Association of RhoGDIα with Rac1 GTPase mediates free radical production during myocardial hypertrophy

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Abstract

Objective: Reactive oxygen species (ROS) contribute to the pathogenesis of myocardial hypertrophy. NADPH oxidase is a major source of ROS production. The small GTPase Rac1 mediates the activation of NADPH oxidase; however, the mechanism of Rac1 activation is incompletely understood.

Methods and results: Transaortic constriction (TAC, C57/Bl6 mice, 360 μm, 21 days) increased the ratio of heart to body weight from [%] SHAM 4.16 ± 0.09 to TAC 7.1 ± 0.37, p < 0.01. Treatment with rosuvastatin prevented pressure-induced cardiac hypertrophy (5.5 ± 0.18, p < 0.05). TAC induced a 4-fold up-regulation of myocardial NADPH oxidase activity as well as Rac1 activity; both effects were absent in statin-treated animals. In cultured rat cardiomyocytes, treatment with angiotensin II (AngII) increased translocation of Rac1 to cell membranes and Rac1 activity. AngII altered neither expression nor tyrosine phosphorylation of GTPase activating protein GAP-p190 and the guanine nucleotide exchange factors Vav and Tiam. Transaortic constriction as well as AngII increased the binding of Rho guanine nucleotide dissociation inhibitor (RhoGDIα) to Rac1. The association of RhoGDIα with Rac1 was mediated by phosphatidylinositol 3-kinase and depended on geranylgeranylation. Statin treatment inhibited RhoGDIα–Rac1 binding both in cultured cardiomyocytes and during myocardial hypertrophy in vivo. Transfection with RhoGDIα siRNA constructs potently reduced RhoGDIα protein expression, decreased AngII-induced superoxide production and lipid peroxidation, and inhibited AngII-induced leucine incorporation.

Conclusions: Myocardial hypertrophy is characterized by activation of Rac1 and NADPH oxidase. The association of the regulatory protein RhoGDIα with Rac1 represents a necessary step in the Rac1-dependent release of ROS. Rac1–RhoGDIα binding may represent a target for anti-hypertrophic pharmacologic interventions, potentially by statin treatment.

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1. Introduction

Left ventricular hypertrophy (LVH) is a major risk factor for the development of heart failure and death [1]. Stimulation of the angiotensin II (AngII) type I receptor or exposure to pressure overload induces cardiac hypertrophy mediated in part via the activation of heterotrimeric G proteins (e.g. Gq) and small G proteins. Specifically, activation of the small G protein Rac1 has been shown to be positioned at the top of a cellular signal cascade leading to cardiac hypertrophy [2–6]. Rac1 activation initiates a cascade of signal amplification involving downstream target proteins such as members of the superfamily of mitogen-activated protein kinases (MAPK), p21-activated kinase (PAK) or focal adhesion kinase (FAK) [2,7]. Furthermore, Rac1 participates in signalling pathways of left ventricular hypertrophy by activating the superoxide generating NADPH oxidase [3,4]. Failing human left ventricular myocardium is characterized by up-regulation of Rac1 activity and superoxide production [5]. NADPH oxidase-dependent production of reactive oxygen species increases...
progressively during hypertrophy in animal studies [8] and is involved in cardiac hypertrophy in response to pressure overload [8,9], stretch [10], angiotensin II-infusion [11] or α-adrenergic stimulation [12].

Rac1 GTPase acts as bimodal relay in the transfer of intracellular signals, combining a cytosol/membrane and a GDP/GTP alternation. Posttranslational isoprenylation is necessary for translocation of Rac1 from the cytosol to the membrane. The capacity to cycle between a GDP-bound conformation and a GTP-bound conformation enables rho proteins to filter, to amplify or to temporise upstream signals. This GDP/GTP switch is controlled by guanine nucleotide exchange factors and GTPase-activating proteins. After stimulation and isoprenylation, Rac1 translocates to the membrane where GDP is exchanged for GTP by guanine nucleotide exchange factors (GEF). However, the detailed molecular mechanism of this activation in cardiac myocytes is not known. At the membrane, GTP-Rac1 interacts with its effector molecules, e.g. p21-activated kinase (PAK). GTP-Rac1 is inactivated by conversion of the GTP to GDP by GTPase activating protein (GAP) and returns into the cytosol completing the activation cycle. Rho guanine nucleotide dissociation inhibitors, RhoGDI, have been shown to negatively regulate rho-family GTPases [13–15]. The RhoGDI subfamily consists of three subtypes. RhoGDIα (p28) and RhoGDIβ (Ly-RhoGDI) are believed to function as dissociation inhibitors for the same spectrum of substrates, whereas RhoGDIγ does not bind to Rac1 [16]. RhoGDIα is believed to be ubiquitously expressed, however, RhoGDIβ is expressed only in hematopoietic tissues and predominantly in B- and T-lymphocyte cell lines [17].

Outside of hematopoietic tissues, RhoGDIα is the relevant GDI of Rac1 GTPase [16,17]. The inhibitory activity of GDI derives both from an ability to bind the carboxy-terminal isoprene of rho family members and extract them from membranes and from inhibition of GTPases cycling between the GTP- and GDP-bound states [18,19]. Experiments using reconstituted isoprenylated rho/RhoGDI complexes show that rho proteins dissociate from RhoGDI prior to their activation. The structure of rho/RhoGDI complexes reveals that the binding site for the guanine nucleotide exchange factors overlaps the interface for RhoGDI supporting the role of RhoGDI as inhibitors of GDP/GTP exchange [13]. Therefore, the current concept of NADPH oxidase activation is that dissociation of Rac1 from RhoGDI is required for membrane translocation and GDP/GTP exchange [20–22].

The importance of Rac1 activity for superoxide release has prompted experiments searching for pharmacologic tools to inhibit Rac1. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), in addition to inhibiting cholesterol synthesis, downregulate Rac1-GTPase activity by reducing isoprenylation and translocation of Rac1 to the cell membrane [3,4,23]. Inhibition of Rac1 by statins decreases NADPH oxidase-related production of reactive oxygen species in cardiac myocytes and reduces cardiac hypertrophy [3–5,24,25].

The aim of this study was to characterize the interaction of Rac1 with its regulatory co-factors during myocardial hypertrophy in vivo and in cultured cardiomyocytes. We hypothesized that exposure to pressure or angiotensin II would induce activation of Rac1 activity potentially by facilitating the release of Rac1 from rho guanine nucleotide dissociation inhibitor.

2. Methods

2.1. Materials

Angiotensin II was purchased from Sigma. LY 294002 and Wortmannin, geranylgeranyltansferase inhibitor (GGTl-286) and farnesyltransferase inhibitor (FTI-276) were obtained from Calbiochem. Rosuvastatin was a gift from AstraZeneca. [3H] Leucine, Hybrid N-lylmembranes and X-ray film were supplied by Amersham Biosciences. 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) was purchased from Molecular Probes (Eugene, OR). Antibiotics, calf serum and cell culture medium were obtained from Invitrogen.

2.2. Transaortic constriction

The study was approved by the animal ethics committee of the Universitat des Saarlandes and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Pub. No. 85-23, revised 1996). Ten-week-old female C57/Bl6 mice were housed under standard conditions. Animals were anesthetized with ketamine (100 mg/kg body weight [BW], i.p.) and xylazine (10 mg/kg, i.p.) for transverse aortic constriction (TAC). After orotracheal intubation using a 20-gauge catheter, the tube was connected to a volume cycled rodent ventilator (Harvard Apparatus) on supplemental oxygen with a tidal volume of 0.2 ml and respiratory rate of 110 per min. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision and aortic constriction was performed by tying a 7-0 nylon suture ligature against a 27-gauge needle to yield a narrowing 360 μm in diameter and a transverse aortic constriction of 65–70%. Control mice underwent a sham operation. After 3 weeks, animals were anesthetized with pentobarbital (100 mg/kg BW, i.p.) and a 1.4 Fr pressure-transducing catheter (Mikro Tip Catheter, Millar instruments) was used for LV-pressure measurements.

2.3. Cell culture

Cardiomyocytes from neonatal Sprague–Dawley rats and H9C2 rat heart myoblasts were isolated and cultured
as described [4,26]. Neonatal cardiomyocytes were grown to confluence in F 10-medium (HAM + glutamine) supplemented with 10% horse serum, 5% fetal calf serum, 100 U/ml of penicillin and 100 µg/ml streptomycin. H9C2 cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% (v/v) fetal calf serum, glutamine (2 mM) and penicillin (100 IU/ml). Neonatal cardiomyocytes showed spontaneous contractions. Cells were kept in serum free medium for 24 h before treatment. Cellular viability under all treatment conditions was determined by cell count, morphology, and trypan blue exclusion. Cellular protein content was quantified by a modified Lowry assay.

2.4. Rac1 GST-PAK pull down assay

A glutathione-S-transferase (GST)-PAK-CD (PAK-CRIB domain) fusion protein, containing the Rac1 binding region from human PAK1B was used to determine Rac1 activity as described [5]. Escherichia coli transformed with the GST-PAK-CD construct were grown at 37 °C to an absorbance of 0.3. Expression of recombinant protein was induced by addition of 0.1 mmol/l isopropylthiogalactoside for 2 h. Left ventricular myocardium or cultured cells were homogenized and resuspended in lysis buffer ((in mmol/l) Tris–HCl 50 (pH 7.4), NaCl 100, MgCl2 2, benzamidine 1, NP-40 1%, glycerol 10%, leupeptin, pepstatin, and aprotinin 1 µg/ml, respectively), and centrifuged at 21,000 rpm, 5 min, 4 °C. Equal amounts of supernatant protein were incubated with the GST-PAK-CD fusion protein bound to glutathione-coupled Sepharose beads at 4 °C, 30 min. Beads were washed three times with lysis buffer, eluted in Laemmli buffer (60 mmol/l Tris (pH 6.8), 2% sodium dodecylsulfate, 10% glycerol, 0.1% bromphenol blue), and analyzed for bound Rac1 by Western blotting.

2.5. Western blotting

Total cell lysates were prepared as described previously [4,5]. For membrane preparation, cells were pelleted by low-speed centrifugation and lysed in HME buffer (in mmol/l) phosphate 50 (pH 7.0), EGTA 1, protease inhibitors (Complete , Roche), sucrose 150, leupeptin 0.005, and NADPH 0.1 as described. Tissue was mechanically lysed using a teflon homogenizer in ice-cold buffer B, lacking leucigenin and substrate. Total protein concentration was adjusted to 1 mg/ml. Lysates of left ventricular myocardium were incubated in the presence or absence of angiotensin II, 1 µM, for 24 h. 6 h before harvest, 1 µCi/ml [3H] leucine was added. Cells were washed 3 x with ice cold PBS and harvested in lysis buffer (100 mM Tris pH 6.8, 40% SDS, 20% Glycerol). The incorporated [3H] leucine was determined by liquid scintillation counting.

2.6. Immunoprecipitation

Left ventricular myocardial tissue from sham- and TAC-hearts as well as cell lysates from neonatal cardiomyocytes and H9C2-cultures, grown in 100-mm dishes, were prepared by addition of cold immunoprecipitation buffer (50 mM sodium glycerolphosphate, pH 7.3, 1 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.1 mM Na2VO4) containing a protease inhibitor mixture. Total protein was isolated and incubated with primary antibodies for 4 h at 4 °C. The following primary antibodies were used to immunoprecipitate proteins from cell lysates: RhoGDIA (A-20): sc-360) (1:250 dilution, Santa Cruz Biotechnology Inc.) and Anti-Rac1, clone 23A8 (cat.# 05-389, Upstate). Immune complexes were collected with protein G/A-agarose beads. Immunoprecipitated protein was pelleted, rinsed, resuspended in gel loading buffer, then boiled and pelleted again. The supernatant was subjected to western analysis.

2.7. [3H] Leucine incorporation

24 h after transfection, 10⁶ cardiomyocytes were re-seeded in 6 well-plates in the presence and absence of angiotensin II, 1 µM, for 24 h. 6 h before harvest, 1 µCi/ml [3H] leucine was added. Cells were washed 3 x with ice cold PBS and harvested in lysis buffer (100 mM Tris pH 6.8, 40% SDS, 20% Glycerol). The incorporated [3H] leucine was determined by liquid scintillation counting.

2.8. NADPH oxidase activity assay

NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay in buffer B containing (in mmol/l) phosphate 50 (pH 7.0), EGTA 1, protease inhibitors (Complete, Roche), sucrose 150, lucigenin 0.005, and NADPH 0.1 as described. Tissue was mechanically lysed using a teflon homogenizer in ice-cold buffer B, lacking lucigenin and substrate. Total protein concentration was adjusted to 1 mg/ml. Lysates of left ventricular myocardium were incubated in the presence or absence of angiotensin II, 1 µmol/l, 10 min, 37 °C. 100 µl aliquots of the protein sample were measured over 10 min in quadruplicates using NADPH as substrate in a scintillation counter (Berthold Lumat LB 9501) in 1 min intervals.

2.9. Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species production in H9C2 was measured by 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescence. Dishes of sub-confluent cells
were washed and incubated in the dark for 30 min in the presence of 10 mmol/l 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). Culture dishes were transferred to an inverted microscope (Eclipse TS 100/TS 100-F, Nikon), equipped with a digital still camera DXM 1200 (Nikon), and reactive oxygen species generation was detected as a result of the oxidation of H2DCFDA (excitation, 488 nm; emission longpass LP515-nm filter set). 512/C2 512 pixel images were collected by single rapid scans and identical parameters, such as contrast and brightness, for all samples. Five groups of 20 cells for each sample were randomly selected from the image and fluorescent intensity was taken. The relative fluorescence intensities are average values of all experiments. DCF-fluorescence was quantified using Confocal Assistant™ (Version 4.02) and Carl Zeiss LSM Beta (Version 3.92) Software and was expressed relative to baseline fluorescence in individual cells.

2.10. Measurement of lipid peroxidation

Cardiomyocytes were homogenized in PBS (pH 7.4) containing butylated hydroxytoluene (4 mmol/l). Lipid hydroperoxides were determined using the Lipid Peroxidation Assay Kit II (Calbiochem) and expressed as μmol/mg protein.

2.11. Small interfering RNA (siRNA) transfection studies

Small interfering RNAs (siRNAs) for RhoGDIα and scrambled control siRNA (scrRNA, Silencer® Negative Control #1 siRNA, Cat.# 4611) were purchased from Ambion Inc. (Austin, Texas). The following sequences specific for RhoGDIα were used: Arhgdia_1 sense 5’GGACUGGAGAAAGGACGAUt3; antisense 5’AUGCUCUUUGCCAGUUCCtgc 3’, Arhgdia_2 sense 5’GGACUGAGAAAGCUCUCGAt3’, antisense 5’UCGGAGGCUUCAUGCUCt3’. Transfections of subconfluent H9C2 cells were performed using the Amoza Nucleofector device (Amoza Biosystems, Gaithersburg, USA) according to the manufacturer’s instructions. One microliter of RhoGDIα siRNA (100 nM) and 100 μl of Amoza nucleofector was used for each well, containing 1 × 10⁵ cells. After transfection, cells were maintained in culture medium for 48 h before Angiotensin II (1 μM/3 h) treatment.

Fig. 1. Effect of transaortic constriction (TAC) to 360 μm in C57/Bl6 mice and treatment with rosuvastatin (Statin, 2 mg/kg, s.c.) on the ratio of heart weight (A) and lung weight (B) to body weight after 21 days. n=18, *p<0.01 vs. sham and #p<0.02 versus transaortic constriction.

Fig. 2. (A) Effect of transaortic constriction (TAC) to 360 μm in C57/Bl6 mice and treatment with rosuvastatin (Statin, 2 mg/kg, s.c.) on Rac1 GTPase activity and (B) on NADPH oxidase activity after 21 days. n=4, *p<0.05 vs. sham.
3. Statistical analysis

Band intensities were analyzed by densitometry. All values are expressed as mean±S.E.M. For all multiple comparisons, ANOVA followed by Bonferroni post-hoc analysis for multiple comparisons was applied. Differences were considered significant at \( p<0.05 \).

4. Results

4.1. Statin treatment prevents myocardial hypertrophy

Transaortic constriction (360 \( \mu \)m, 21 days, \( n=18 \) per group) increased the ratio of heart to body weight from 4.16±0.09 in sham operated mice to 7.1±0.37 post transaortic constriction, \( p<0.01 \). Animals with aortic constriction exhibited increased left ventricular systolic pressure (124±5 mm Hg in TAC vs. 95±7 mm Hg in sham operated mice, \( p<0.05 \)). Left ventricular systolic pressure after transaortic constriction in the statin group was not different from vehicle treated mice (132±7 mm Hg, \( p=\text{n.s.} \) vs. TAC). Rosuvastatin (2.0 mg/kg, s.c., 21 days) prevented pressure induced cardiac hypertrophy (5.5±0.18, \( p<0.05 \)) (Fig. 1A). Treatment with statin reduced the increase of lung weight induced by transaortic constriction as an indicator of improved clinical outcome (Fig. 1B). Rosuvastatin (2.0 mg/kg, s.c., 10 days) did not affect serum cholesterol levels (119±2.5 mg/dl vs. 112±10 mg/dl, \( n=6, \ p=\text{n.s.} \)).

![Fig. 3. (A) Representative Western blots (\( n=4–8 \)) of H9C2- and rat neonatal cardiomyocytes treated with angiotensin II (Ang, 1 \( \mu \)M, 3 h) and rosuvastatin (Statin, 1 \( \mu \)M, 16 h). (B) Representative examples and quantifications of expression of the Rac1–RhoGDI\( \alpha \) complex in the membrane (\( n=6, \ p<0.05 \) vs. C) and the cytosol (\( n=4, \ p=\text{n.s.} \) vs. C) after treatment with angiotensin II (Ang, 1 \( \mu \)M, 3 h) as well as GAPDH control. C, control; IP, immunoprecipitation; WB, Western blot.](image-url)
4.2. Myocardial hypertrophy is associated with activation of Rac1 and NADPH oxidase

GST-PAK pull-down assays demonstrated a 5-fold up-regulation of Rac1 activity in pressure-induced left ventricular hypertrophy (567±146% of sham operated animals, p<0.05). Fig. 2A shows that treatment with rosuvastatin completely prevented the increase of Rac1 activity mediated by aortic constriction (144±51% of sham). Activation of Rac1 in hypertrophic left ventricles was associated with a 4-fold increase of NADPH oxidase activity (412±137% of sham, p<0.05). Rosuvastatin prevented afterload-induced NADPH oxidase activity (163±52% of sham) (Fig. 2B).

4.3. Angiotensin II and hypertrophy induce the association of Rac1 with RhoGDIα

To further characterize the mechanism of Rac1 activation, H9C2- and neonatal cardiomyocytes were treated with angiotensin II (AngII). As expected from our previous studies, angiotensin II (1 μM, 24 h) increased translocation of Rac1 to cell membranes as well as Rac1 activity (data not shown, [4]). We and others have previously demonstrated that Rac1 mediates Angiotensin II-induced up-regulation of superoxide release and hypertrophy of cardiomyocytes [3,4]. We hypothesized that co-factors of rho protein activation may be involved in Rac1 activation [27,28]. However, Angiotensin II (1 μM, 30 min–24 h) did not significantly alter expression nor tyrosine phosphorylation of GTPase activating protein GAP-p190 and the guanine nucleotide exchange factors Vav and Tiam (data not shown).

Treatment with angiotensin II (1 μM, 24 h) increased Rac1 total protein (159±28%, p<0.05), whereas RhoGDIα...
protein expression was not altered. Angiotensin II increased the binding of rho guanine nucleotide dissociation inhibitor α (RhoGDIα) to Rac1 (immunoprecipitation) (279±40% of control, p<0.05, n=6) (Fig. 3A). Angiotensin II increased the abundance of the Rac1–RhoGDIα complex not only in total cell lysates but also in isolated cell membranes (251±35% of control p<0.05, n=6). Angiotensin II did not significantly increase Rac1–RhoGDIα in the cytosol (133±22% of control, n=4) (Fig. 3B). Treatment with rosuvastatin (1 μM, 24 h) prevented up-regulation of RhoGDIα–Rac1 by angiotensin II (116±30% of control). Treatment with rosuvastatin alone did not significantly alter RhoGDIα–Rac1 association (89±18% of control). Experiments using rat neonatal cardiomyocytes showed the same results (Fig. 3).

Fig. 4 shows that treatment (24 h) with statin or treatment with a geranylgeranyl-transferase inhibitor, but not with a farnesyl-transferase inhibitor prevented AngII-induced Rac1–RhoGDIα association. In vascular smooth muscle cells, phosphatidylinositol-3-(PI3) kinase is an upstream mediator of AngII-stimulated NADPH oxidase [29]. We therefore tested the effect of two inhibitors of PI3 kinase. Wortmannin and LY 294002 completely prevented Rac1–RhoGDIα binding in the presence of AngII (Fig. 4). RhoGDIα protein expression was not significantly changed under any of these conditions (n=4–12).

4.4. Association of Rac1 with RhoGDIα depends on PI3-kinase and isoprenylation

Fig. 6. Effects of transfection of H9C2 cardiomyocytes with scrambled siRNA (scr) and transfection with RhoGDIα-1 (S1) and RhoGDIα-2 (S2) siRNA and treatment with angiotensin II (Ang, 1 μM, 3 h). Free radical production was assessed by 2,7-dichlorodihydrofluorescein (DCF) fluorescence and quantification of lipid peroxidation. (A) Effect of transfection with RhoGDIα siRNA on total protein expression of RhoGDIα (representative Western blot). (B) Representative images of 2,7-dichlorodihydrofluorescein (DCF) fluorescence and (C) quantification of superoxide production (n=5, *p<0.01 vs. C, #p<0.01 vs. scr) and lipid peroxidation (n=4, *p<0.05 vs. scr) (D).
4.5. Transaortic constriction induces association of Rac1–RhoGDI\(\alpha\) in vivo

On the basis of the cell culture data, the association of Rac1 with RhoGDI\(\alpha\) was studied in vivo. Similar to the data in cultured cardiomyocytes, Fig. 5 shows that Rac1 expression was increased after TAC to 130±14\%, RhoGDI expression was not altered, and Rac1–RhoGDI binding markedly increased to 246±39\% in the hearts of transaortic constricted animals compared to sham operated mice \((n=5, \ p<0.05)\). Treatment with rosuvastatin completely prevented this effect (95±16\% of sham, \(n=5, \ p<0.05)\).

4.6. Inhibition of RhoGDI\(\alpha\) by si-RNA transfection inhibits free radical production in cardiomyocytes

To test for a causal role of RhoGDI\(\alpha\) for AngII-induced free radical release, H9C2-9C2 cardiomyocytes were transfected with two RhoGDI\(\alpha\) siRNA constructs. Western analysis confirmed that the siRNA transfection protocol potently reduced RhoGDI\(\alpha\) protein expression by 65±12\%, \(p<0.01\), compared to cells transfected with scrambled siRNA (scrRNA) \((n=5, \text{ Fig. 6A})\). Fig. 6B (representative cells) and Fig. 6C (quantification) show that inhibition of RhoGDI\(\alpha\) expression by siRNA transfection completely prevented AngII-induced intracellular free oxygen radical production \((n=5\text{ for each of the two siRNA constructs})\). Similarly, specific inhibition of RhoGDI\(\alpha\) prevented the AngII-induced increase of lipid peroxidation \((\text{Fig. 6D})\).

4.7. Inhibition of RhoGDI\(\alpha\) prevents AngII-induced protein synthesis in cardiomyocytes

Protein synthesis is a prerequisite of cardiomyocyte hypertrophy. In control-transfected H9C2-cells (scrRNA), angiotensin II induced a 2-fold increase of leucine-incorporation \((197\pm64\%, \ n=10, \ p<0.05)\). However, in cells transfected with RhoGDI\(\alpha\) siRNA angiotensin II did not increase leucine uptake \((\text{Fig. 7})\).

5. Discussion

These experiments identify the rho guanine nucleotide dissociation inhibitor \(\alpha\) as a mediator of both agonist- and pressure induced hypertrophy in cultured cardiac myocytes and in vivo. The association of RhoGDI\(\alpha\) with Rac1 is a necessary step in the Rac1-dependent release of ROS and may represent a novel target for anti-hypertrophic pharmacologic interventions, potentially by statin treatment.

We show that pressure-induced myocardial hypertrophy in mice is characterized by activation of Rac1- and NADPH oxidase activity. These data support the fundamental role of Rac1 GTPase to regulate the assembly of the NADPH oxidase complex and superoxide release which has been observed not only in neutrophils but in several cell types including vascular and cardiac cells \([4,5,20,22,23,30]\). However, the molecular mechanism of Rac1 activation and the interaction of Rac1 with rho protein co-factors in cardiac cells remain incompletely understood.

Guanine nucleotide exchange factors are determinants of downstream signalling specificity for members of the rho GTPase family. Specifically, Vav and Tiam have been shown to regulate Rac1 function in several cell types \([27,28,31]\). However, we did not observe a significant regulation of expression nor tyrosine phosphorylation or co-immunoprecipitation with Rac1 of Vav and Tiam by angiotensin II in cultured cardiomyocytes nor in mouse myocardium after transaortic constriction. As with any negative result, technical limitations can never be completely excluded, but extensive studies in our hands did not suggest a significant contribution of Vav and Tiam during hypertrophy. Similarly, we did not see a significant alteration of expression nor tyrosine phosphorylation of the GTPase activating protein GAP-p190.

In addition to GEF and GAP, rho guanine nucleotide dissociation inhibitors contribute to the tight control of small G proteins \([13,18,27]\). Based on experiments with reconstituted Rac1/RhoGDI complexes and analysis of their crystal structure, the current concept of NADPH oxidase activation is that dissociation of Rac1 from RhoGDI is required for Rac1 membrane association and GDP/GTP exchange \([20–22]\). For example, Michaelson et al. have shown that co-overexpression of RhoGDI\(\alpha\) with high amounts of GFP-Rac1 shifted the localization of GFP-Rac1 from the membrane to a cytosolic pattern in MDCK cells, however, the effect of RhoGDI\(\alpha\) on wildtype Rac1 was not studied \([19]\). In contrast to this study, RhoGDI\(\alpha\) expression was not increased in hypertrophied mouse hearts or after treatment with angiotensin II. Importantly, Michaelson et al. stress that the subcellular localizations of Rho

![Fig. 7. Effects of transfection of H9C2 cardiomyocytes with scrambled siRNA (scr) and transfection with RhoGDI\(\alpha\)-1 (S1) siRNA and treatment with angiotensin II (Ang, 1 \(\mu\)M, 24 h) on protein synthesis (leucine incorporation) \((n=10, \ *p<0.05\text{ vs. scr})\).](image)
GTPases are diverse, dynamic and cell type dependent [19]. Indeed, several groups show that RhoGDIs function by shutting rho proteins between membranes [14,15]. Whether RhoGDI serve in this process as subsidiary transporters of inactive Rac1 or may potentially facilitate Rac1 activity has been a matter of discussion—complicated by the in vitro nature of most studies of membrane extraction and delivery [13]. In contrast to our initial working hypothesis, we observed that stimulation with angiotensin II increased the binding of RhoGDIα to Rac1 in cardiomyocytes. This robust finding was observed in H9C2 cells as well as rat neonatal cardiomyocytes. The observation was tested in vivo: mice with pressure-induced myocardial hypertrophy were characterized by a marked increase of binding of RhoGDIα to Rac1. As expected from our previous studies [4,5], angiotensin II increased Rac1 protein expression which may facilitate the formation of RhoGDIα–Rac1 association. RhoGDIα expression remained unchanged by angiotensin II as well as in hypothyroid left ventricles. Since upregulation of the complex exceeded the increase of Rac1 quantitatively, the increase of Rac1 expression only partially explains to the upregulation of RhoGDIα–Rac1 association. In the presence of angiotensin II, Rac1–RhoGDIα association increased in the membrane fraction of cardiomyocytes. In order to test the role of RhoGDIα for free radical release, a protocol was established to effectively inhibit RhoGDIα expression in cardiomyocytes via transfection with RhoGDIα siRNA constructs. Inhibition of RhoGDIα expression prevented angiotensin II induced superoxide production as well as lipid peroxidation. Furthermore, specific inhibition of RhoGDIα inhibited angiotensin-induced protein synthesis, a prerequisite for hypertrophy. Taken together, these data demonstrate that RhoGDIs is necessary for Rac1-mediated free radical release and hypertrophy in cardiomyocytes. Although at first glance surprising, these data are in agreement with observations in cell-free systems showing that GDP-Rac1 complexed to RhoGDIα isolated from macrophage cytosol functions as a potent activator of NADPH oxidase, potentially facilitating the three-dimensional co-localization with NADPH oxidase subunits [32–34].

In agreement with previous studies using other statin drugs, the HMG-CoA reductase inhibitor rosuvastatin effectively prevented cardiac hypertrophy, Rac1 activity, NADPH oxidase activation and the release of myocardial free oxygen radicals both in cultured cardiomyocytes and in mice [3–5,24,25]. Since statins do not lower serum cholesterol in rodents nor in the cell culture medium, these effects are independent of serum cholesterol. The model of aortic banding in wild-type mice excludes anti-atherosclerotic statin effects. The mechanism of inhibition of rho protein function by statins is the prevention of posttranslational attachment of geranylgeranylpyroshophosphate, which is necessary for translocation of rho proteins to the membrane [35]. The presented data clarify a significant detail of this process. The association of RhoGDIα with Rac1 is mediated by phosphatidylinositol-3-(P13) kinase and depended on geranylation. Rosuvastatin treatment completely inhibited RhoGDIα–Rac1 binding both in cultured cardiomyocytes and during myocardial hypertrophy in vivo. Structural studies show that RhoGDIα has a modular organization with an N-terminal helix–loop–helix domain and a C-terminal sandwich domain that carries a hydrophobic binding site for the geranylgeranyl group attached to Rac1 [13,36]. Therefore the cell culture and the in vivo data suggest that inhibition of the isoprenoid-dependent binding of Rac1 to RhoGDIα and subsequent association of this complex with NADPH subunits at the cell membrane is an important molecular mechanism of the anti-oxidative effects of statin treatment. Ongoing clinical trials in patients with heart failure (CORONA, GISSI-HF) will help to understand if and to which extent cholesterol-independent mechanisms may contribute to the clinical effects of lipid-lowering by statins. Furthermore, Rac1–RhoGDIα binding may represent a novel target for anti-hypertrophic pharmacologic interventions.

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