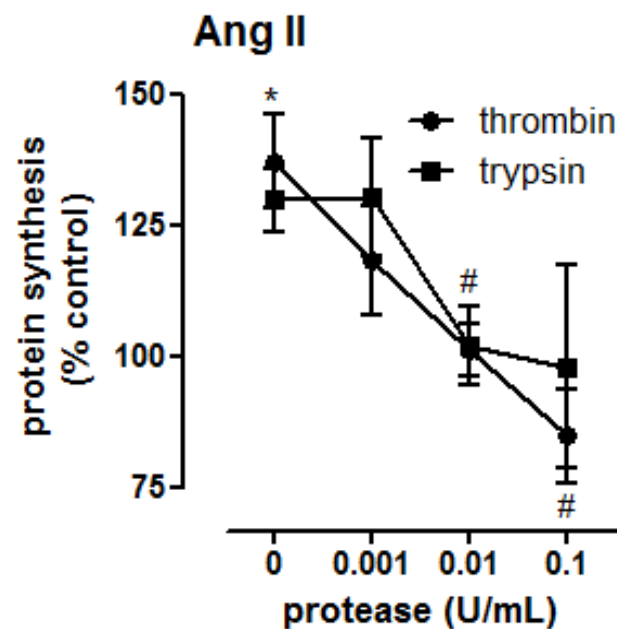
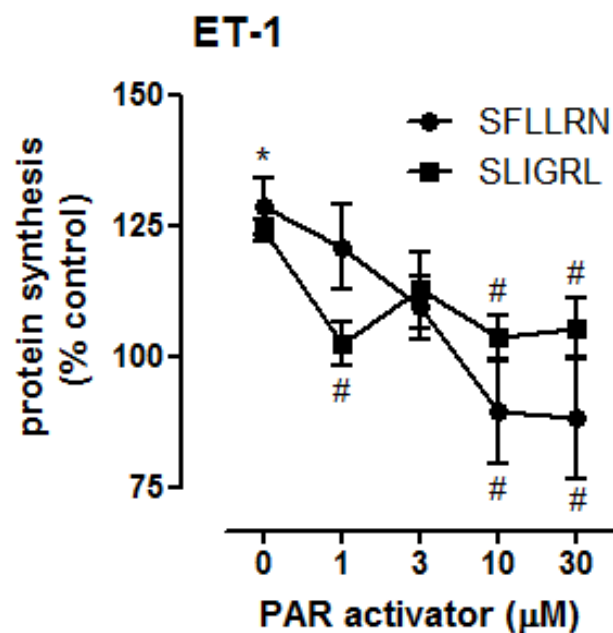


b



a**b****c****d**



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