3.) Signalpfade der Hypertrophie von Kardiomyozyten nach Hypoxie/Reoxygenierung

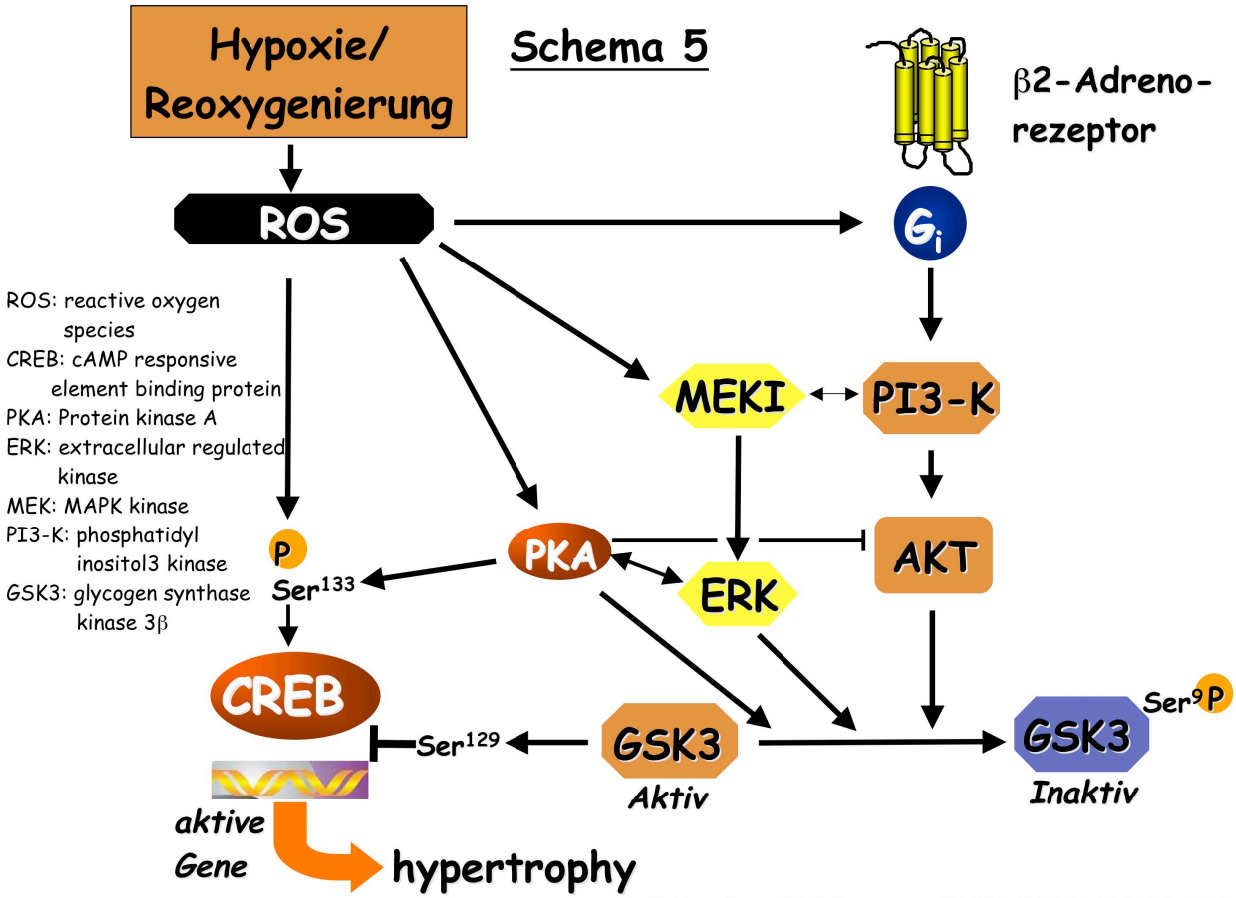
Nach einem Myokardinfarkt kommt es im Laufe einiger Monate zum sogenannten „Remodelling“ der linken Herzkammer; dieser Begriff fasst die Ausbildung der Narbe an Stelle des untergegangenen Myokardgewebes sowie eine Hypertrophie der verbleibenden Herzmuskelzellen zur Aufrechterhaltung der Auswurfleistung zusammen. Die bei der myokardialen Hypertrophie durch Druckbelastung involvierten Signaltransduktionswege sind durch eine Vielzahl von Untersuchungen an genetisch veränderten Mäusen gut charakterisiert. Offensichtlich spielt die Calcium-abhängige Regulation der Signalmoleküle Calcineurin sowie des Transkriptionsfaktors NF-AT hier eine entscheidende Rolle (Molkentin, 2000; Molkentin et al., 1998).

Die Signaltransduktionswege, die die Hypertrophie des vitalen Myokardgewebes nach einem Herzinfarkt regulieren, sind hingegen bisher nur wenig untersucht worden. Aus klinischer Sicht können die Aktivierung des Renin/Angiotensin Systems sowie der β-Adrenorezeptoren als belegt gelten, da eine Therapie mit ACE-Hemmern und β-Blockern das postinfarzielle ventrikuläre Remodelling positiv beeinflusst (Pfeffer et al., 2003; Yaoita et al., 2002). Unsere Arbeitsgruppe hat nun vor diesem wissenschaftlichen Hintergrund in vitro die Hypothese getestet, dass eine sechsstündige Hypoxie gefolgt von einer kompletten Reoxygenierung direkt die Hypertrophie von Kardiomyozyten induziert. Dieses Modell entspricht dem klinischen Ablauf des Ischämie/Reperfusions-Schadens bei Infarkt. Interessant ist dabei die Beobachtung, dass die Reperfusion auch klinisch einen entscheidenden Stimulus sowohl im Hinblick auf die Apoptose als auch die Hypertrophie von Kardiomyozyten darstellt.

Die ersten Experimente an neonatalen Rattenkardiomyozyten zeigten im Vergleich zu Kontrollzellen einen verstärkten Einbau von radioaktiv markiertem Leucin 48h nach Hypoxie/Reoxygenierung. Darüber hinaus war die Zelloberfläche vergrößert sowie „atrial natriuretic factor“ mRNA als Marker der Hypertrophie in erhöhter Menge nachweisbar (El Jamali et al., 2004). Die beteiligten Signaltransduktionswege wurden zunächst durch pharmakologische Untersuchungen charakterisiert. Die Inhibition des β2-Adrenorezeptors, Gi-gekoppelter heterotrimerer Rezeptoren, des PI-3-Kinase-Pfades, der MAPK ERK sowie von Sauerstoffradikalen und der Protein Kinase A unterdrückte die Hypertrophie von

Kardiomyozyten in diesem Model (Schema 5). Die Untersuchung der Aktivierung einer Reihe von Transkriptionsfaktoren mit Hilfe von DNA-Bindungsuntersuchungen ergab den interessanten Befund, dass die Hypoxie zunächst eine Vielzahl von Faktoren wie NF-κB, GATA-4, CREB und HIF 1α aktiviert; bis auf die DNA-Bindung des Transkriptionsfaktors CREB sind diese Aktivitäten jedoch schon 30 Min. nach Reoxygenierung nicht mehr nachweisbar. Vorversuche mit verschiedenen Inhibitoren hatten jedoch gezeigt, dass die Reoxygenierung entscheidend für die Hypertrophie der Kardiomyozyten ist. Die weiteren Untersuchungen konzentrierten sich daher auf CREB. Darüber hinaus wurde dieser Ansatz dadurch bestätigt, dass die spezifische Inhibition des Transkriptionsfaktors NF-κB durch Überexpression der inhibitorischen Untereinheit IκBαΔN in diesem Modell keinen Effekt auf die Hypertrophie hatte. Interessanterweise differenziert dieses Ergebnis das Hypoxie/Reoxygenierungsmodell von der Angiotensin-induzierten Hypertrophie. Die Inhibition von NF-κB vermindert in vitro die Hypertrophie von Kardiomyozyten nach Angiotensin II Stimulation signifikant (Purcell et al., 2001).

Es wurde im Folgenden der Aktivierungspfad von CREB untersucht. Interessanterweise hatten die Inhibitoren der Hypertrophie in diesem Modell keinen Effekt auf die Phosphorylierung des CREB - Serinrestes 133, der die Transaktivierung des Transkriptionsfaktor-komplexes steuert. Hingegen fand sich eine deutliche Verminderung der CREB DNA Bindung, die mit dem Effekt auf die Hypertrophie korrespondierte. Nach älteren Veröffentlichungen wird die CREB DNA-Bindung durch die Aktivität der GSK3β reguliert (Cross et al., 1995). Diese Hypothese wurde daraufhin in dem verwendeten Modell untersucht: tatsächlich fand sich nach Hypoxie/Reoxygenierung eine Phosphorylierung der GSK3β, einhergehend mit einer verminderten Kinaseaktivität, und einer Unterdrückung der Phosphorylierung an dem CREB-Serinrest 129, der durch die Aktivität der GSK3β kontrolliert wird. Die gesteigerte DNA-Bindung von CREB wurde durch Anti-oxidantien, Inhibitoren des PI-3 Kinase Pfades sowie Blocker des β2-Adrenorezeptors unterdrückt – ähnlich der Liste von Inhibitoren, die die Hypertrophie in diesem Model unterdrückt. Die Hypothese einer zentralen Rolle des GSK3β/CREB Signalpfades wurde zudem durch Versuche mit der im DNA-bindenden Bereich mutierten CREB-Variante, „Killer CREB“, belegt: nach Transfektion von K-CREB war die Hypoxie/Reoxygenierungs - induzierte Hypertrophie signifikant vermindert. Das sich aus diesen Versuchen ergebende Schema ist in Abbildung 5 zusammen gefasst.



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Reoxygenation after severe hypoxia induces cardiomyocyte hypertrophy in vitro: activation of CREB downstream of GSK3β

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ABSTRACT

In vivo, left ventricular remodeling after myocardial infarction involves hypertrophy generally attributed to increased cardiac workload. We hypothesized that hypoxia/reoxygenation directly induces cardiomyocyte hypertrophy and studied several participating kinases and transcription factors in isolated cardiomyocytes. Hypoxia for 6 h followed by 42 h reoxygenation induced cardiomyocyte hypertrophy assessed by ^{3}H leucine incorporation and immunohistochemistry. Inhibition of reactive oxygen species (ROS), serine/threonine kinase AKT, and ERK abolished reoxygenation-induced hypertrophy. In addition, a β2-adrenergic receptor (β2-AR) antagonist, as well as Gi inhibitor pertussis toxin, blocked reoxygenation-induced hypertrophy. Hypoxia for 6 h increased transcription factors CREB, NF-κB, and GATA DNA binding activities. However, only CREB DNA-binding was sustained during reoxygenation. Inhibition of PI3-kinase, ERK, and PKA abrogated reoxygenation-induced CREB DNA-binding without affecting CREB serine-133 phosphorylation. These same pathways were found to regulate hypoxia/reoxygenation-induced GSK3β kinase activity and CREB serine-129 de-phosphorylation. GSK3β mutants resistant to phosphorylation blocked the stimulation of CRE-dependent transcription induced by hypoxia/reoxygenation. Transfection of cardiomyocytes with a dominant-negative mutant of CREB abrogated hypoxia/reoxygenation-induced hypertrophy. We suggest that hypoxia/reoxygenation induces cardiomyocyte hypertrophy through CREB activation. Inactivation of GSK3β by hypoxia/reoxygenation, possibly integrating PI3-kinase and ERK pathways downstream of β2-AR and ROS, is a prerequisite for CRE-dependent transcription. Transient hypoxia may contribute to cardiac hypertrophy in ischemic heart disease independent of cardiac workload.

Key words: cardiac hypertrophy • heart failure • CRE-dependent transcription

Left ventricular (LV) remodeling after ischemia/reperfusion involves interstitial fibrosis, cardiomyocyte apoptosis, and hypertrophy. The workload increase for the surviving myocardium may activate signaling cascades that induce cardiac hypertrophy, functional deterioration, and failure (1). Hypertrophy is an adaptive response to compensate for impaired cardiac function. Despite the diversity of stimuli that lead to hypertrophy, a common molecular

response involves increased cell size, protein synthesis, and enhanced sarcomere organization accompanied by partial reactivation of the fetal gene program. In addition to the Ca^{2+}-induced activation of calcineurin followed by nuclear translocation of the transcription factor NF-AT, activation of the mitogen activated protein kinases ERK, p38, phosphorylation of the serine/threonine kinase AKT, and inactivation of glycogen synthase kinase-3β (GSK3β) were all shown to be involved in hypertrophy (2–5). Downstream to these signaling molecules, several transcription factors were found to contribute to cardiac remodeling including GATA 4 and NF-κB (6, 7). β-adrenoreceptor (β-AR) blockade was shown to improve cardiac remodeling in ischemic myocardium (8). Although β1-AR stimulation promotes cardiomyocyte apoptosis, β2-AR stimulation activates survival pathways (9). The latter effect has been attributed to activation of the PI3-kinase/AKT pathway by β2-AR (10). Interestingly, AKT was shown to be an important mediator of compensatory hypertrophy in the context of ischemia/reperfusion (11). In addition, different cardiac phenotypes associated with ventricular either β1 or β2-AR overexpression confirmed the fundamental difference in their myocardial signaling function (12).

Hypoxia adaptation has been characterized both in cardiac tissue and in tumor growth. A major component is the gene expression regulation by the hypoxia-inducible transcription factor 1α (HIF1α; 13). Hypoxia-induced effects in the heart include reperfusion-induced apoptosis and protection from apoptosis by ischemic preconditioning (14, 15). The late phase of preconditioning is mediated in part by activation of the transcription factor NF-κB downstream of reactive oxygen species (ROS; 15). ROS are mainly generated during reperfusion and contribute to signaling pathways activated in ischemia/reperfusion injury (16). ROS have also been shown to activate AKT and ERK, two major signaling proteins involved in cardiomyocyte hypertrophy (17). We tested the hypothesis that hypoxia/reoxygenation directly affects cardiomyocyte growth in vitro and analyzed the potential role of β-AR signaling. Our results suggest that hypoxia/reoxygenation induces cardiomyocyte hypertrophy through CREB activation. This effect involves β2-AR-dependent pathways in the control of CREB activity downstream of GSK3β.

MATERIALS AND METHODS

Cell culture

Primary culture of cardiac ventricular myocytes from 2- to 3-day-old Wistar rats were prepared as previously described (18) with minor modifications. In brief, minced ventricles were digested with collagenase type II (0.14 mg/ml; Worthington Biochemicals, Cellsystems, St. Katharinen, Germany) and pancreatin (1.2 mg/ml) in ADS buffer (116 mmol/L NaCl, 20 mmol/L HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid), 1 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, pH 7.35). Myocytes were purified by discontinuous Percoll gradient centrifugation. Cells were plated on gelatin-coated dishes at a density of 0.95 ´ 10^5 cells/cm^2 and cultured in DMEM/F12 supplemented with 5% horse serum, 3 mmol/L sodium pyruvate, 20 µmol/L L-glutamine, 100 µmol/L ascorbic acid, 2 g/l bovine serum albumin, penicillin (100 U/ml)/streptomycin (100 µg/ml), and NST supplement media (Sigma, Munich, Germany). Culture media were changed to serum-free media without NST 24 h before stimulation. As revealed by anti-α-sarcomeric actin staining, cultures consistently contained >95% cardiomyocytes. Cells were placed in an oxygen-regulated incubator (Labotect, Hannover, Germany) for 6 h (0% O2, 5% CO_2) followed by reoxygenation in standard cell conditions (21%

O_2, 5% CO_2) or stimulated with hydrogen peroxide (H_2O_2 100μM/L, Sigma) for the indicated times. Inhibitors of PKA: H89 (10 μmol/L) + PKI (50 μM/L), PI3-kinase: LY294002 (10 μmol/L), ERK: PD98059 (10 μmol/L) or p38: SB 203580 (10 μM/L) (Calbiochem, Bad Sodden, Germany), as well as antagonists of β2-adrenergic receptor: ICI118,551 (100 nmol/L), β1-adrenergic receptor: CGP20712A (300 nmol/L; Sigma) and α-adrenergic receptor: prazosin (100 nM/L) were added at the beginning of reoxygenation. Further inhibitors used in this study included ROS-antagonist N-acetyl-cysteine (NAC; 10 mM/L) and diphenylene-iodonium (DPI; 10 μM/L; Calbiochem) as well as G_i inhibitor pertussis toxin (400 ng/ml; Sigma) and GSK3β inhibitor II (10 μM; Calbiochem). All culture reagents and additives were purchased from Gibco, Invitrogen, Karlsruhe, Germany unless otherwise stated. PC12 cells were cultured on collagen-coated culture dishes in RPMI 1640 medium supplemented with 10% horse serum and 10% fetal calf serum as described previously (19).

Western blot

At the end of the culture period, cells were washed twice, scraped off in ice-cold PBS and pelleted by centrifugation. Cells lysis was achieved in buffer A (20 mmol/L HEPES, pH 7.9; 350 mmol/L NaCl, 0.5 mmol/L EDTA; 0.5 mmol/L EGTA; 1 mmol/L MgCl2, 10% glycerol, 1% Nonidet P-40, 10 mmol/L NaF, 0.1 mmol/L NaV, 8 mmol/L β-glycerophosphate, and 1 tablet protease inhibitors cocktails/50 ml buffer; Roche, Mannheim, Germany). Protein extract was recovered by centrifugation. Aliquots of proteins (10–20 μg) were separated on 12.5% sodium dodecyl sulfate polyacrylamide gels (SDS/PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Immunoblots with primary antibodies (P-AKT, AKT, P-GSK3β, P-CREB serine-133 and CREB; (Cell Signaling, Frankfurt, Germany), P-CREB serine-129/133 (Biosource), GSK3β (Santa Cruz), HIF1-α (Novus Biologicals), or anti-α-sarcomeric-actin (Sigma) was performed as previously described (18). The autoradiographic intensity of each band was scanned and analyzed by NIH imager program, which gives integrated optical density. Values were normalized to total levels of phosphorylated and unphosphorylated proteins (AKT, CREB, or GSK3β).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described for NF-κB EMSA in cardiomyocytes (18). In brief, 5 to 10 μg of total cellular protein was incubated at room temperature with radioactive double-strand oligonucleotide (30000 cpm/sample) in buffer C (20 mmol/L HEPES, pH 8.4; 60 mmol/L KCl; 4% Ficoll 400) supplemented with 0.05 μg/μl poly dI-dC, 0.5 mg/ml bovine serum albumin (BSA), 5 mmol/L DTT for 30 min. Samples were run on native polyacrylamide gels (4%) in TBE buffer, pH 8.3 (50 mmol/L Tris Base, 50 mmol/L boric acid, 1 mmol/L EDTA). Gels were dried and exposed to hyperfilm (Amersham) overnight at –80°C. We used the following oligonucleotides: CREB: 5'-GATCCTGGAAGGGGAGTGACATCAGTCATATA-3'; NF-κB: 5'-GATCCAGGGCTGGGGATTCCCCATCTCCACAGG-3'; GATA: 5'GATCTGTGGCTGATAAATCAGAGACTAGAC-3'; OCT-1: 5'-GATCCCAACTCTTCACCTTATTTGCATAAGCGATTCTATAG-3'. GATA and CREB oligonucleotides were designed from the human BNP promoter sequence (*20*). Protein loading was controlled by EMSA using the OCT probe (*21*). DNA-protein complexes of interest were identified by supershift analysis with specific antibodies against CREB (Cell Signaling), GATA

4 and p65 (Santa Cruz) incubated with protein extract 2 h prior to incubation with the radiolabelled-probe.

Protein synthesis assays

^{3}H leucine (2 μCi/ml) was added for the final 18 h before harvesting. Cellular proteins were precipitated, washed twice with 5% trichloroacetic acid, once in 100% ethanol, and finally dissolved in 0.5 M NaOH. Incorporated ^{3}H leucine was measured by liquid scintillation counter, each measurement performed in triplicate. In order to discriminate true hypertrophy from any proliferation in the cell culture, values were normalized to DNA content determined by optical densitometry at 260 nm.

Immunfluorescence analysis

Cells were grown on glass coverslides with laminin in 24-well culture dishes. At the end of the treatment, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton 100 for 15 min at 4°C. Cells were subjected to immunostaining by using antibodies against α-sarcomeric actin (Sigma) and rhodamine-conjugated secondary antibody (Sigma). α-sarcomeric actin expression was visualized on a fluorescent microscope (Zeiss).

Northern blots

Total RNA was extracted from cardiomyocytes with Quiagen RNeasy kit following manufacturer's instructions. RNA (10 μg) separated by electrophoresis was transferred to Hybond N nylon filters as previously described (22). Hybridization was performed with the Quickhyb buffer (Stratagene) for 3 h at 68°C in the presence of 5'-end-labelled oligonucleotide probe (2´10^{6} cpm/ml). Filters were then washed to a final stringency of 0.2´ SSC, 0.1% SDS at 68°C and subjected to autoradiography. The ANP oligonucleotide probe sequence used was 5'-AATGTGACCAAGCTGCGTGACACACCACAAGGGCTTAGGATCTTTTGCGATCTGCTC AAG–3'.

Transient transfection

AMAXA transfection

Cardiomyocytes were transfected with Rc/RSV vector expressing K-CREB (K287L), where the CREB DNA-binding domain has been mutated (kindly provided by Richard H. Goodman; Vollum Institute, Portland, OR; *23*). We used the newly available nucleofector technique. Conditions were optimized for neonatal cardiomyocytes in collaboration with the manufacturer (AMAXA, Cologne, Germany). Briefly, 2´ 10^{6} cells were resuspended in 100 μl of nucleofector^{TM} solution, mixed with 4 μg of DNA, and electroporated. FACS analysis of cells transfected with control plasmid EGFP-N1 (Clontech, Palo Alto, CA) revealed transfection efficiencies of 35–40%.

Recombinant adenovirus vectors

Ad5-IκBαΔN replication-defective E1 and E3 adenovirus contains an N-terminal deleted IκBα cDNA (amino acid residues 71–317) flanked by RSV 3'LTR and the bovine growth hormone

polyadenylation signal (kindly provided by P. Loeser, MDC). Ad5-control virus contains the transcriptional control element of the former virus but no coding sequence. Cells were infected as previously described at a multiplicity of infection of 40 plaque-forming units per cell (18). Subsequently, the cells were cultured in serum-free medium for an additional 24 h before treatments.

Reporter gene assay

The luciferase reporter plasmid CRE-luciferase contains four copies of the CRE site located in front of the minimal rat insulin I promoter fused to the firefly luciferase reporter gene (kindly provided by Willhart Knepel; 24). The pcDNA 3.1 expression vector encoding GSK3βS9A mutant was kindly provided by C. Badorff (Frankfurt, Germany) and previously described (25).

PC12 cells were seeded in 24-well dishes, and transient transfection was performed the next day using 3 µl of Tfx50 reagent (Promega) and 1 µg of total DNA plasmid. Transfection efficiency was around 10% as controlled by GFP-transfection experiments. phRL-TK vector (Promega) expressing the renilla luciferase under the control of herpes simplex virus thymidine kinase promoter (HSV-TK promoter) was transfected with the test plasmids to control for the variability in transfection efficiency. At 48 h cells were harvested in 100 µl of passive lysis buffer and assayed for renilla and firefly luciferase activities as described by manufacturer (Promega).

Statistical analysis

Data are mean ± SEM values and were normalized to controls. Statistical analysis was performed using student's *t*-test. A value for $P < 0.05$ was accepted as significant.

RESULTS

Hypoxia/reoxygenation induces cardiomyocyte hypertrophy

To assess the efficiency of the hypoxia treatment, Western blot analysis of HIF1α protein was performed on cells treated for 1, 3, or 6 h under hypoxia or 6 h hypoxia followed by 5 min reoxygenation. As described before, the HIF1α protein was stabilized during hypoxia and rapidly degraded during reoxygenation, while total CREB was unchanged (Fig. 1*A*; 13). To test the hypothesis that hypoxia/reoxygenation affects protein synthesis, ^{3}H leucine incorporation was determined on cells cultured under hypoxia for 1, 3, or 6 h followed by reoxygenation to complete 48 h of culture. Protein synthesis was increased in correlation with prolonged culture under hypoxia. A maximum of 1.7±0.15 fold increase was observed for 6 h hypoxia followed by 42 h reoxygenation (Fig. 1*B*). No siginificant difference concerning DNA content was observed between control cells and cells subjected to hypoxia/reoxygenation at this late time point, excluding major differences in total cell count between hypoxia-treated dishes and control dishes. As ^{3}H leucine was only present during the final 18 h of the experiment, any nonspecific effect in relation to hypoxia/reoxygenation or inhibitors could be excluded as this would occur earlier. Consistent with the ^{3}H leucine data, immunohistochemical analysis showed that hypoxia/reoxygenation at these time points induced an increase of cell size and α-sarcomeric actin expression (Fig. 1*C*). We next determined the expression levels of ANP by Northern blot. The mRNA levels were increased by hypoxia/reoxygenation (Fig. 1*D*). Collectively, these data demonstrate that hypoxia/reoxygenation induces cardiomyocyte hypertrophy in vitro.

Hypoxia/reoxygenation-induced hypertrophy involves reactive oxygen species, PI-3 kinase, MAPK ERK, and β2-adrenergic receptor-dependent, pertussis toxin sensitive pathways

We next analyzed the effect of various inhibitors known to be involved in G-protein coupled receptor-induced hypertrophy. Cells were cultured for 6 h under hypoxia followed by 42 h of reoxygenation. Antagonists were added at the time of reoxygenation as reperfusion was shown to increase ROS production, which we hypothesized to be important for hypoxia/reoxygenation-induced hypertrophy (16). ROS in turn have been shown to activate the PI3-kinase/AKT pathway involving Gαi-protein coupled receptors (17). We did not observe toxic effects of inhibitors or vehicles when administered at the beginning of the reoxygenation period. The ROS-inhibitor NAC almost completely abolished hypoxia/reoxygenation induced hypertrophy (1.14±0.110). The β2-AR antagonist ICI 118,551 inhibited hypertrophy (1.20±0.11), while the β1-AR antagonist CGP20712A (1.53±0.11) or the α-AR blocker prazosin had no significant effect (1.44±0.24). Inhibition of both β1-AR and β2-AR did not enhance the inhibitory effect of ICI 118,551 alone (1.25±0.07; Fig. 2*A*). As β2-AR but not β1-AR couple to Gi we tested the effect of the Gi inhibitor pertussis toxin (PTX). PTX completely abolished hypoxia/reoxygenation (0.85±0.08) induced hypertrophy.

We further analyzed the activation of signaling pathways possibly activated by ROS and β2-AR, where pharmacological inhibition was most potent concerning hypertrophy. Cells were harvested for Western blot after 30 min of reoxygenation. Hypoxia/reoxygenation increased the phosphorylation of AKT (2.7±0.5 fold) and MAPK ERK (1.4±0.1 fold; Fig. 2*B*, *C*). To determine the relevance of these signaling proteins in hypertrophy, protein synthesis was measured after hypoxia 6 h/reoxygenation 42 h in the presence of PI3-kinase – (LY294002), ERK – (PD 98059), or PKA – inhibitors (H89 and PKI) added at the beginning of reoxygenation. The hypoxia/reoxygenation-induced hypertrophy was completely inhibited by LY294002 and PD98059, whereas PKA-inhibition exerted only a partial effect (Fig. 2*A*). Inhibition of the PI3-kinase was most potent. Interestingly, inhibition of PI3-kinase as well as PKA diminished MAPK ERK phosphorylation (Fig. 2*B*). Furthermore, AKT phosphorylation by hypoxia/reoxygenation was down-regulated by MEK1 inhibition (PD 98059) but enhanced after PKA inhibition (Fig. 2*C*). An inhibitory role for PKA on AKT activation has been described before involving the cAMP receptor EPAC (26). Furthermore, cross-talk between the MAPK ERK pathway downstream of Raf/MEK1 and PI3-kinase/AKT is also a well-known phenomenon (27). The results suggest an important role for PI3-kinase/AKT as well as ERK signaling pathways possibly downstream of β2-AR/Gi in hypoxia/reoxygenation—induced hypertrophy.

DNA-binding activity of CREB, GATA, and NF-κB under hypoxia/reoxygenation

Because transcription factors GATA and NF-κB have been implicated in cardiomyocyte hypertrophy, these transcription factors were included in the investigation in addition to CREB as a downstream target of β-AR's (6, 7). Supershift experiments identified the migrating complex involving p65, CREB or GATA4 (Fig. 3*A*). EMSA analysis showed that 6 h of hypoxia increased CREB, NF-κB, and GATA DNA-binding activity by 2- to 4-fold. Already 30 min of reoxygenation decreased binding activities of NF-κB and GATA. In contrast, CREB DNA-binding even increased after 30 min of reoxygenation (Fig. 3*B*) and was still enhanced after 2 h of reoxygenation (Fig. 3*C*).

Hypoxia/reoxygenation-dependent hypertrophy does not require NF-κB activation

As NF-κB has been implicated in cardiac hypertrophy and was shown to mediate the late phase of preconditioning in the context of ischemia/reperfusion, the effect of NF-κB inhibition on hypoxia/reoxygenation-induced hypertrophy was analyzed (7, 15). Cardiomyocytes were infected either with the Ad5-control virus or Ad5-IκBαΔN virus expressing an undegradable mutant of NF-κB inhibitory subunit IκBα. Western blot analysis confirmed IκBαΔN expression in cardiomyocytes transfected with Ad5-IκBαΔN virus (Fig. 4*A*). EMSA analysis showed that IκBαΔN expression effectively abolished NF-κB DNA-binding activity induced by TNFα or 6 h of hypoxia in cardiomyocytes (Fig 4*B*). Protein synthesis induced by hypoxia/reoxygenation was not affected by the specific inhibition of NF-κB (Fig. 4*C*). Unexpected enhancement of protein synthesis was observed in untreated Ad5-IκBαΔN-transfected-cells, where NF-κB DNA binding was down-regulated below baseline levels (Fig. 4*B*, *C*). Whether this is an adaptive response of the cells due to the lack of constitutive NF-κB activity remains unclear. Nevertheless, these data demonstrate that hypoxia/reoxygenation-induced hypertrophy does not require NF-κB activation.

Regulation of CREB phosphorylation and DNA binding activity by differential pathways involving ROS and GSK3β

Robust phosphorylation and sustained increase of its DNA-binding activity suggested that CREB is important in hypoxia/reoxygenation-induced hypertrophy. Therefore, we further analyzed the regulation of this transcription factor by hypoxia/reoxygenation. Because the phosphorylation of CREB at serine-133 is mediated by a variety of kinases, including PKA (28), $p90^{RSK2}$ downstream of ERK, and AKT (29), we tested the effect of H89, PD98059, and LY294002, respectively, on CREB serine-133 phosphorylation. None of these inhibitors blocked CREB serine-133 phosphorylation, which was induced 2.8±0.3-fold in hypoxia/reoxygenation-treated cells (Fig. 5*A*). However, CREB phosphorylation induced by 10 μmol/L forskolin (30 min) was inhibited by H89 (data not shown). These data indicate that phosphorylation of CREB at serine-133 could involve an additional pathway as suggested previously in PC12 cells possibly linked to ROS (30). Indeed, we observed increased serine-133 phosphorylation after treatment with ROS (100 μM H_2O_2, Fig. 6*B*) known to be released in reoxygenation.

CREB activity has been described to depend on GSK3β inactivation, as a secondary phosphorylation at serine-129 targeted by GSK3β inhibits CREB DNA binding and CRE-dependent transcription (28). GSK3β in turn was recently shown to be a central mediator of cardiomyocyte hypertrophy in vivo, as inactivation of this kinase by phosphorylation of serine-9 is required for cardiac hypertrophy (*5*, *25*). We therefore analyzed CREB serine-129- and GSK3β serine-9 phosphorylation. Interestingly, hypoxia/reoxygenation down-regulated CREB serine-129 phosphorylation as well as GSK3β kinase activity (Fig. 5*B*, *C*). Western blot analysis demonstrated a 2.5±0.4-fold increased phosphorylation of GSK3β on serine-9 in cells subjected to hypoxia for 6 h followed by reoxygenation for 30 min sensitive to inhibition of PI3-kinase, ERK, and PKA (Fig. 5*C*). Furthermore, this effect was abolished by ROS-inhibition (NAC + DPI) as well as blockade of β2-AR/Gi (ICI + PTX; data not shown). Specific pharmacologic inhibition of GSK3β down-regulated CREB serine-129 phosphorylation to a similar level as hypoxia/reoxygenation, whereas both inhibitor and h/r almost abolished phosphorylation at this

site (Fig. 5*B*). As CREB 129 phosphorylation was not totally abolished, additional kinases might regulate phosphorylation of this site like $Ca2^{+}$-dependent CaMK II previously implicated in CRE-dependent transcription independent of CREB serine-133 phosphorylation (31).

To further characterize CREB activation in the context of hypoxia/reoxygenation-treatment, EMSA analysis was performed. Hypoxia/reoxygenation enhanced the CREB DNA-binding activity by threefold; this effect was significantly counteracted by inhibitors of PI3-kinase, MAPK ERK and PKA (Fig. 5*D*). In addition, β2-AR and Gi inhibition reduced CREB DNA binding as well as antagonists of ROS (NAC) and the NADH/NADPH oxidase inhibitor DPI, whereas inhibition of p38 had no effect. In summary, regulation of CREB DNA-binding and phosphorylation by hypoxia/reoxygenation involves independent pathways. PI3-kinase/AKT, ERK, and PKA possibly downstream of ROS-formation and β2-AR dependent Gi activation regulate CREB DNA-binding activity, whereas a currently unknown pathway downstream of ROS mediates CREB phosphorylation at serine-133. CREB DNA binding corresponds inversely to GSK3β activity regulating a secondary phosphorylation site at serine-129.

Phosphorylation of AKT, ERK, GSK3β, and CREB by hydrogen peroxide

To evaluate the potential role of ROS, we stimulated cardiomyocytes with H_2O_2 (100 μM). We observed robust phosphorylation of AKT, which was down-regulated by β2-AR antagonist, Gi inhibitor, and surprisingly by the GSK3β inhibitor II (Fig. 6*A*). MAPK ERK was also strongly activated; however, this activation is only slightly inhibited by β2-AR antagonist and unaffected by Gi inhibition. These data extend previously published data showing ERK and AKT activation by ROS through $G\alpha_o$ and $G\alpha_i$ heterotrimeric proteins. (17) Next, we analyzed GSK3β serine-9 phosphorylation by H_2O_2. In contrast to the findings after hypoxia/reoxygenation (Fig. 5*C*), the strong GSK3β phosphorylation was unaffected by PI-3 kinase and ERK inhibition but sensitive to PKA inhibition. Although CREB serine-133 phosphorylation showed a similar pattern, CREB serine-129 was regulated in the opposite direction: corresponding to GSK3β serine-9 phosphorylation meaning kinase inactivity, serine-129 was phosphorylated only in the absence of H_2O_2. This effect was unaltered by β2-AR and Gi inhibitor. The specific GSK3 inhibitor II reduced CREB serine-129 phosphorylation while being less potent than H_2O_2. The data demonstrate GSK3β to integrate several signaling pathways towards regulating CREB serine-129 phosphorylation.

Inactivation of GSK3β activity is required for enhanced CREB transcriptional activity

To evaluate the potential role of GSK3β on CREB activity, reporter gene assays with a CRE-luciferase vector were performed (24). The CRE-construct was not induced by forskolin in cardiomyocytes after liposomal transfection (efficiency: 5%) possibly relating to cell-type specific effects concerning this construct. Therefore, these experiments were done in PC12 cells, which, similar to cardiomyocytes, activate the PI3-kinase/AKT/GSK3β pathway under hypoxia (32). PC12 cells transiently transfected with a CRE-luciferase reporter construct cotransfected with either pcDNA3.1 or GSK3βS9A were subjected to hypoxia/reoxygenation. We observed a 2.5±0.3 fold increase of CRE-dependent luciferase expression by hypoxia/reoxygenation. This effect was completely abolished in cells cotransfected with GSK3βS9A, a mutant of GSK3β that cannot be inactivated by phosphorylation (Fig. 6*B*). A similar effect was observed in PC12 cells

treated with forskolin (Fig. 6*B*). These results demonstrate that CRE-dependent transcription is regulated by GSK3β and point to a hierarchical two-step process for full activation of CREB after hypoxia/reoxygenation: phosphorylation of CREB through ROS and inactivation of GSK3β mediated by PI3-kinase/AKT and possibly ERK and PKA.

Requirement of CREB for hypoxia/reoxygenation-induced hypertrophy

Next, we tested the hypothesis that CREB activation was essential for the development of hypertrophy in hypoxia/reoxygenation. Protein synthesis was determined in cells transfected with either the control vector or a dominant-negative CREB mutant, K-CREB. K-CREB can dimerize with wild-type CREB but lacks the DNA-binding domain thereby preventing gene activation by titration of the endogenous CREB (23). Transfection efficiency and K-CREB function were assessed by EMSA. Hypoxia 6 h/reoxygenation 30 min enhanced CREB DNA-binding activity in cells transfected with the control vector, whereas K-CREB expression partially blocked CREB DNA-binding activity (Fig. 7*B*). ^{3}H leucine incorporation experiments showed that hypoxia 6 h/reoxygenation 42 h could increase protein synthesis in cells transfected with the control vector (1.51±0.1, $P<0.05$), whereas hypoxia/reoxygenation-induced hypertrophy was abrogated in K-CREB-transfected cells (0.9±0.2; Fig. 7*C*). These findings demonstrate that CREB is essential for hypoxia/reoxygenation-induced hypertrophy.

DISCUSSION

The important findings in our study were that hypoxia for 6 h followed by 42 h reoxygenation resulted in cardiomyocyte hypertrophy. The hypertrophy depended upon ROS-release, PI3-kinase as well as ERK and PKA activation. The kinases appear to be downstream to signaling via β2-adrenoreceptor/Gi coupling, since β2-AR blockade and direct Gi inhibition diminished the hypertrophic effect, whereas β1-AR—or α-AR—blockade did not. The transcription factor CREB, but not NF-κB, was essential for cardiomyocyte hypertrophy. Inactivation of GSK3β in control of CREB serine-129 phosphorylation was important for hypoxia/reoxygenation-induced CRE-dependent transcription.

Hypoxia/reoxygenation and cardiomyocyte hypertrophy

We observed an increased cell size, increased protein synthesis, increased α-sarcomeric actin, and enhanced ANP expression following reoxygenation. This effect was blocked by the antioxidant N-acetyl cysteine. Direct stimulation of cardiomyocytes with hydrogene peroxide activated several signaling pathways related to cardiomyocyte hypertrophy. These data compare favorably with the observation that reoxygenation activates ROS generation from mitochondria, xanthine oxidase, and NAD(P)H oxidase (16). ROS in turn have been implicated in cardiomyocyte hypertrophy induced by mechanical stretch earlier. Low concentrations of H_2O_2 (10–30 μM) were shown to induce protein synthesis in adult cardiomyocytes via MAPK ERK (33). However, there seem to be subtle differences in hypoxia/reoxygenation and H_2O_2 signaling in cardiomyocytes: In our study, H_2O_2 and hypoxia/reoxygenation treatment induced phosphorylation of GSK3β-serine-9 and dephosphorylation of CREB serine-129. Although hypoxia-/reoxygenation-induced signaling was sensitive to Gi, PI3-kinase, and ERK inhibition, H_2O_2-induced GSK3β and CREB regulation was not. As several upstream pathways regulated GSK3β (34), this state-of-affairs might be due to the high H_2O_2 concentration used (100 μM)

which activates additional pathways substituting for the above-mentioned pathways. In addition, previous studies have also found the H_2O_2 induced AKT and ERK activation, which we hypothesize to be upstream of CREB serine-129 phosphorylation, to be PTX-insensitive (17).

Aside from cardiomyocytes, several cell types demonstrate ROS-sensitive growth. Norepinephrine induces vascular smooth muscle cell (VSMC) growth through ROS generated by activation of NAD(P)H–dependent oxidase (35). PDGF-dependent stimulation of NAD(P)H oxidase in VSMC occurs by association of $G\alpha_i$ with PDGF-α receptor (36). In cardiac fibroblasts, angiotensin II stimulation increases intracellular ROS, which induce transcriptional up-regulation of IL-6 levels by activation of CREB (37). Hypoxia/reoxygenation directly induces proliferation of endothelial cells (38). This effect was attributed to Ca^{2+}-dependent signaling upstream of ERK. CREB activity is potently activated by Ca^{2+} upstream of ERK, PKA, and Rsk2 providing a potential link to the above mentioned pathway (39). In addition, calcium/calmodulin protein kinase II has been shown to regulate CREB phosphorylation at another site than serine-133, which inhibits cAMP-induced CRE-dependent transcription (31).

Role of β-adrenoreceptors in hypoxia/reoxygenation induced hypertrophy

In neonatal cardiomyocytes, ROS were shown to activate ERK and AKT through stimulation of Gi proteins, but not Gs or Gq (17). This Gi-dependent activation of ERK and AKT requires activation of PI3-kinase and tyrosine kinase Src. Only β2-AR couple to Gi thereby activating PI3-kinase and ERK, whereas β1 adrenergic receptors activate the classical Gs/adenyl cyclase/cAMP pathway (10). Indeed, we found that ERK, AKT, and GSK3β were phosphorylated by hypoxia/reoxygenation. GSK3β phosphorylation as well as hypoxia/reoxygenation induced hypertrophy were potently blocked by Gi-antagonist pertussis toxin and partially by the β2-AR inhibitor ICI118,551. The α-AR antagonist prazosin, which also couples to Gi, had no effect. We hypothesize the β2-AR antagonist to affect intracellular Gi activity by conformational changes of the receptor. Clearly, direct inhibition of Gi was more potent than specific β2-AR antagonism, which indicated direct activation of Gi by ROS independent of β2-AR to contribute to hypoxia-/reoxygenation-induced hypertrophy. Interestingly, Gi activation itself was recently shown to induce ROS generation by activation of NAD(P)H oxidase (36). Once activated, this pathway could therefore amplify itself thereby explaining the effect on hypertrophy at 48 h after hypoxia.

GSK3β as a central mediator of hypoxia/reoxygenation induced hypertrophy

Protein synthesis experiments demonstrated that reoxygenation-induced activation of PI3-kinase/AKT, ERK, and PKA pathways were necessary to induce hypertrophy. Inhibitors of these pathways as well as antioxidant NAC blocked GSK3β kinase inactivation by preventing serine-9 phosphorylation. GSK3β kinase activity was closely correlated to CREB serine-129 phosphorylation and CREB DNA binding. Consistent with these results, activation of AKT, as well as overexpression of a constitutively active mutant of MEK1 acting upstream of ERK, has been shown to induce cardiac hypertrophy in vivo (4, 40). The inactivation of GSK3β by hypoxia/reoxygenation dependent on PI3-kinase, ERK, and PKA is in agreement with previous studies demonstrating GSK3β to integrate several, independent upstream pathways (5). Suprisingly, pharmacological inhibition of GSK3β did not completely abolish CREB serine-129 phosphorylation. This may be due to a different EC50 of this newly available inhibitor in

cardiomyocytes compared with other cell types. Alternatively, other previously described pathways negatively regulating CRE-dependent transcription might be involved. Particularly Ca^{2+}-induced activation of CaMK II has been implicated in CREB regulation (31). Recent data demonstrate that GSK3β is predominately inactivated by the PI3-kinase/AKT pathway and less by the canonical Wnt-pathway in cardiomyocytes (41). In vivo studies have shown that GSK3β inactivation is a prerequisite for stress-induced cardiac hypertrophy (5, 25). These studies as well as our findings support the idea that GSK3β represents a point of crosstalk and convergence of disparate hypertrophic signaling pathways.

Transcription factors and cardiomyocyte hypertrophy

Several transcription factors downstream of the above-mentioned signaling pathways have been implicated in hypertrophy (6, 7). In the present study, 6 h of hypoxia induced NF-κB, GATA, and CREB DNA-binding activity, and stabilized HIF1α protein. Because inhibitors added at the beginning of reoxygenation, where HIF1α, GATA4, and NF-κB are already down-regulated, abrogated the hypertrophy, we concluded that these transcription factors were not primarily involved in reoxygenation-induced hypertrophy. This hypothesis is supported by our experiments, which excluded NF-κB as being a mediator of hypoxia/reoxygenation-induced hypertrophy. Nevertheless, NF-κB was shown to be essential for late preconditioning and Gq-coupled-receptor induced hypertrophy (7, 15, 42). The transcription factors necessary for Gq-coupled-receptor and reoxygenation-induced hypertrophy seem to differentiate these two model systems.

Regulation of CREB transcriptional activity by hypoxia/reoxygenation

Western blot experiments demonstrated strong phosphorylation of CREB by reoxygenation for merely 30 min, independent of PI3-kinase, ERK, and PKA. Similar observations have been reported in PC12 neuroblastoma cells, where hypoxia was shown to induce CREB phosphorylation via a novel signaling pathway independent of PKC, PKA, Ca^{2+}, MAPKAP2, p38 MAPK, and RSK2 (30). Recent data show that ROS is involved in CREB phosphorylation at serine-133 (19). Consistent with these studies, we observed that H_2O_2 induces CREB serine-133 phosphorylation independently of the above-mentioned pathways in cardiomyocytes. In contrast to the data concerning CREB phosphorylation, EMSA analysis revealed that hypoxia/reoxygenation regulates CREB DNA-binding through PI3-kinase and ERK activation. Although serine-133 phosphorylation has been thought to be sufficient for CREB responsive element (CRE)-mediated gene activation, several studies have shown that these events can be dissociated. In both PC12 cells and in primary neurons, depolarization activates the cFos–CRE in a manner that is blocked by inhibitors of PKA despite the fact that CREB phosphorylation is maintained (43). Phosphorylation of CREB at serine-133 creates the consensus sequence SXXXS(P) for subsequent CREB phosphorylation by GSK3β at serine-129 (44). Phosphorylation of CREB at serine-129 has been shown to inhibit CREB DNA-binding activity (28, 45). Indeed, hypoxia 6 h followed by reoxygenation for 30 min inactivated GSK3β kinase activity, reduced CREB serine-129 phosphorylation, and induced CREB DNA-binding depending on the same upstream pathways in our study. This observation suggests that active GSK3β inhibits CREB DNA-binding through CREB serine-129 phosphorylation. Reporter gene assays performed in PC12 cells confirmed that a constitutive active mutant of GSK3β abrogates CRE-dependent gene transcription induced by forskolin or 6 h hypoxia followed by 24 h

reoxygenation. A similar model of a sequential activation of CREB by PKA and GSK3β in control of DNA binding affinity has been described (28). In summary, hypoxia/reoxygenation regulates CRE-dependent transcription by two independent signaling pathways. Both CREB serine-133 phosphorylation and inactivation of GSK3β, in order to diminish CREB serine-129 phosphorylation, are required for hypoxia/reoxygenation-induced CREB activation and CRE-dependent transcription.

Role of CREB in hypertrophy

^{3}H leucine incorporation experiments demonstrated that CREB activation was essential for hypoxia/reoxygenation and the resultant cardiomyocyte hypertrophy. Consistent with our data, previous reports in vascular smooth muscle cells suggested that CREB downstream of ERK may be important in AngII-induced hypertrophy (46). However, CREB activity was assessed only by serine-133 phosphorylation in that study. In vivo, heart-restricted dominant-negative CREB expression leads to dilated cardiomyopathy (47). Mice lacking the CREM– gene (cAMP responsive element modulator) demonstrate impaired cardiac contraction due to decreased SERCA expression (48) These in vivo data demonstrate the functional importance of the CREB/CREM family concerning cell hypertrophy and survival in end-differentiated cell types like neurons and adult cardiomyocytes. Consistent with our conclusions, overexpression of the inducible cAMP early repressor (ICER), an endogenous inhibitor of CRE-mediated transcription up-regulated by β-adrenoreceptor stimulation, was recently shown to attenuate isoproterenol-induced cardiomyocyte hypertrophy in vitro (49).

The pure in vitro character of the experiments as well as the specificity of the inhibitors and phosphorylation-specific antibodies used may limit our study. Although inhibitor concentrations were carefully chosen based on previous publications, we cannot exclude the possibility that some of the observed effects are due to nonspecific side effects on other signaling cascades. Concerning the effects of CREB and GSK3β mutants, adenoviral delivery would have resulted in more efficient transfection. However, adenoviral transfection might also induce unexpected cellular effects as observed with the NF-κB inhibitory subunit IκBΔN. The proposed model of a central role for CREB serine-129 phosphorylation downstream of GSK3β in cardiac hypertrophy warrants further investigation in other models of heart hypertrophy. Whether or not the prominent role of transcription factor CREB is a characteristic feature in ischemic remodeling remains to be determined in vivo. A comparison of the hypertrophic signaling pathways induced by hypoxia/reoxygenation and those mediated by Gq-coupled receptors should provide interesting information. Such comparisons may add new insights to address the ongoing debate whether a specific hypertrophic signaling pathway or whether an alteration in the intrinsic balance of signaling pathways are involved in the transition from cardiac hypertrophy to heart failure.

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Fig. 1

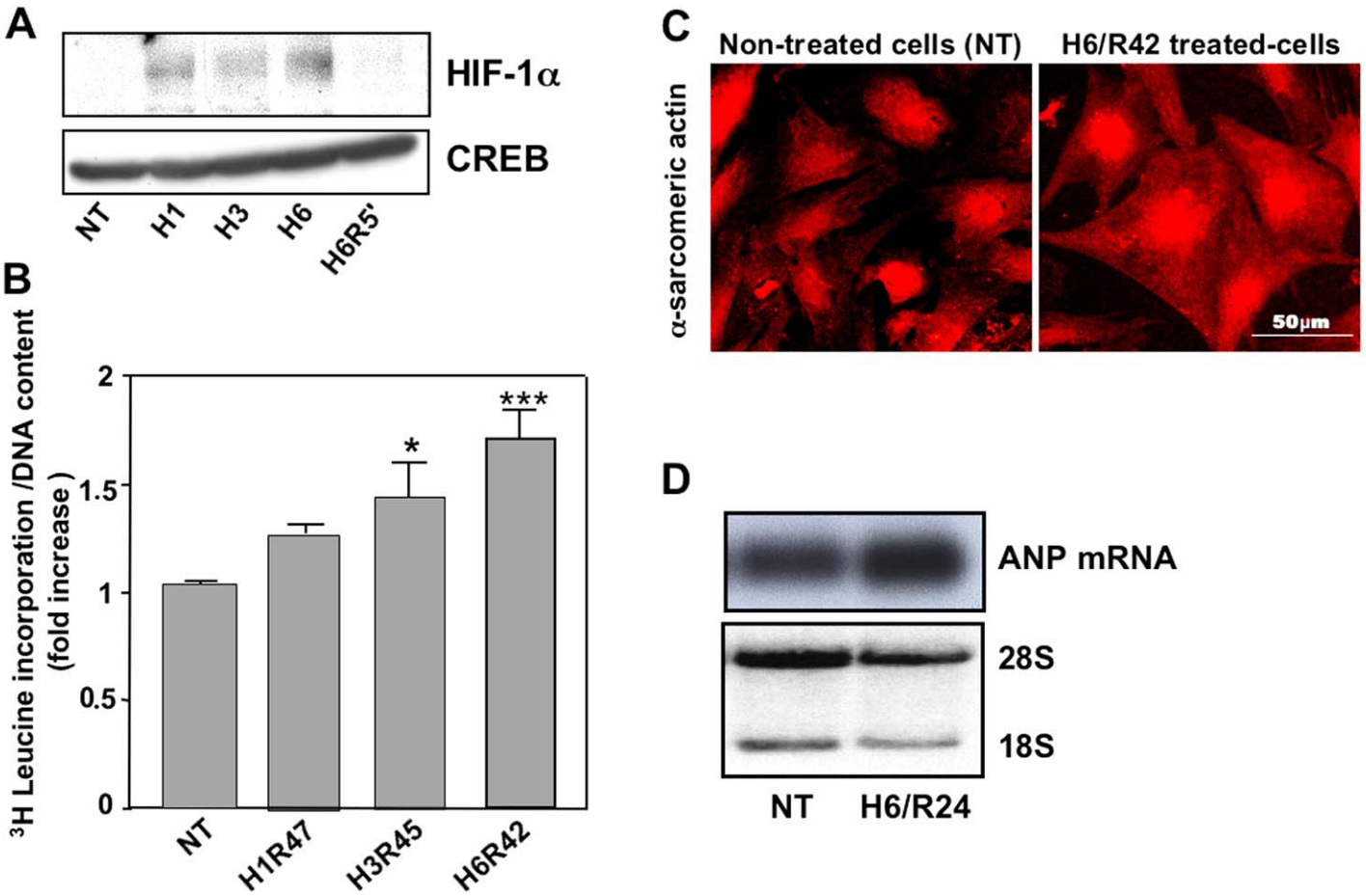

Figure 1. Hypoxia/reoxygenation induces hypertrophy. ***A)*** Activation of transcription factors by hypoxia was confirmed by increased HIF1-α protein levels rapidly down-regulated by reoxygenation. Cardiomyocytes were cultured under severe hypoxia (0% O_2, 5% CO_2, 95% N_2) for 1, 3, or 6 h (H1, H3, H6) or under 6 h hypoxia followed by 5 min reoxygenation (H6R5'). Total CREB levels were unaltered by hypoxia. ***B)*** For quantitative hypertrophy assays, cardiomyocytes were cultured under various times of hypoxia followed by reoxygenation to complete 48 h of culture (H1R47, H3R45, H6R42, respectively). Protein synthesis as determined by ^{3}H leucine incorporation normalized to DNA content revealed a significant increase in protein synthesis. Data indicate fold induction of protein synthesis versus control cells and are mean ± SEM of 3 to 6 independent experiments from as many independent cell preparations. *$P < 0.05$; ***$P < 0.005$. ***C)*** Immunofluorescence staining of α-sarcomeric actin confirmed hypertrophy in cardiomyocytes after hypoxia 6 h and reoxygenation 42 h. ***D)*** Northern blot experiments with ANP probe demonstrated increased ANP-levels. Top: ANP autoradiogram, Bottom: Loading control by ethidium bromide staining of the ribosomal RNA 18S and 28S.

Fig. 2

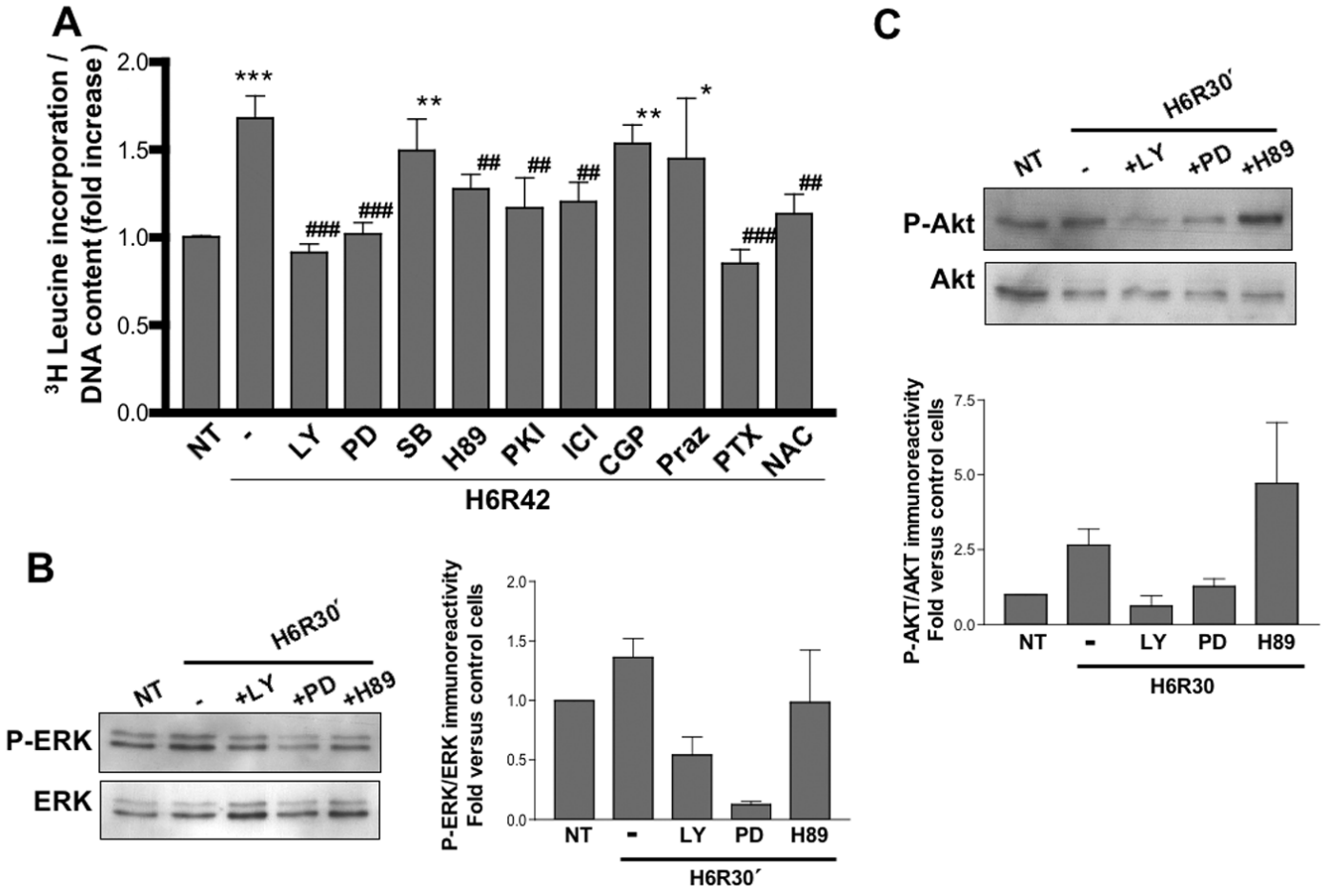

Figure 2. Signaling pathways involved in hypoxia/reoxygenation-induced hypertrophy. *A*) Inhibition of PI-3 kinase, MAPK ERK, β2-adrenoreceptor, G_i, and reactive oxygen species (ROS) abrogated H/R-induced hypertrophy. Cardiomyocytes were exposed to hypoxia 6 h/reoxygenation 42 h. Inhibitors were added during reoxygenation (PI3-kinase-inhibitor: LY294002 (LY); ERK-inhibitor: PD98059 (PD); p38 MAPK: SB203580 (SB); PKA-inhibitor: H89 (each 10 μM) and PKI (50 μM); β2-AR antagonist: ICI118,551 (ICI) (100 nM); β1-AR antagonist : CGP20712A (CGP; 300 nM); α-AR antagonist prazosin (Praz), 100 nM; G_i inhibitor: pertussis toxin (400 ng/ml); ROS-inhibitor: N-acetyl cysteine (NAC, 10 mM). ***B)*** Hypoxia 6 h/reoxygenation 30 min (H6R30`) induced phosphorylation of ERK and (***C***) AKT as detected by Western blot analysis of phosphorylated protein. The effect of PI3-kinase – (LY), MEK1 – (PD), and PKA – inhibition (H89) was analyzed. Results are representative of at least three independent experiments.

Fig. 3

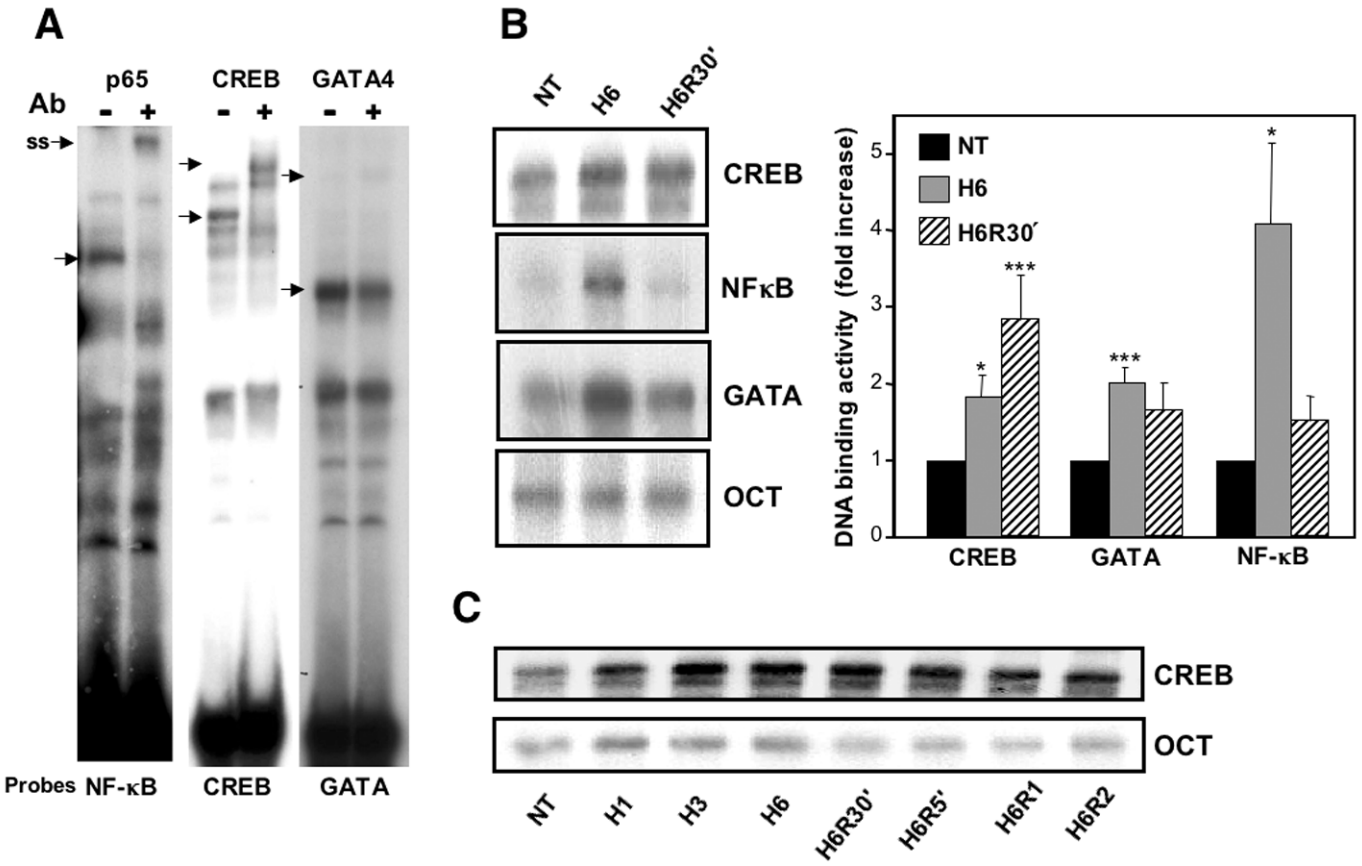

Figure 3. Sustained activation of transcription factor CREB but not GATA or NF-κB by hypoxia/reoxygenation. ***A)*** To confirm EMSA specificity, supershift analysis was performed for CREB, GATA, and NF-κB transcription factors in cardiomyocyte cell extracts harvested after 6 h of hypoxia. DNA-protein complexes involving the p65, GATA4, or CREB protein are marked with an arrow (ss=supershifted complexes). ***B)*** Only CREB DNA binding was sustained after reoxygenation, whereas NF-κB and GATA binding activities were rapidly down-regulated. Autoradiograms of representative EMSA experiments performed on cells treated by hypoxia 6 h (H6) or hypoxia 6 h, 30 min reoxygenation (H6R30′) are shown. DNA binding activities were normalized to the corresponding OCT band. Data indicate fold increase versus control cells (NT) and are mean ± SEM values of 4 to 8 independent experiments. **P*<0.05; ****P*<0.001. ***C)*** A representative EMSA autoradiogram (*n*=3) shows the sustained effect of hypoxia/reoxygenation on CREB DNA binding activity.

Fig. 4

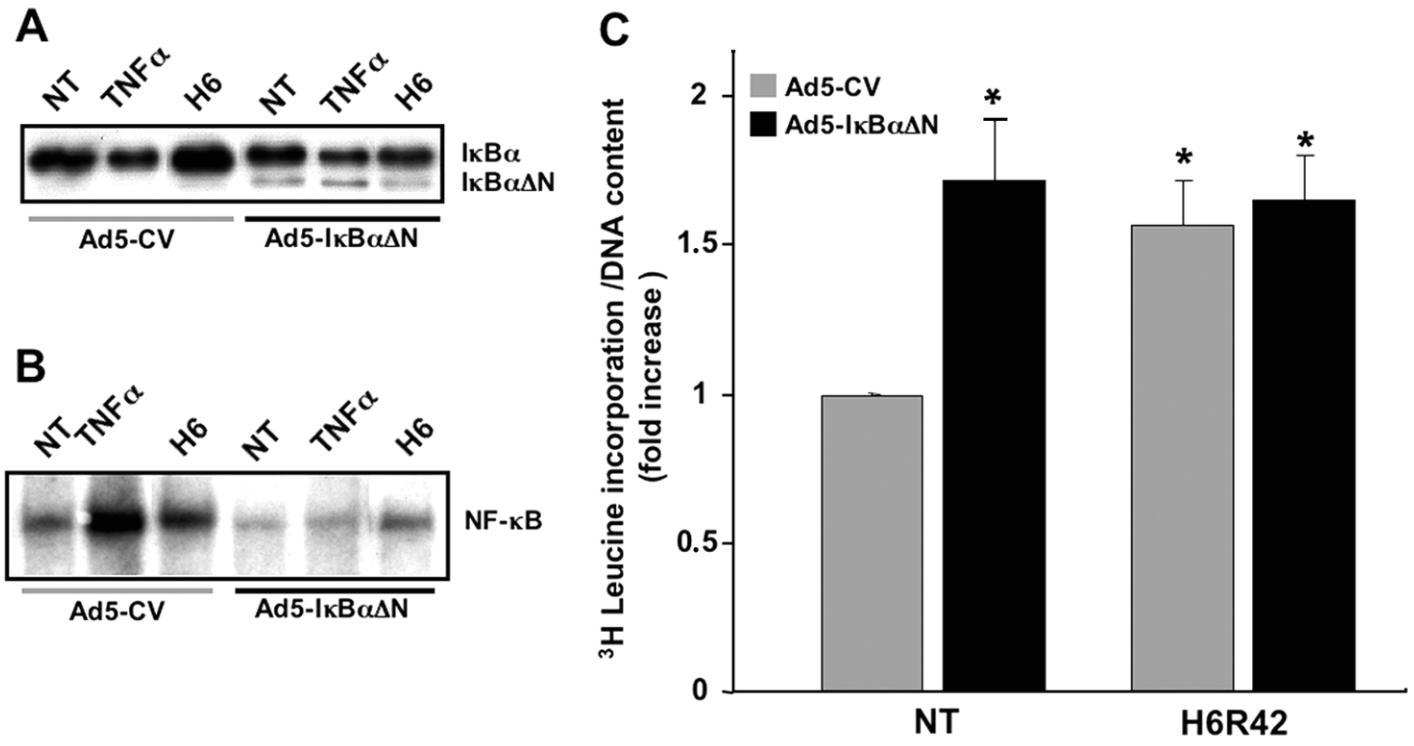

Figure 4. Hypoxia/reoxygenation-induced hypertrophy does not require NF-κB activation. Specific inhibition of transcription factor NF-κB by IκBαΔN does not inhibit hypoxia/reoxygenation-induced hypertrophy. ***A)*** Expression of mutated IκBα (IκBαΔN) in cardiomyocytes down-regulates endogenous IκBα levels at baseline and after TNF-α (30 min, 10 ng/ml) or 6 h of hypoxia. ***B)*** EMSA analysis using the NF-κB oligonucleotide probe demonstrates total inhibition of NF-κB binding by IκBΔN. ***C)*** Protein synthesis is increased after hypoxia/reoxygenation in control-vector infected cardiomyocytes as well as in IκBαΔN-infected cells. Expression of IκBαΔN up-regulates protein synthesis at baseline. Data are mean ± SEM of three independent experiments. **P*<0.05 (***C***).

Fig. 5

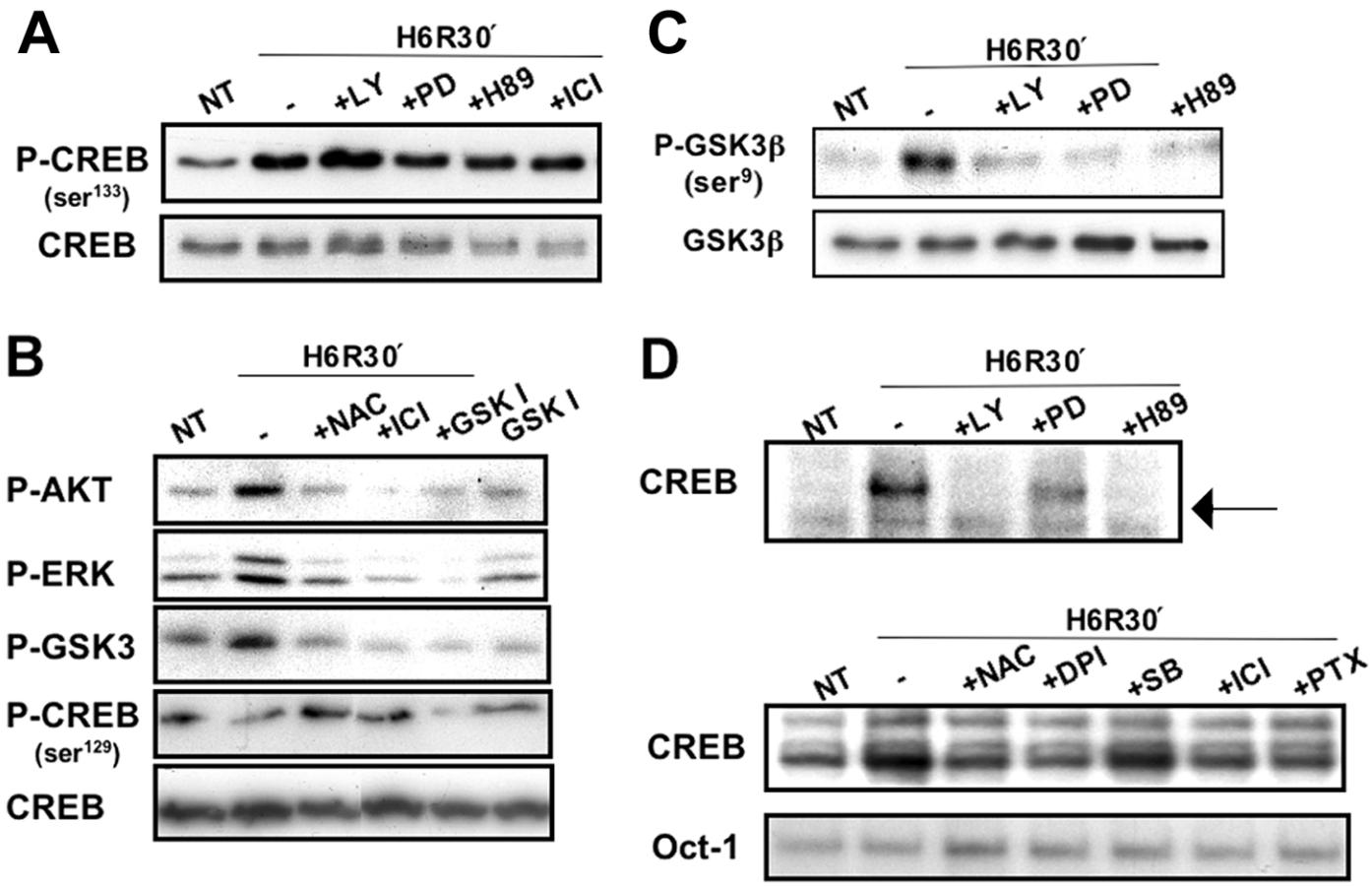

Figure 5. Regulation of CREB by hypoxia/reoxygenation. Inhibition of PI3-kinase, MAPK ERK, and PKA have no effect on hypoxia/reoxygenation-induced CREB serine-133 phosphorylation but inhibit CREB DNA binding. Inhibition of DNA binding correlates inversely to CREB serine-129 phosphorylation and directly to GSK3β serine-9 phosphorylation. Cardiomyocytes were subjected to hypoxia 6 h/reoxygenation 30 min with inhibitors present during reoxygenation as described (**Fig. 2*B***). ***A)*** Up-regulation of CREB serine 133 phosphorylation occurs independent of PI3-kinase, ERK, PKA, or β2-AR. ***B)*** CREB serine-129 phosphorylation is reduced by hypoxia 6 h/reoxygenation 30 min in correlation to AKT, ERK, and GSK3β phosphorylation. ROS-inhibitor NAC and β_2-AR inhibitor ICI antagonize this effect. The GSK3β inhibitor II (GSK I) blocks CREB 129 phosphorylation both after H/R as well as under baseline conditions. ***C)*** PI3-kinase, MAPK ERK, and PKA regulate phosphorylation of GSK3β at serine-9. ***D)*** CREB DNA binding is blocked by inhibition of PI3-kinase, MAPK ERK, PKA, ROS, β_2-AR, and Gi. Data represent at least three independent experiments.

Fig. 6

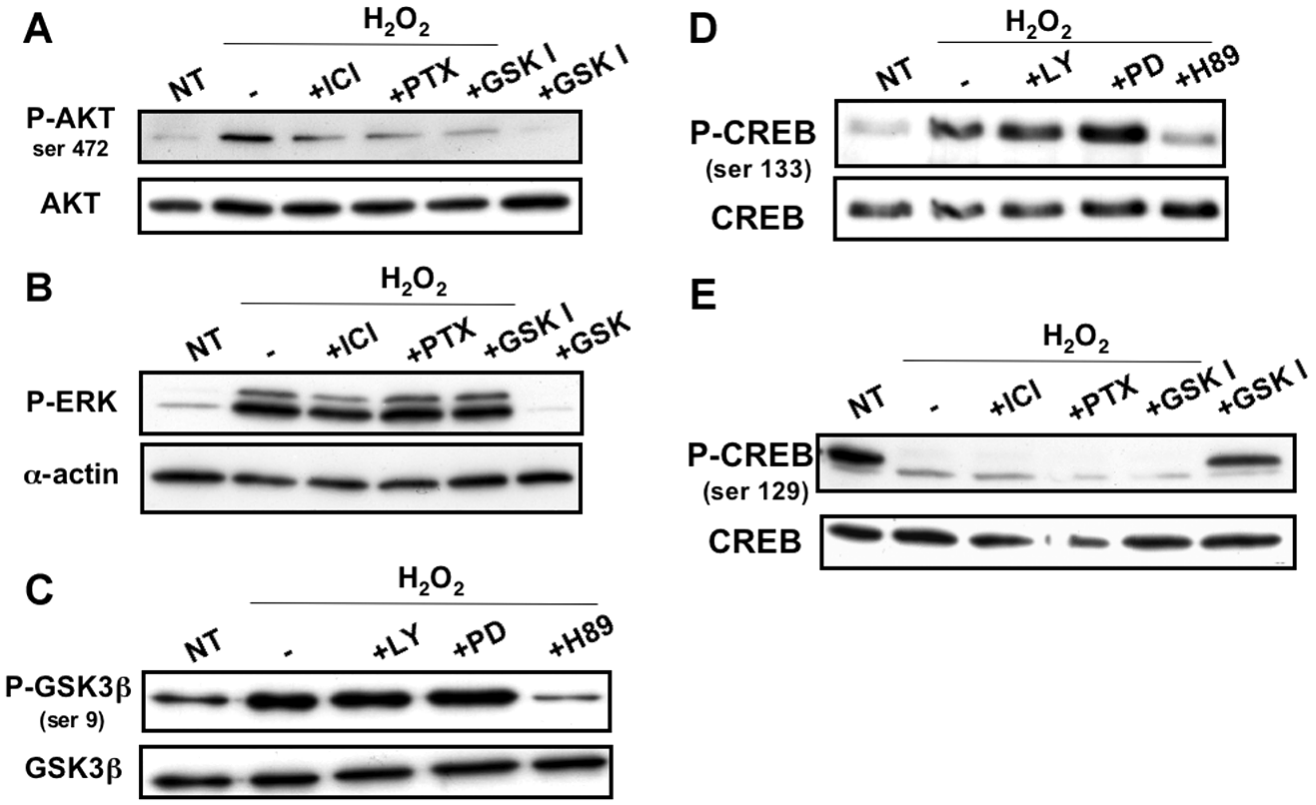

Figure 6. Activation of AKT, ERK, and CREB phosphorylation by H_2O_2. Cardiomyocytes stimulated with H_2O_2 (100 μM, 30 min) show increased AKT serine-472, ERK, GSK3β serine-9, and CREB serine-133 phosphorylation while relieving CREB serine-129 phosphorylation. ***A)*** AKT serine-472 phosphorylation is down-regulated by inhibitors of β2-AR, Gi, and GSK3β kinase activity. Data are mean ± SEM of three independent experiments. MAPK ERK phosphorylation (***B***), GSK3β serine-9 phosphorylation (***C***), CREB serine-133 (***D***), and CREB serine-129 (***E***) phospho-status regulated by H_2O_2 is insensitive to inhibitors effective in hypoxia/reoxygenation. PKA antagonist H89 inhibits GSK3β serine 9 phosphorylation (***C***). The GSK3β inhibitor type II (GSK I) partially down-regulates CREB 129 phosphorylation under baseline conditions (***E***).

Fig. 7

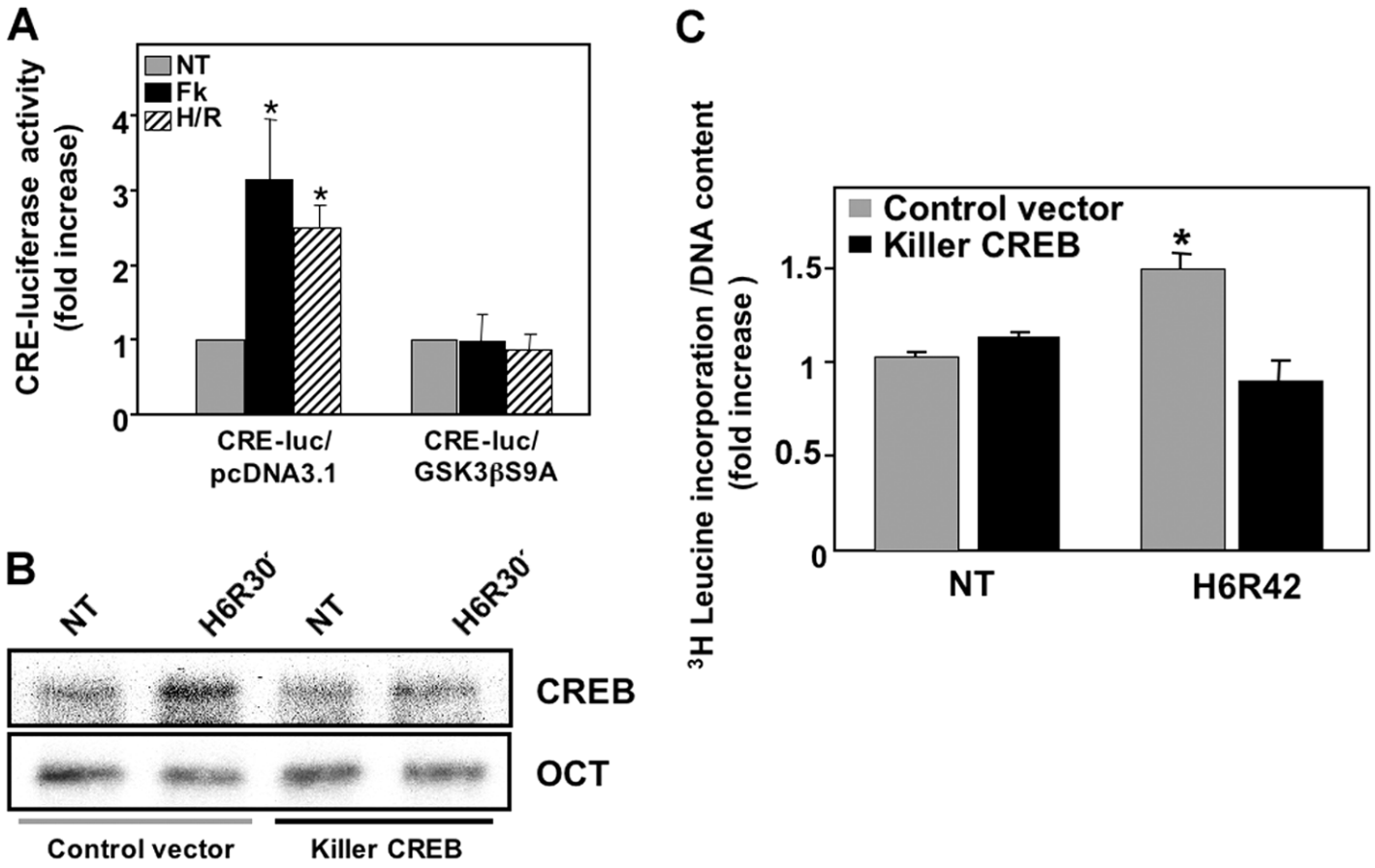

Figure 7. CRE-dependent transcription is regulated by GSK3β and necessary for cardiomyocyte hypertrophy. ***A)*** Forskolin and hypoxia/reoxygenation-induced CRE-dependent transcription is blocked by GSK3β mutant S9A. PC12 cells transfected with CRE-luciferase reporter gene and pcDNA3.1 (control vector) or GSK3β mutant (GSK3βS9A) were treated with forskolin (10 μM, 24 h) or hypoxia 6 h /reoxygenation 24 h. Relative luciferase activity normalized to renilla activity was measured. Data are expressed as mean ± SEM representing *n*=5 independent experiments. ***B***, ***C)*** Transfection of K-CREB, a mutant lacking the DNA binding domain of CREB, abrogated CREB DNA binding and inhibited cardiomyocyte hypertrophy induced by hypoxia/reoxygenation. ***B)*** Down-regulation of CREB DNA binding after transfection of K-CREB. Cardiomyocytes were transfected with pcDNA3.1 or K-CREB vector. CREB DNA-binding activity was determined by EMSA analysis on transfected cells treated by hypoxia 6 h/reoxygenation 30 min. ***C)*** Hypoxia/reoxygenation does not increase protein synthesis in K-CREB transfected cardiomyocytes. Data are expressed as mean ± SEM of three independent experiments **P*< 0.05.