

PRE-CLINICAL RESEARCH

Beneficial Effects of Mammalian Target of Rapamycin Inhibition on Left Ventricular Remodeling After Myocardial Infarction

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- Objectives** The extent of adverse myocardial remodeling contributes essentially to the prognosis after myocardial infarction (MI). In this study we investigated whether inhibition of “mammalian target of rapamycin” (mTOR) attenuates left ventricular (LV) remodeling after MI.
- Background** Therapeutic strategies to inhibit remodeling are currently limited to inhibition of neurohumoral activation. The mTOR-dependent signaling mechanisms are centrally involved in remodeling processes and provide new therapeutic opportunities.
- Methods** Everolimus (RAD) treatment was initiated on the day after or 3 days after induction of myocardial infarction (MI) in rats.
- Results** After 28 days, RAD-treated animals had reduced post-MI remodeling, with improved LV function and smaller LV end-diastolic diameters (8.9 ± 0.3 mm vs. 11.4 ± 0.2 mm, $p < 0.05$), end-diastolic volumes (304 ± 30 μ l vs. 414 ± 16 μ l, $p < 0.05$), and cardiac myocyte size (-40% vs. vehicle, $p < 0.05$). Infarct size was significantly reduced compared with vehicle-treated animals. The mTOR inhibition increased autophagy and concomitantly decreased proteasome activity in the border zone of the infarcted myocardium. Measurement of autophagic flux demonstrated that RAD did not decrease autophagosome clearance. When RAD treatment was initiated 3 days after MI, adverse remodeling was still attenuated and increased autophagy was still present. Sustained improvement of LV function was observed 3 months after MI, even when RAD treatment was discontinued after 1 month.
- Conclusions** Inhibition of mTOR is a potential therapeutic strategy to limit infarct size and to attenuate adverse LV remodeling after MI. (J Am Coll Cardiol 2009;54:2435–46) © 2009 by the American College of Cardiology Foundation

Ischemic heart disease is one of the leading causes of morbidity and mortality worldwide (1,2). An increase in ventricular volume is a primary predictor of mortality after myocardial infarction (MI) (3). Thus, one of the most

important factors for improving the prognosis after MI is the attenuation of adverse myocardial remodeling (4,5). Due to improvement of therapeutic strategies after MI, mortality decreased significantly in the past decades. Consecutively, there is also an increasing number of patients whose prognosis depends on optimal treatment after MI. Despite the widespread use of angiotensin-converting enzyme inhibitors, beta-blockers, and aldosterone-antagonists, the incidence of heart failure as the end-stage of left ventricular (LV) remodeling still remains high.

Underlying mechanisms of remodeling are manifold, including activation of physical and neurohumoral processes as well as activation of growth factors and the protein translation machinery (6,7). Despite its well-known role in the myocardial remodeling process, protein synthesis has not been investigated for its potential to influence the remodeling process. One

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**Abbreviations
and Acronyms**

- AMC** = 7-amino-4-methylcoumarin
- EDD** = end-diastolic diameter
- ESD** = end-systolic diameter
- LV** = left ventricle/ventricular
- MDC** = monodansylcadaverine
- MI** = myocardial infarction
- mTOR** = mammalian target of rapamycin
- NFκB** = nuclear factor-kappa B
- RAD** = everolimus
- TTC** = triphenyl tetrazolium chloride

important molecule related to protein synthesis is the “mammalian target of rapamycin” (mTOR), which is mainly activated via the Akt/PI3K pathway. This signaling mechanism is centrally involved in physiological hypertrophy but also takes part in pathological remodeling of the heart (8–10). The mTOR inhibitors such as rapamycin or everolimus (RAD) have an inhibitory effect on protein synthesis via direct inhibition of mTOR. Due to its immunosuppressant actions, mTOR inhibitors thus far have mainly been used in transplantation medicine. However, they are also being used as proliferation inhibitors for coating drug-eluting stents to reduce restenosis after coronary angioplasty. Sirolimus is even effective to prevent

restenosis when given orally (11). In the context of LV hypertrophy, recent studies indicate that rapamycin has an inhibitory effect on development of LV hypertrophy in vivo (8,10). In this study, we tested the hypothesis of whether treatment with mTOR inhibitors can prevent adverse LV remodeling after MI.

Methods

The investigation conforms to the guide for the care and use of laboratory animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the authorities of the Regierungspräsidium Karlsruhe, Germany.

Study design. After surgery, animals were randomized for treatment with RAD (3.0 mg/kg/day) or vehicle by oral gavage starting after recovery from surgery. The RAD treatment with a dose of 3.0 mg/kg/day has been established to be efficacious and well-tolerated in previous studies (12,13). For basic characterization, including echocardiography and hemodynamic measurements, animals were killed 28 days after MI. For protein analysis, a subgroup of animals was killed at days 1, 3, 14, and 28 after surgery. In addition, we investigated the effect of delayed treatment with RAD (3.0 mg/kg/day) starting 3 days after induction of MI. In an additional experimental series, we examined whether 1-month treatment with RAD (starting on day 1 after MI) would have sustained effects on LV function after 3 months. For killing, anesthesia was induced by intraperitoneal injection of ketamine (70 mg/kg) and xylazine (2 to 5 mg/kg), and echocardiographic and invasive measurements were performed. The heart was then arrested in diastole by injection of saturated potassium chloride solution. After dissecting the LV, myocardial samples from

different regions of the LV (infarcted area, border zone, and remote area) were snap frozen for biochemical measurements or fixed in formalin for further histological evaluation.

MI. For the MI model we used male Wistar rats with a weight of 200 g (Charles River Laboratories, Sulzfeld, Germany). Animals were anesthetized by IP injection of ketamine (70 mg/kg) and xylazine (2 to 5 mg/kg). After orotracheal intubation and ventilation, the thorax was opened left parasternally, and MI was induced by ligating the left anterior descending coronary artery just below the left atrial appendage. The quality of the infarction was confirmed visually by the change of the color of the myocardium.

Echocardiography. Transthoracic echocardiography was performed in a modified setting as previously described in detail (14). Studies were recorded with a dynamic focused 10-MHz probe with an ATL 5000 echocardiography machine. The M-mode measurements of LV dimensions were averaged from more than 3 cycles. The investigator who conducted the echocardiography was blinded for the treatment status.

LV pressure–volume measurements. For the invasive assessment of pressure–volume relationships, rats were anesthetized as described in the preceding text. The LV was catheterized retrogradely via the right carotid artery with a 2.0-F impedance-micromanometer catheter (Millar Instruments, Inc., Houston, Texas). The raw conductance volumes were corrected for parallel conductance by the hypertonic saline dilution method. For absolute volume measurements, the catheter was calibrated with known volumes of heparin-treated rat blood. Data were recorded with a sampling rate of 1,000 Hz with the Chart software (ADInstruments, Colorado Springs, Colorado). For subsequent analysis of pressure–volume loops PVAN software (Millar Instruments, Inc.) was used.

Pathology. Histological studies were conducted with formalin-fixed, paraffin-embedded hearts from animals of all groups. Cross sections of the LV obtained midway between base and apex were stained with hematoxylin/eosin, and myocyte size was measured with the ImageJ software (ImageJ, NIH, Bethesda, Maryland). To assess macrophage infiltration of the infarcted area, immunohistochemistry was performed with ED1 antibody. Infarct size 28 days after MI was assessed by triphenyl tetrazolium chloride (TTC) staining and by histology with Masson’s trichrome staining. The tissue slices were photographed, and the infarcted area was calculated as percentage of the whole LV with ImageJ software (ImageJ).

Autophagic flux. We investigated autophagic flux in vivo, according to the previously described method by Iwai-Kanai et al. (15). After surgery, the animals were randomized for treatment with RAD (3.0 mg/kg/day) or vehicle. Animals were killed 72 h after infarction. Four hours before the animals were killed, we injected chloroquine (10 mg/kg IP), which inhibits lysosomal activity. The administration of monodan-

Table 1	Hemodynamic Parameters of Vehicle and RAD-Treated Animals 28 Days After Myocardial Infarction		
	Sham (n = 5)	Vehicle (n = 15)	RAD 3.0 (n = 9)
Heart rate (beats/min)	411 ± 10	379 ± 15	357 ± 26
End-systolic volume (μl)	142 ± 21	349 ± 14*	215 ± 30†
End-diastolic volume (μl)	304 ± 23	414 ± 16*	304 ± 30†
End-systolic pressure (mm Hg)	147 ± 17	120 ± 5*	120 ± 6
End-diastolic pressure (mm Hg)	3.2 ± 0.6	9.6 ± 0.6*	4.6 ± 0.7†
Stroke volume (μl)	183 ± 17	87 ± 7*	105 ± 9
Ejection fraction (%)	58 ± 6	20 ± 1*	35 ± 4†
dP/dt max (mm Hg/s)	11,308 ± 990	6,982 ± 597*	8,086 ± 743
dP/dt min (mm Hg/s)	-11,013 ± 1,094	-5,305 ± 364*	-5,345 ± 410
Tau _w (ms)	8.6 ± 0.3	14.7 ± 0.7*	13.8 ± 1.3

Results are presented as average ± SEM. *p < 0.05 versus sham; †p < 0.05 versus vehicle. RAD = everolimus.

sylcaverine (MDC) (1.5 mg/kg) was done via IP injection 1 h before the animals were killed. MDC is known to label acidic endosomes, lysosomes, and autophagosomes (16,17). One hour after MDC injection, animals were killed and cardiac tissue was harvested immediately. The tissues were fixed in 10% formalin for preparation of paraffin-embedded sections and examined for cardiac autophagy under a fluorescence microscope.

Western blot analysis. For further biochemical analysis, animals were killed 1, 14, or 28 days after MI. Analysis of

RAD effects was performed with RAD treatment starting 1 and 3 days after MI. LV protein lysates (100 μg) were prepared from the LV tissue of the remote area. Western blot analysis of lysates of the rat hearts were performed as described previously (18). Protein-loading was confirmed by Coomassie blue staining. Primary antibodies used were 4EBP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, California), phospho 4E-BP1 (Cell Signaling Technology, Inc., Beverly, Massachusetts), p70/S6 (Santa Cruz Biotechnology, Inc.), phospho p70/S6 (Cell Signaling Technology

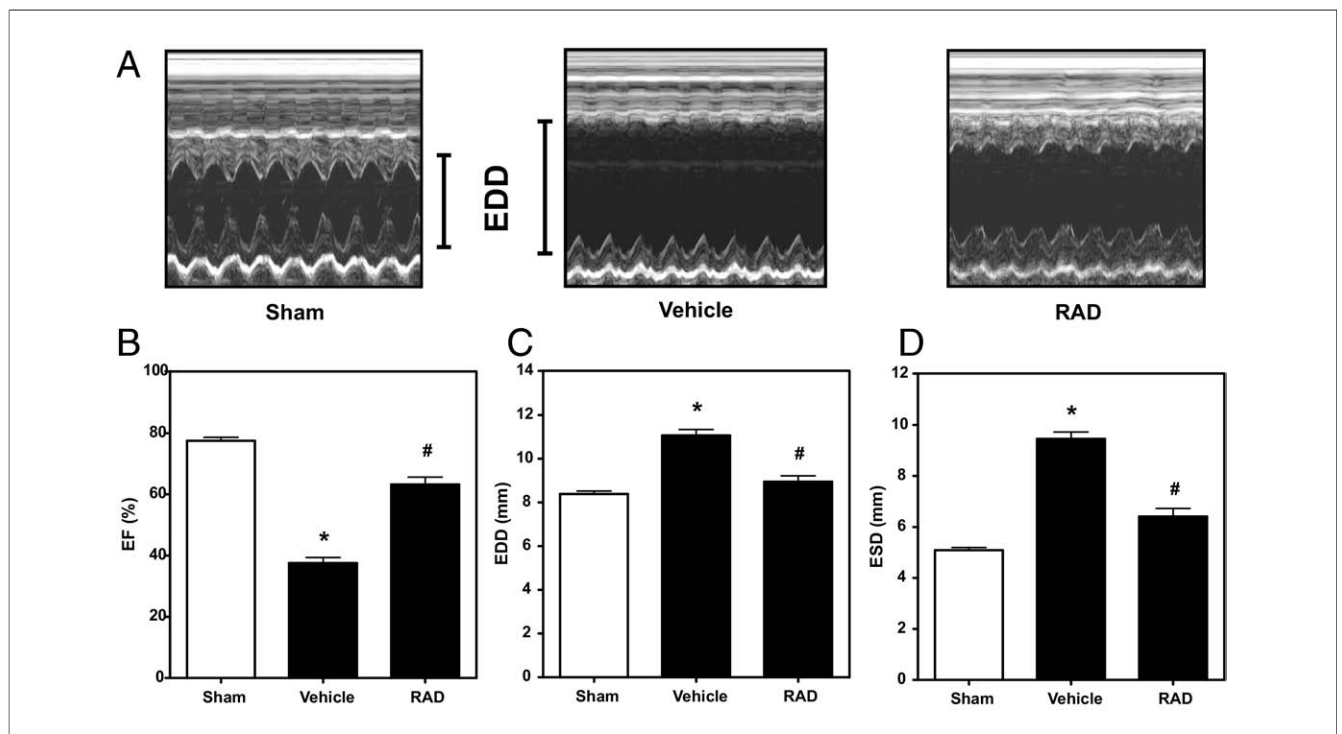


Figure 1 mTOR Inhibition Attenuates Myocardial Remodeling After MI

(A) Transthoracic echocardiographic M-Mode images recorded from the parasternal short axis on the level of the papillary muscles of the left ventricle (LV) in sham-, vehicle-, and everolimus (RAD)-treated (3.0 mg/kg/day) animals after 28 days. (B) Ejection fraction (EF) was significantly higher in the RAD-treatment group after 28 days as compared with vehicle. (C) End-diastolic diameter (EDD) and (D) end-systolic diameter (ESD) of the LV estimated from transthoracic echocardiographic M-Mode images. The EDD and ESD were significantly reduced in RAD-treated animals (3.0 mg/kg/day) after 28 days. *p < 0.05 versus sham; #p < 0.05 versus vehicle. Sham n = 6, vehicle n = 14, RAD n = 14. MI = myocardial infarction; mTOR = mammalian target of rapamycin.

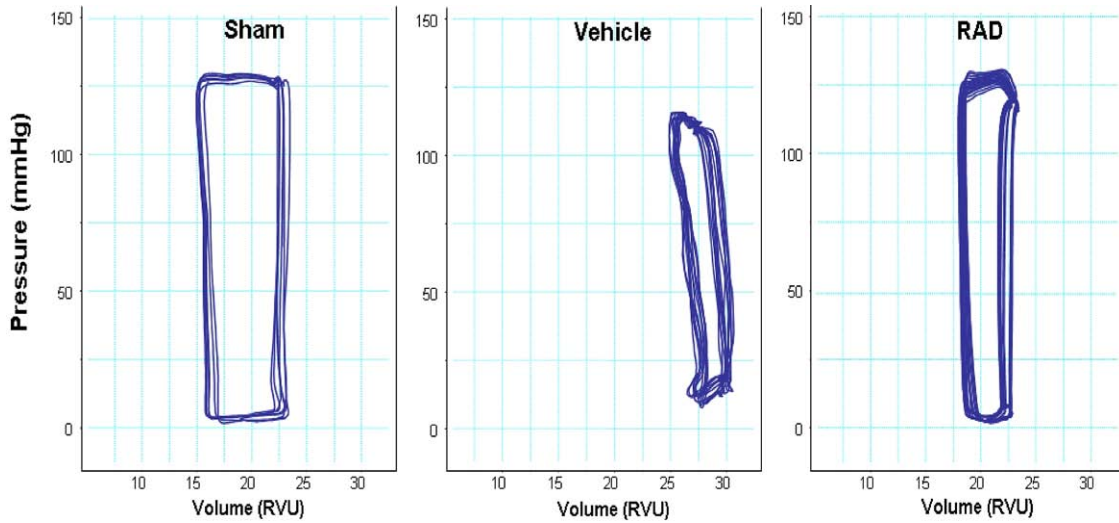


Figure 2 Representative Pressure-Volume Loops During Steady-State Conditions in Sham-, Vehicle-, and RAD-Treated Rats 28 Days After MI

A significant rightward shift of the pressure volume loops is observed in the vehicle-treated animals, which can be reduced ("leftwarded") by treatment with RAD 3.0 mg/kg/day. RVU = relative volume unit; other abbreviations as in Figure 1.

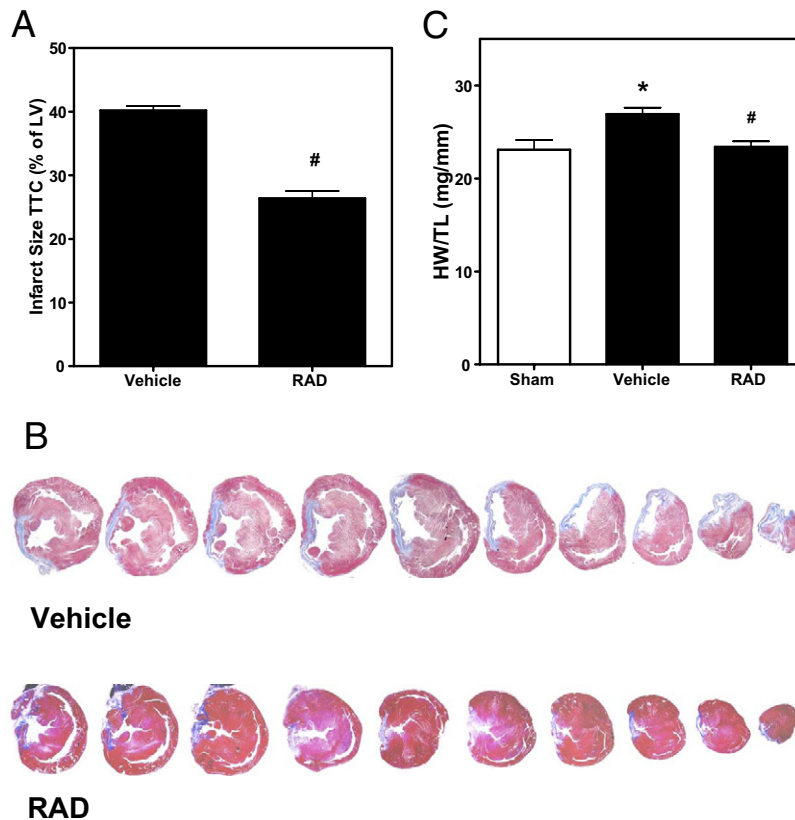


Figure 3 Effects of RAD Treatment on Infarct Size and Myocardial Hypertrophy

(A) Quantitative measurement of infarct size by triphenyl tetrazolium chloride (TTC) staining. Infarct size was significantly smaller in RAD-treated animals as compared with vehicle-treated animals 28 days after MI. (B) Representative examples of Masson's trichrome staining of infarcted hearts. Smaller infarct size was confirmed by histology in the RAD-treatment group. (C) The increased ratio of heart weight (HW) to tibia length (TL) in vehicle-treated MI-animals was significantly reduced after RAD treatment. * $p < 0.05$ versus sham; # $p < 0.05$ versus vehicle. Abbreviations as in Figure 1.

Inc.), ribosomal S6 (Santa Cruz Biotechnology, Inc.), phospho ribosomal S6 (Cell Signaling Technology, Inc.), LC3B (Cell Signaling Technology, Inc.), ubiquitin (P4D1, Cell Signaling Technology, Inc.), and p65 (Cell Signaling Technology Inc.).

Proteasome activity assay. The commercially available "Proteasome Activity Assay Kit" (Millipore, Bedford, Massachusetts) was used for assaying the proteasome activity that recognizes the substrate LLVY (19). The assay is mainly based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC. The free AMC fluorescence was quantified with a 380/460 nm filter set in a fluorometer.

Gene expression. Real-time polymerase chain reaction was performed with the TaqMan assay. Structural modifications of the dNTPs in the sense of a locked nucleic acid allow relatively high annealing temperatures. During the elongation period the deoxyribonucleic acid polymerase destroys the TaqMan-probe through its 5'-exonuclease activity and separates the quencher from the fluorescence molecule, allowing the fluorescence signal to be registered.

Electrophoretic mobility shift assay for nuclear factor-kappa B (NFκB). Activity of NFκB was assessed with electrophoretic mobility shift assay as described previously (20). In brief, tissue from the border zone of the infarction

was homogenized in 400 μl of hypotonic buffer. Nuclear fractions were obtained and resuspended in 50 μl ice-cold buffer C. Nuclear extracts (10 μg each) were incubated with labeled oligonucleotide probes and 2 μg of poly(deoxyinosine-deoxycytidine)-poly(deoxyinosine-deoxycytidine) in 20 μl of binding buffer. The sequences of the oligonucleotides for NFκB were used (Santa Cruz Biotechnology). Binding reactions were resolved on a 4% native polyacrylamide gel and exposed to X-ray film for 12 to 24 h.

Statistics. The results are expressed as mean ± SEM. Statistical analysis was performed with the Graph-Pad Prism Software Package (GraphPad, Inc., San Diego, California). Differences between groups were tested by 1-way analysis of variance with post hoc comparisons by Dunnett's post hoc test and paired or unpaired Student *t* test where appropriate. The differences between groups in the long-term observation were tested by 2-way analysis of variance.

Results

mTOR inhibition attenuates myocardial remodeling after MI. After 1 month, echocardiographic and invasive hemodynamic measurements demonstrated a reduction of LV systolic and diastolic function in vehicle-treated animals as

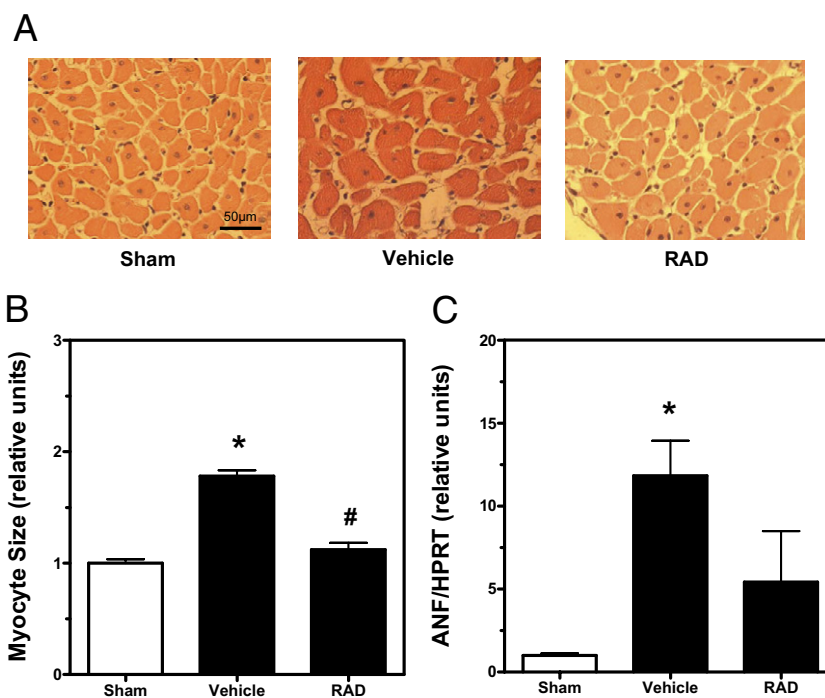


Figure 4 Effects of RAD Treatment on Cardiac Myocyte Size and ANF Expression

(A) Exemplary hematoxylin eosin stainings of a section of the remote area of the MI in sham, vehicle, and RAD-treated animals after 28 days. (B) Quantitative measurement of the myocyte size 28 days after MI. Myocyte size was significantly smaller in RAD-treated animals as compared with vehicle-treated animals. Sham *n* = 5, vehicle *n* = 5, RAD *n* = 13. (C) Gene expression of atrial natriuretic factor (ANF) normalized to the house-keeping gene *HPRT* was significantly increased in vehicle MI-animals but not in the RAD-treatment group. **p* < 0.05 versus sham; #*p* < 0.05 versus vehicle. Abbreviations as in Figure 1.

compared with sham-operated animals (Table 1). The RAD treatment (3.0 mg/kg/day) led to an attenuation of cardiac remodeling. Ejection fraction as determined by echocardiography was significantly reduced in the vehicle group ($37 \pm 2\%$ vs. $77 \pm 1\%$), whereas in the RAD group, ejection fraction was higher as compared with the vehicle group ($63 \pm 2\%$) (Figs. 1A and 1B). LV end-diastolic diameter (EDD) and end-systolic diameter (ESD) dimensions were markedly enlarged in vehicle-treated animals (EDD 11.4 ± 0.2 mm, and ESD 9.7 ± 0.2 mm) as compared with RAD-treated animals (EDD 8.9 ± 0.3 mm, and ESD 6.4 ± 0.3 mm) (Figs. 1C and 1D). Hemodynamic measurements confirmed improvement of systolic and diastolic dysfunction in the RAD group as compared with vehicle-treated rats with MI (Table 1, Fig. 2). Ejection fraction as assessed by pressure-volume measurements was $35 \pm 4\%$ in the RAD group and $20 \pm 1\%$ in vehicle-treated animals ($p < 0.05$). Compared with vehicle-treated animals, RAD treatment caused a substantial leftward shift of the

LV pressure-volume curves (Fig. 2). In addition, RAD treatment reduced the increase in LV end-diastolic volume ($304 \pm 30 \mu\text{l}$ vs. $414 \pm 16 \mu\text{l}$ for RAD- and vehicle-treated rats, respectively, $p < 0.05$) (Table 1).

Effects of RAD on infarct size and myocardial hypertrophy. The RAD treatment (3 mg/kg/day) after induction of MI led to a significant decrease of infarct size as compared with vehicle treatment as assessed by TTC staining (Fig. 3A), whereas area at risk was similar to vehicle-treated animals. Histological examination also showed a reduction of infarct size ($30 \pm 2\%$ vs. $21 \pm 4\%$) (Fig. 3B). By this time the increase in the ratio of LV weight to tibia length observed in vehicle-treated animals with MI was significantly blunted in the RAD-treatment group (Fig. 3C). Next, we evaluated the effect of RAD on cardiac myocyte size in the vital area remote from the infarction. After 4 weeks, there was a significant increase in cardiac myocyte size in the MI group as compared with sham-operated animals, whereas myocytes from MI animals treated with RAD were approxi-

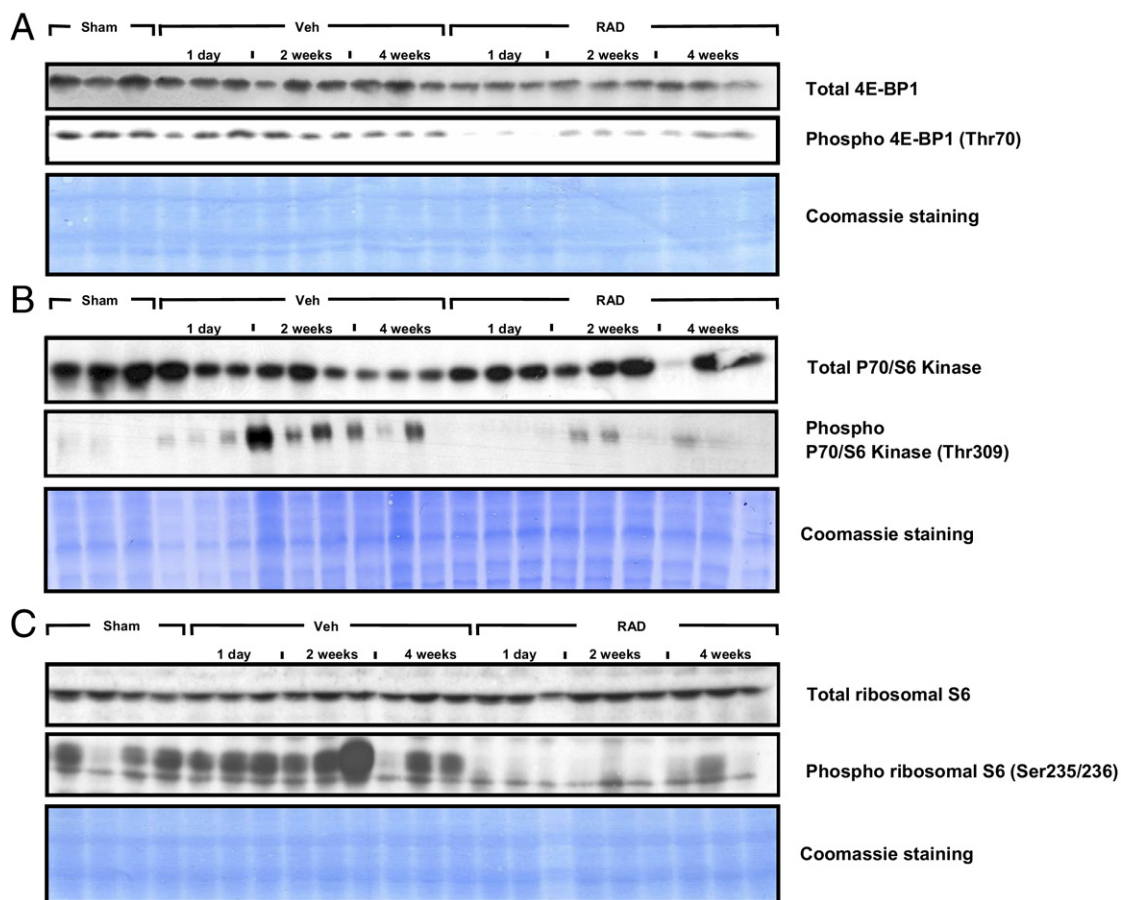


Figure 5 Protein Levels and Phosphorylation Levels of mTOR-Related Regulators of Protein Synthesis

(A) Western blot analysis revealed a significant decrease of phosphorylation of 4E-BP1 (Thr 70) after administration of RAD after MI. (B) Protein levels of phospho-p70/S6K after MI; p70/S6K, 1 of the main downstream targets of mTOR—which mediates cellular growth—displayed a significant decrease of phosphorylation after administration of RAD after MI. (C) Phospho ribosomal S6K is significantly reduced by mTOR-inhibition. Coomassie blue staining confirms similar loading of protein. Veh = vehicle; other abbreviations as in Figure 1.

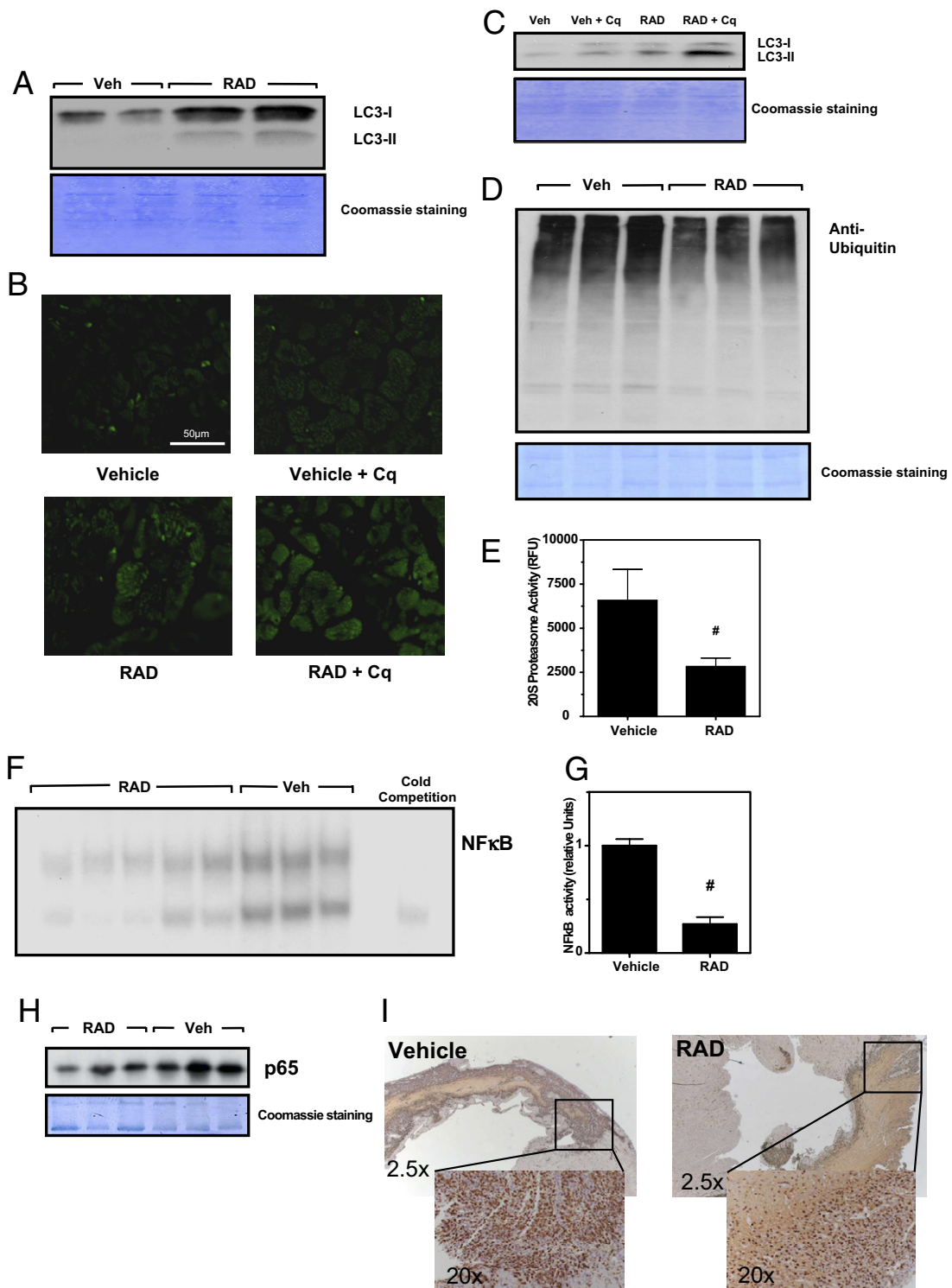


Figure 6 Protein Degradation Mechanisms Are Regulated by mTOR After MI

(A) LC3, a marker of autophagy, is up-regulated in the border zone 3 days after MI in RAD-treated animals. (B and C) Detection of autophagosomes *in vivo* by monodansylchloride (MDC) 3 days after MI in vehicle (Veh)- and RAD-treated animals (B). Activity of LC3 corresponded to autophagous flux assessed by MDC (C). (D and E) Concomitant down-regulation of the ubiquitin proteasome system is observed. (F) Electrophoretic mobility shift assay blot for nuclear factor-kappa B (NFκB), demonstrating a reduced activity in MI animals after RAD-treatment. (G) Densitometric quantification of NFκB clearly demonstrates the significant reduction of NFκB in the border zone of the infarction. (H) Western blot of the p65 subunit also showed significant reduction due to treatment with RAD. (I) In the border zone of the infarction, a reduced macrophage invasion was observed. # $p < 0.05$ versus Veh. Cq = chloroquine; RFU = relative fluorescence units; other abbreviations as in Figure 1.

mately 40% smaller as compared with the untreated MI group (Figs. 4A and 4B). Gene expression of atrial natriuretic factor was significantly increased in vehicle-treated MI animals, whereas RAD-treated MI animals displayed no significant difference compared with sham-operated animals (Fig. 4C).

Inhibition of protein synthesis through RAD. As previously described (8,10), mTOR inhibitors such as RAD block the protein kinase mTOR (especially TORC1, the mTOR-raptor complex), which phosphorylates molecules involved in the regulation of protein synthesis such as S6 kinase and 4EBP-1 (Fig. 5). The 4EBP-1, which negatively regulates protein synthesis, was less phosphorylated in the RAD group, indicating increased restraint on protein synthesis (Fig. 5A). Interestingly, in vehicle-treated animals with MI that were killed 1, 14, and 28 days after MI, we found a significant increase in phosphorylation of p70/S6-kinase, indicating activation of protein translation after MI (Fig. 5B). However, in RAD-treated animals with MI, phospho p70/S6-kinase as well as its downstream target ribosomal S6 were less phosphorylated leading into inactivation of these molecules (Fig. 5C). Total levels of p70/S6K and ribosomal S6 remained unchanged (Figs. 5B and 5C). **mTOR regulates autophagy and proteasomal degradation and attenuates inflammatory response after MI.** Because mTOR is one of the essential regulators of autophagy,

a process that has recently been associated with myocardial ischemia, we investigated whether inhibition of mTOR led to increased autophagy. LC3, a marker of autophagy, was significantly increased in the border zone of the infarction in RAD-treated animals 3 days after MI (Fig. 6A). To examine whether increase of LC3 was due to increased autophagosome formation or impairment of lysosomal fusion, we additionally measured autophagic flux in vivo with MDC as a marker of autophagosomes 3 days after MI (Fig. 6B). Fluorescence microscopy revealed an increase of autophagosomes in RAD-treated animals and a further increase after administration of chloroquine, which indicates that RAD does not lead to decreased clearance of autophagosomes. In concordance with these findings, increased activity of LC3 was found in the same experimental setting with chloroquine (Fig. 6C). Interestingly, with RAD treatment, decreased activity of the ubiquitin-proteasome system was observed, demonstrated by a decrease of ubiquitinated proteins (Fig. 6D) and a decrease of proteasomal activity (Fig. 6E). Next, we investigated whether proteasome inhibition results in decreased NFκB activity. NFκB is known to be pro-inflammatory with important impact on cardiovascular remodeling (21) and was found to have a pivotal role in infarct size (22). Treatment with RAD led to a significant reduction of NFκB in the border zone of MI as compared with vehicle-treated animals (Figs. 6F and 6G).

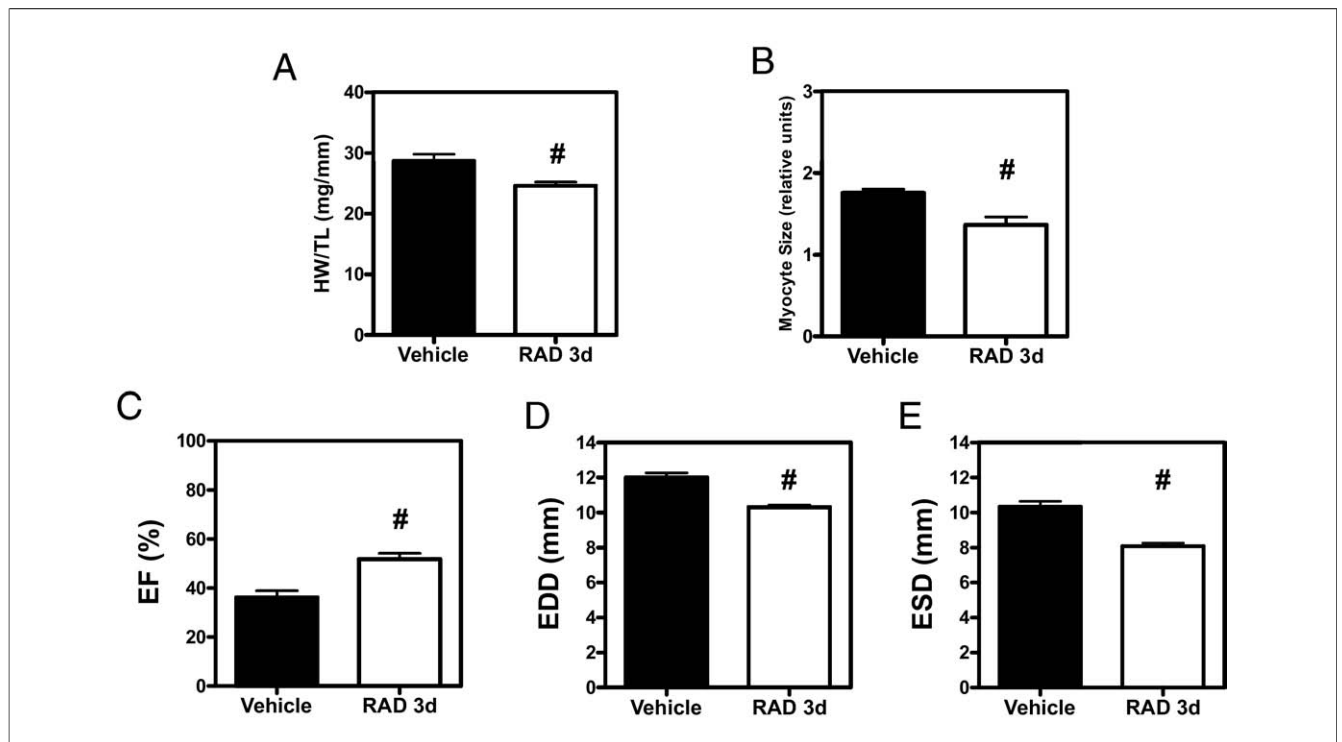


Figure 7 Morphology and Echocardiography After Initiation of RAD Treatment 3 Days After MI

(A) The increased HW/TL ratio in vehicle-treated MI-animals was significantly reduced after RAD treatment (3.0 mg/kg/day) starting 3 days after infarction. (B) Quantitative measurement of the myocyte size 28 days after MI. Myocyte size was significantly smaller in RAD-treated animals as compared with vehicle-treated animals. (C to E) Transthoracic echocardiography. (C) The EF was significantly higher in the RAD-treatment group after 28 days as compared with vehicle. (D and E) The EDD and ESD of the LV showed a significant reduction due to RAD treatment compared with vehicle. #p < 0.05 versus vehicle. Abbreviations as in Figures 1 and 3.

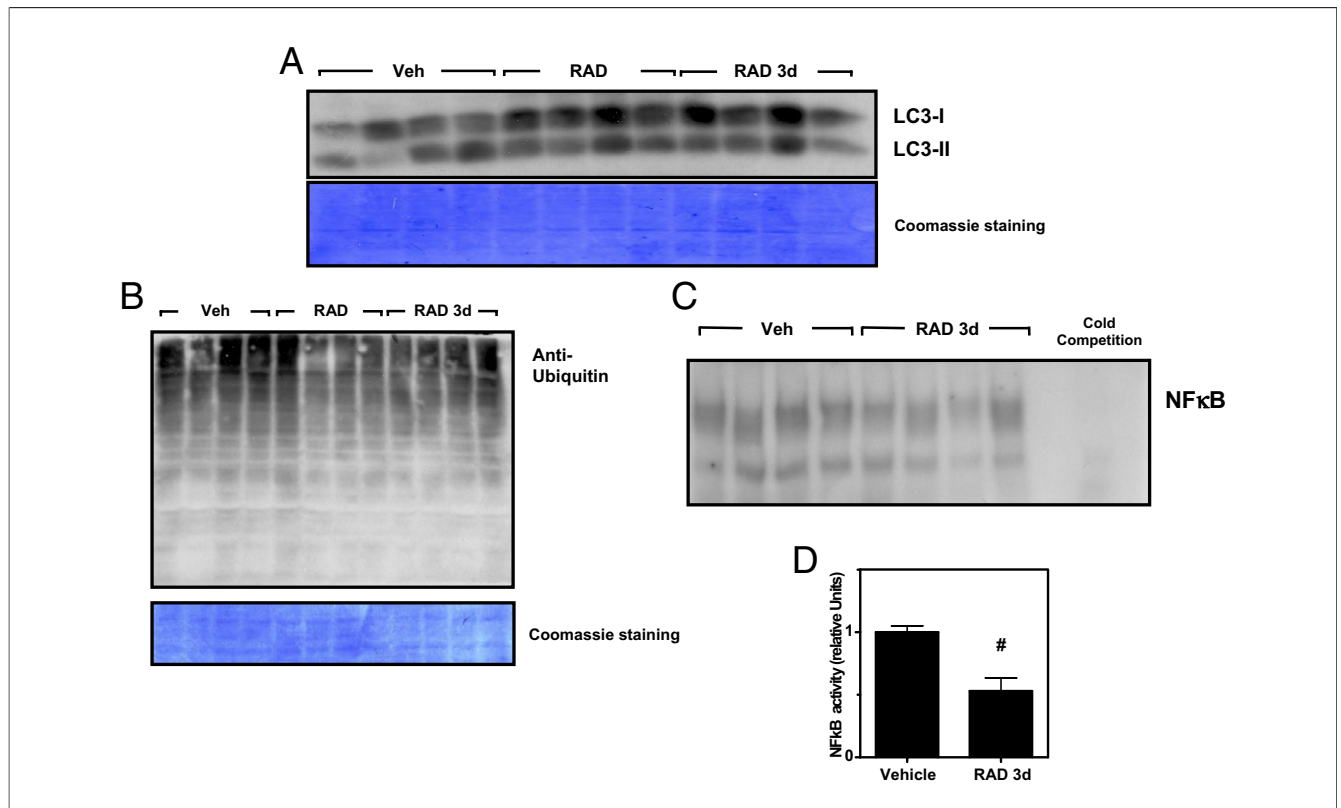


Figure 8 Autophagy and NFκB Activation 28 Days After MI With RAD Treatment Starting 1 or 3 Days After MI

(A) LC3, a marker of autophagy, is still up-regulated in the remote area 28 days after MI in RAD-treated animals after 1 and 3 days (3.0 mg/kg/day). (B) The concomitant down-regulation of the ubiquitin proteasome system is still observed. (C and D) Electrophoretic mobility shift assay blot demonstrating reduced activation of NFκB in MI animals with RAD treatment initiated 3 days after MI in the remote area of the myocardium. Densitometric quantification is shown in D. #p < 0.05 versus vehicle. Abbreviations as in Figures 1 and 6.

Immunoblotting of the p65 subunit also showed a clear reduction (Fig. 6H), in concordance with these findings. Macrophage invasion into the infarcted area, representing an essential step of inflammatory response, was also diminished 72 h after MI (Fig. 6I). These data suggest that—besides inhibition of protein synthesis—increases of autophagy, proteasome inhibition, and reduced inflammation contribute to the beneficial effects of RAD after MI.

Timing of mTOR inhibition. Because patients in the clinical setting frequently present in subacute stages of MI, we investigated whether RAD favorably affects myocardial remodeling in the later phase of MI. Therefore, we initiated RAD treatment (3.0 mg/kg/day) or vehicle treatment 3 days after MI. Infarct size was not significantly altered in the late-treatment RAD group as compared with the vehicle-treated group (37% vs. 40%). Interestingly, significant attenuation of myocardial remodeling was still observed in this RAD group, although the beneficial effect was smaller as compared with the earlier administration, starting within 1 day after MI (Figs. 7A and 7B). In addition, a reduction in relative myocyte size by 25% as compared with vehicle-treated animals was still observed (Fig. 7B). Rats were also characterized by transthoracic echocardiography after a treatment period of 28 days (Figs. 7C to 7E). Again,

significant attenuation of myocardial remodeling was clearly visible. Increase of LC3 and a decrease of ubiquitinated proteins as well as reduced NFκB activity were still present in the remote area 28 days after MI in the early-treatment group as well as in the late-treatment group (Figs. 8A to 8C).

We further investigated whether RAD treatment exerts beneficial effects when given only temporarily (treatment started on day 1 after MI and continued for 1 month). In animals that were killed 3 months after MI (i.e., 2 months after discontinuing RAD treatment), the improvement of LV function was still clearly detectable (Fig. 9). Thus, the mTOR inhibitor RAD exerts long-term beneficial effects on adverse remodeling.

Discussion

The main finding of this study is that targeting the FRAP/mTOR pathway systemically can prevent adverse LV remodeling and limit infarct size after MI. Of note, medication in this study was initiated on the day after induction of MI, thus closely resembling a frequent clinical situation. When RAD was given 3 days after MI, infarct size was similar to vehicle-treated animals, although attenuation of adverse myocardial remodeling and consecutive

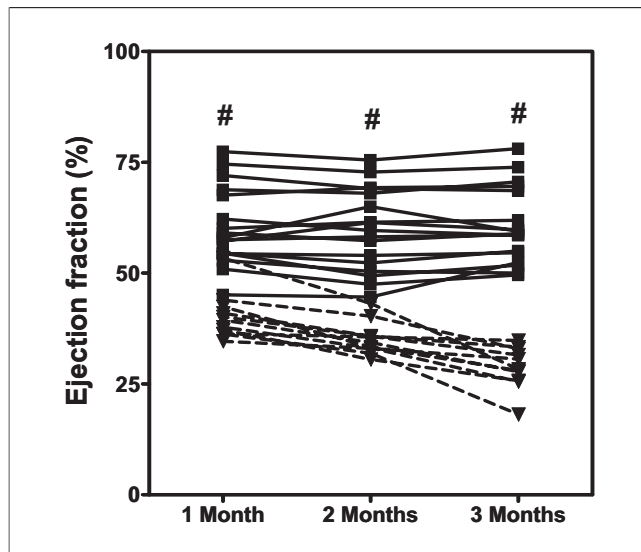


Figure 9 Long-Term Effects of RAD Treatment

The RAD treatment (starting on day 1 after MI) for 28 days led to sustained improvement of LV function 3 months after MI (squares, solid lines), whereas vehicle-treated animals display a progressive deterioration of LV ejection fraction (triangles, dashed lines). Data from serial echocardiography in the same animals are depicted. #p < 0.05 versus vehicle. Abbreviations as in Figure 1.

improvement of LV function were still detectable. At a clinically relevant dose, RAD effectively attenuated infarction-induced remodeling.

Molecular mechanisms of mTOR-inhibition leading to reduced myocardial remodeling. It is known that mTOR plays a key role in regulating cellular growth and development (23,24). An inhibitory effect of rapamycin on development of LV hypertrophy in the setting of aortic banding in mice has been demonstrated previously (8,10). Khan et al. (25) have recently reported protective effects of rapamycin in the context of ischemia/reperfusion. In this setting, the infarct size reduction was mainly attributed to opening of mitochondrial adenosine triphosphate channels. In contrast, Kis et al. (26) demonstrated that administration of mTOR inhibitors before onset of ischemia diminishes the cardioprotective effect of ischemic pre-conditioning. However, these findings were derived from a Langendorff model of ischemia/reperfusion. In the present study we examined the role of mTOR inhibition in an *in vivo* model, which allows investigation of the long-term course and chronic remodeling effects. In contrast to Kis, we applied mTOR inhibitors after myocardial ischemia, as is the case in a clinical setting.

P70/S6K is one of the main downstream targets of mTOR. Interestingly, p70/S6K was activated significantly in the hearts of infarcted animals, whereas treatment with RAD completely suppressed infarct-related p70/S6K activation. Activation of p70/S6K is associated with enhanced protein synthesis, which leads to myocardial hypertrophy (27–29). Rapamycin inhibits the p70/S6K effects as well as myocardial hypertrophy in several animal models (8,10). In contrast, deletion of ribosomal S6K1 and S6K2 does not

alter the myocardial growth response of physiological or pathological stimuli, indicating that these kinases do not seem to be mandatory for the development of cardiac hypertrophy (27). However, this model has not been tested in MI. Even if activation of p70/S6K seems to be a critical factor for the development of cardiac hypertrophy in response to MI, other pathways play a role in mediating the effect of mTOR inhibitors. Another downstream target of mTOR, 4E-BP1, an inhibitor of protein synthesis—which is inactivated by mTOR—also modulates this response. Phosphorylation of 4E-BP1 is known to accelerate the release of eIF4E, allowing increased formation of the eIF4F translation factor complexes (30). As noted in the preceding text, 4E-BP1-phosphorylation is reduced in RAD-treated animals, thereby dis-inhibiting its anti-growth properties. Obviously, these effects that are modulated by RAD can protect the heart from adverse remodeling effects.

Importantly, the physiological response to cellular stress involves down-regulation of mTOR (31). In addition it has been demonstrated that autophagy as an energy-recovering process of protein degradation is associated with mTOR inhibition in the context of myocardial ischemia (32). Up-regulation of autophagy acts as a protective mechanism in the failing heart (33). These findings are consistent with the inhibition of mTOR. Because myocardial blood flow is permanently discontinued in the central region of the infarct zone due to ligation of the corresponding coronary artery, it is obvious that reduction of infarct size must be the result of a specific process in the border zone. The results of this study demonstrate increased autophagy in the critical border zone upon mTOR inhibition. Moreover, a relevant down-regulation of the ubiquitin proteasome system was observed. Such a counter-regulation was demonstrated previously in another context (34). These results underline that mTOR inhibition after MI mimics a conserved process for survival of mammalian cells. Iwai-Kanai et al. (15) have demonstrated that the mTOR inhibitor rapamycin increases autophagosome formation, in line with the results from this study. Concomitant with proteasome inhibition, NFκB activity was reduced in the border zone of the infarct in RAD-treated animals, and macrophage invasion was reduced. Palombella et al. (35) previously demonstrated that the activity of the ubiquitin proteasome system is required for activation of NFκB. Also, in the heart, proteasome inhibition blocks activation of NFκB after myocardial ischemia (36). Inhibition of NFκB activity by the mTOR inhibitors rapamycin as well as RAD has also been found in other cell types (37,38). It is well-known that inflammation is an important factor in myocardial remodeling spreading of the infarction zone (39). Reduced MI size in mice after *in vivo* transfection of cis element decoy against NFκB has previously been shown (22). Frantz et al. (40) demonstrated that the deletion of the p50 subunit of NFκB leads to reduced infarct size in mice. In addition, Li et al. (41) demonstrated that overexpression of A20, which inhibits NFκB, reduces post-infarct remodeling. However, another

study showed that pharmacological inhibition of NFκB starting 24 h after MI for 28 days did improve LV remodeling and cardiac dysfunction but did not reduce infarct size (42). Therefore, inhibition of the NFκB pathway might contribute to improving adverse LV remodeling, but it is not clear whether reduced inflammation is also a contributing factor for infarct size reduction after RAD treatment.

Conclusions and Clinical Perspective

Despite the widespread use of therapeutics interfering with the neurohumoral axis, the incidence of heart failure as the end-stage of LV remodeling and cardiac hypertrophy remains high. The mTOR inhibitors are successfully used for prevention of restenosis. The results of this study provide evidence that the mTOR inhibitor RAD can prevent LV remodeling after MI.

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