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ORIGINAL ARTICLE

Intracellular signaling pathways control mitochondrial events associated with the development of ischemia/reperfusion-associated damage

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Summary

Ischemia (I) and reperfusion (R) trigger a series of events, which culminate in severe injury to the transplanted organ. Cell death resulting from the formation of mitochondrial reactive oxygen species (ROS) coupled with the perturbation of mitochondrial Ca^{2+} homeostasis is central to the development of IR-associated tissue damage. We and others have shown recently that intracellular signaling pathways critically control these mitochondrial changes, making them potential targets for therapeutic intervention. Using a heterotopic murine heart transplant model as well as primary and immortalized cardiomyocyte cells we established the activity patterns of mitogen-activated protein kinases (MAPKs) ERK, JNK, and p38 during IR, and probed into their role in the perturbation of mitochondrial ROS and Ca^{2+} homeostasis, which are necessary for cardiomyocyte death. Our results showed a strong activation of all three MAPKs as well as a rise in mitochondrial ROS and Ca^{2+} during early reoxygenation. Inhibiting p38 kinase most efficiently prevented ROS production, Ca^{2+} overload and cell death, suggesting that targeting this signaling molecule may provide a possible strategy to limit the effects of IR.

Introduction

Interruption and restoration of oxygen and nutrient supply are unavoidable during solid organ transplantation [1,2]. Reperfusion not only causes the resumption of metabolic functions but also initiates a series of processes, which lead to severe transplant damage [2]. Mitochondria-derived reactive oxygen species (ROS) abundantly generated during reperfusion have been linked to mitochondrial Ca^{2+} overload, which ultimately leads to cell death [2]. In clinical settings, ROS toxicity can be controlled through the use of scavenging agents [3,4]. In the future, ROS-metabolizing enzymes may also be applied and small molecule mimetics of these proteins have been developed [5,6]. However, as ROS production is the prime initiating event leading to IR-associated injury (IRI), strategies to limit the effects of ROS already produced will have limited benefit and novel approaches are desirable, which prevent their generation.

We have shown the ability of the RAS-RAF-MEK-ERK or cytoplasmic pathway [7] to prevent unphysiologically high mitochondrial ROS and Ca^{2+} levels which otherwise cause cell death [8]. As ROS production occurs early during reperfusion, modulation of intracellular signaling

may provide the unique chance to halt the development of IRI at a very early time point. Evidence for the activation of intracellular signaling cascades during IR has been accumulated over the last several years; however, in most cases, a detailed picture of the pathways involved and the timing of these events is missing. Early on expression profiling has identified numerous genes regulated in response to reperfusion [9], and for several a link to IRI could be confirmed [10,11]. Transcriptional changes occur very early and require the activation of upstream signaling pathways. ERK1,2 constitute the prototypic mitogen-activated protein kinases (MAPKs), but related kinases have been described, which function in similarly structured pathways, but initially were shown to respond to unphysiological stimuli leading in many cases to cell death. These MAPKs, also termed stress kinases, comprise the c-Jun N-terminal kinases (JNK1-3) as well as the p38 family [12–14]. The importance of MAPKs in cell fate determination and the availability of small molecular weight inhibitors targeting them [12,15], prompted us to check into the activation status of MAPK signaling under IR *in vivo* and hypoxia/reoxygenation (HR) *in vitro* and to gain insights into their roles in regulating mitochondrial ROS and Ca^{2+} homeostasis.

Material and methods

Cardiac transplant model and experimental design for the induction of ischemia and reperfusion

Male inbred BALB/c mice weighing 24–29 g were kept with unlimited access to water and standard laboratory chow according to local guidelines and the Austrian Animal Care Law. For heart transplantations, anesthesia was induced by intramuscular injection of Xylazine (5–10 mg/kg; Xylasol®, Clinipharm, Switzerland) and Ketamine (Ketanest S®, 100 mg/kg; Pfizer Corporation, Vienna, Austria). Hearts were transplanted heterotopically into the neck region of recipients as described previously [11,16,17]. Isotonic NaCl (1–4 °C) was used for a cold flush (5 ml) and grafts were transplanted immediately thereafter. Warm ischemia time upon anastomosis was kept at 45 min.

Hypoxia (H) and reoxygenation (R) studies using HL-1 cells and mouse primary cardiomyocytes

Cardiac muscle cell line HL-1cells were derived from AT-1 mouse atrial cardiomyocytes [18,19] and have been used as an *in vitro* model for studying many aspects of cardiac biology [18,19]. Cells were maintained in Claycomb cell culture medium as described previously [19]. For hypoxia/reoxygenation experiments, cells were placed into a serum- and glucose-free Claycomb medium and exposed to 0.5% O_2 at 37 °C for 45 min (hypoxia, H), using a Modular Incubator Chamber (Billups-Rothenberg, Del Mar, CA, USA). During subsequent reoxygenation (R), cells were kept in standard culture medium. Primary mouse cardiomyocytes were prepared from 19-day-old BALB/c mice embryos according to a previously reported method [20]. Hypoxia (H) and reoxygenation (R) were simulated as described for HL-1 cells.

Preparation of protein lysates

Tissue sections were snap-frozen in liquid nitrogen (N_2) immediately after harvest. For the preparation of protein lysates, samples were placed in ice-cold lysis buffer [50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.25% (w/v) sodium deoxycholate, 1.0% (v/v) NP-40, 1.0 mM ethylene diamine tetraacetic acid (EDTA), and Protease Inhibitor Cocktail Set I; Calbiochem] and homogenized with an Ultra-Turrax (IKA, Staufen, Germany). The resulting suspension was transferred to a micro-centrifuge tube and centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was used in all further analyses. The concentration of solubilized proteins was measured using the Lowry method (Bio-Rad protein assay kit; Bio-Rad Laboratories, Hercules, CA USA), adjusted to 2.0 mg/ml with an appropriate amount of lysis buffer and 40 μg total protein were loaded per lane. HL-1 cells and primary cells were directly taken up in lysis buffer following two washes with phosphate-buffered saline (PBS).

Western blotting

Protein separation and immunoblotting were performed as previously described [21]. Immunoblots were probed with the following antibodies: phospho-ERK (sc-7383; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho JNK (AF-1205; R&D Systems, Minneapolis, MN, USA) or phospho-p38 (9211S, Cell Signaling, Danvers, MA, USA). Total ERK, JNK and p38 proteins were detected using the antibodies sc-94 (Santa Cruz Biotechnology), 9252S (Cell Signaling), and 9213S (Cell Signaling) respectively. The relative densities of protein bands were analysed by the IMAGE J software (Scion Corporation, Frederick, MD, USA), and the relative density of each protein band was normalized to that of β-actin (A5441; Sigma-Aldrich, St Louis, MO, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AM4300; Ambion, Austin, TX, USA).

Confocal imaging and measurement of mitochondrial ROS and Ca^{2+}

For mitochondrial ROS and Ca^{2+} measurements, $10–20 \times 10^3$ HL-1 cells were plated in Lab-Tek chambered coverglass (Nalge Nunc, Rochester, NY, USA) and exposed to 45 min of hypoxia (0.5% O_2 at 37 °C for 45 min). ROS

production and changes in mitochondrial matrix Ca^{2+} were measured at the commencement of reoxygenation (≤2 min) using fluorescent probes. In order to analyse mitochondrial ROS production, cells were incubated with MitoTracker Red CM-H_2XRos (0.2 μM; Invitrogen Molecular Probes, Eugene, OR, USA) for 30 min, added directly to the cell culture medium. To analyse the level of mitochondrial matrix calcium Ca^{2+} cells were preloaded with fluorescent Ca^{2+}-specific probe Rhod-2-AM (5 μM; Molecular Probes). Data acquisition and analyses were performed as described previously [8].

Flow cytometry

Apoptosis was assessed following propidium iodide (PI)-staining of the cells and the analysis of cell cycle distribution using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells with hypodiploid DNA content were taken as apoptotic. A total of 1×10^6 HL-1 cells were pelleted by centrifugation and resuspended in 0.5 ml of PBS supplemented with 1% (v/v) fetal calf serum. Fixation was done by drop-wise addition of 5 ml of ice-cold ethanol. PI-staining, FACS and data analyses were performed as described previously [22].

Statistical analysis

All data are presented as mean values ± SEM. Statistical analyses were performed using Student's *t*-test and $P < 0.05$ was defined as the level of significance.

Results

Alterations in MAPK activity associated with ischemia and reperfusion *in vivo*

To assess the activation pattern of MAPK during ischemia and subsequent reperfusion, syngeneic mouse hearts were transplanted heterotopically into the neck region of BALB/c mice. Warm ischemia time upon revascularization was strictly limited to 45 min. Hearts were retrieved at the indicated time points and processed for further analysis. Activation of ERK (ERK1,2), JNK (JNK1,2) and p38 was monitored using phosphorylation-specific antibodies, which recognize the activated forms of these kinases. To control for equal protein loading, blots were re-probed with an antibody detecting total MAPK protein and GAPDH. Three animals were operated and analysed for each time point and the response observed was consistent among different grafts (Fig. 1a,b). Non ischemic control hearts displayed low levels of ERK1,2 activity, while phosphorylated forms of p38 or JNK were undetectable (Fig. 1a,b). Ischemia lead to an increase in stress kinase activities (p38: 4.6-fold; $P < 0.05$ vs. control; JNK: 9.4-fold, $P < 0.05$ vs. control) but left ERK1,2 unaffected. Hearts analysed at an early time point (10 min) after reperfusion displayed significantly elevated ERK activity (ERK1,2: 8.4-fold, $P < 0.01$ vs. control), and a further increase in p38 (p38: 9.4-fold increase, $P < 0.05$ vs. control) and JNK (JNK1,2: 57.7-fold increase, $P < 0.01$ vs. control) activity. MAPKs were also analysed at 2, 12, 24 and 48 h after the start of reperfusion. At 2 h, JNK and p38 activity had ceased, while ERK (ERK1,2: 4.8-fold increase) activity still had not returned to prereperfusion levels and stayed above levels in control hearts until the end of the observation period (ERK1,2: 2.7-fold increase). Thus, all three groups of MAPKs showed distinct time courses of activation and inactivation during ischemia and reperfusion and the main difference between ERK1,2 and the stress kinases was seen in the duration of the signal and in the lack of ERK1,2 activation as a result of ischemia.

Reoxygenation *in vitro* is linked to MAPK activation

As cardiomyocytes are the critical targets for IRI, we wanted to confirm our *in vivo* observations in pure populations of these cells. The mouse cardiomyocyte cell line HL-1 has been described recently and is of true cardiomyocyte origin based on an extensive physiological characterization [18,19]. We subjected these cells to a hypoxia and reoxygenation protocol that mirrored *in vivo* conditions in our mouse studies and analysed them for the activation of MAPKs. As observed *in vivo*, the transition from hypoxia to reoxygenation was marked by a substantial increase in the activity of all three signaling pathways (ERK: 11-fold increase, $P < 0.01$; JNK: 44-fold increase, $P < 0.01$, p38: 59-fold increase, $P < 0.01$) (Fig. 1c,d).

In addition, we also prepared primary cardiomyocytes from 19-day-old mouse embryos following an established protocol (see Materials and Methods). The time course of MAPK activation (Fig. 1e) during hypoxia/reoxygenation was comparable to what had been seen *in vivo* as well as with HL-1 cells. Taken together these data demonstrated that (i) the transition from hypoxic to normoxic conditions was marked by a pronounced increase in MAPK signaling, and (ii) that in the *in vivo* and the *in vitro* systems share a strikingly similar pattern of MAPK activation. Our results also suggested that the signaling activity found in the hearts preferentially originated from cardiomyocytes or, alternatively, that all cells respond uniformly to the stimuli generated under IR and hypoxia/reoxygenation.

Hypoxia/reoxygenation result in elevated mitochondrial ROS and Ca^{2+} concentrations

Prolonged hypoxia and subsequent reoxygenation cause severe damage to cardiomyocytes [2]. During this time,

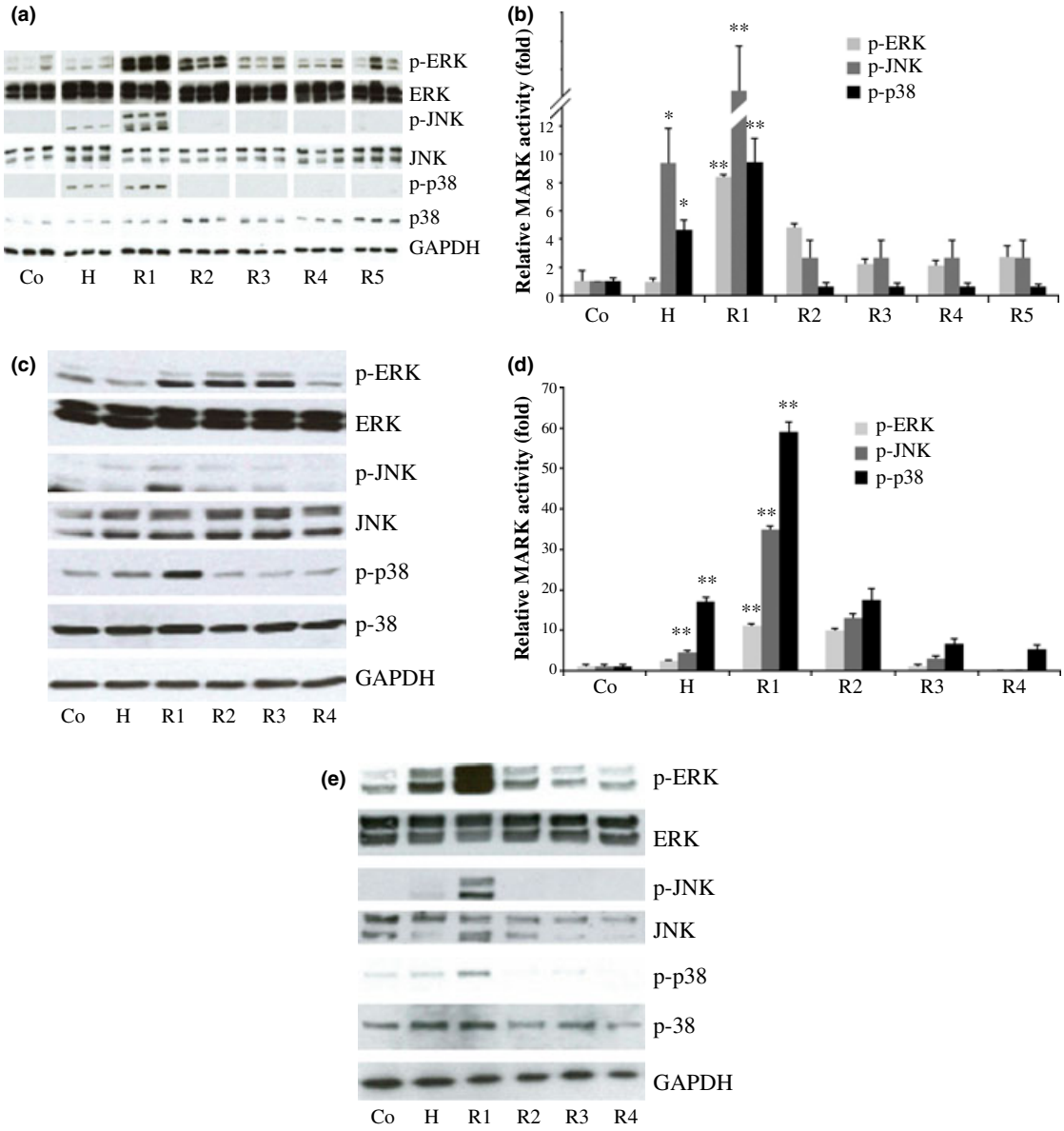

Figure 1 Alterations in MAPK activity are associated with IR or HR. Representative immunoblots (a, c, e) and a summary graph (b, d) are shown. (a, b). Three animals undergoing heterotopic heart transplantation were analysed in each group. Treatment conditions were as follows: Co, untreated animals; H, 45 min of ischemia; R1-R4, hearts exposed to 45 min of ischemia and either 10 min (R1), 2 h (R2), 12 h (R3), 24 h (R4) or 48 h (R5), of reperfusion. BALB/c mouse heart lysates display low levels of ERK1,2 activity in the control group (Co). 45 min of ischemia increased stress kinase activity (p38: 4.6-fold increase, $*P < 0.05$ vs. control; JNK: 9.4-fold increase, $*P < 0.05$ vs. control) but left ERK 1,2 unaffected. 10 min of reperfusion (R1) caused a significant increase in ERK activity (ERK1,2: 8.4-fold increase, $**P < 0.01$ vs. control), and a further increase in p38 (p38: 9.4-fold increase, $**P < 0.01$ vs. control) and JNK (JNK1,2: 57.7-fold increase, $**P < 0.01$ vs. control) activity. After 2 h of reperfusion (R2) JNK (JNK1,2: 2.6-fold increase) and p38 (p38: 0.6-fold) activity had returned to control (Co) levels, while ERK (ERK1,2: 4.8-fold increase) activation still did not return to prereperfusion levels and stayed above levels in control hearts until the end of the observation period (ERK1,2: 2.73-fold increase). Data are expressed as mean ± SEM (of $n = 3$). (c–e) Changes in MAPK signaling in HL-1 cells and primary BALB/c cardiomyocytes subjected to 45 min of hypoxia (0.5% O_2, 37 °C, serum-/glucose-free medium) and up to 48 h of reoxygenation. The treatment conditions were as follows: Co, untreated cells; H, 45 min of hypoxia R1-R4: hypoxia (45 min) followed by reoxygenation in growth medium for 10 min (R1), 2 h (R2), 24 h (R3) or 48 h (R4), respectively. 45 min of hypoxia increased activity of all three MAPKs, 10 min of reperfusion lead to a further increase in their activities. After 2 h of reperfusion JNK and p38 activity had ceased while ERK activity still had not returned to control levels. Data are expressed as mean ± SEM ($n = 3$, all $**P < 0.01$). In the case of primary cardiomyocytes a single experiment was performed.

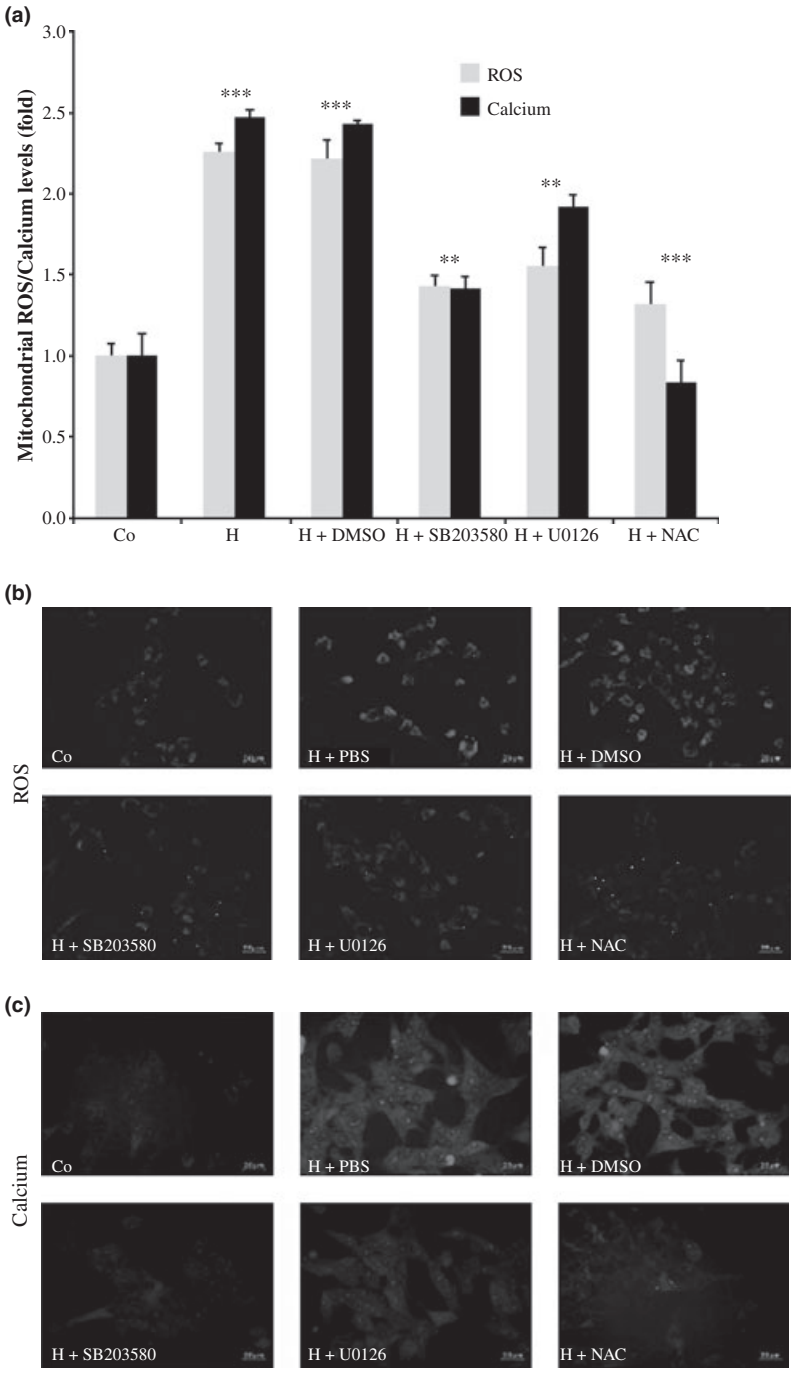

Figure 2 Intracellular signaling affects mitochondrial ROS and Ca^{2+} levels. Hypoxia resulted in elevated mitochondrial ROS (2.3-fold increase, ***$P < 0.001$ vs. control; a, b) and $[Ca^{2+}]_m$ (2.5-fold increase, ***$P < 0.001$ vs. control; a, c). HL-1 cells, pretreated with inhibitors of MEK (UO126) or p38 (SB203580) or the ROS scavenger *N*-acetyl-cysteine (NAC), were exposed to the following hypoxia/reoxygenation protocol: hypoxia: (45 min, 0.5% O_2, 37 °C, serum-/glucose-free Claycomb medium) and reoxygenation (normoxic atmosphere, 37 °C, supplemented Claycomb medium) and ROS production and mitochondrial $[Ca^{2+}]_m$ concentrations were monitored at the earliest time point possible after reoxygenation (≤2 min) using Mito Tracker Red CM-H2XRos (b) or Rhod-2 (c) as probes. Pretreatment with kinase inhibitors [ROS: H+SB203580: 0.8-fold decrease, $[Ca^{2+}]_m$: H+SB203580: 1.1-fold decrease, **$P < 0.01$ vs. hypoxia (H); ROS: H+U0126: 0.7-fold decrease, $[Ca^{2+}]_m$ H+U0126: 0.6-fold decrease, **$P < 0.01$ vs. hypoxia (H)], or NAC (10 μM) resulted in a significant decrease in ROS (a, b) production and mitochondrial $[Ca^{2+}]_m$ (a, c) concentrations compared to untreated cells (ROS: H+NAC: 0.9-fold decrease, $[Ca^{2+}]_m$ H+NAC: 1.6-fold decrease ****$P < 0.001$ vs. hypoxia). Data are expressed as mean ± SEM ($n = 6$).

the increased concentration of ROS and changes in mitochondrial Ca^{2+} levels (calcium overload) are important contributors to the initiation of cardiomyocyte death [2]. To analyse the effects of hypoxia and reoxygenation on the perturbation of ROS and Ca^{2+} homeostasis, HL-1 cells were exposed to hypoxia/reoxygenation and ROS levels were monitored at the earliest time point after reoxygenation using MitoTracker Red CM-H_2XRos as fluorescent probe. This dye accumulates in mitochondria depending upon membrane potential. As shown in Fig. 2, increased mitochondrial ROS levels resulted from this treatment (ROS: 2.3-fold, $P < 0.01$ vs. control). Similar results were obtained following staining with MitoSOX, which also specifically detects mitochondrial ROS (data not shown). Predominant mitochondrial localization of the ROS produced in these HL-1 cells has been observed before in colocalization studies using a mitochondrial marker [23]. Notably, this increase in ROS production was paralleled by a similar change in mitochondrial Ca^{2+} levels (Fig. 2, Ca^{2+}: 2.5-fold, $P < 0.01$ vs. control). Pretreatment of cells with the anti-oxidant *N*-acetyl cysteine (NAC) reversed both, elevated ROS levels (ROS: 0.9-fold, $P < 0.01$ vs. hypoxia control) and the increase in mitochondrial Ca^{2+} (Ca^{2+}: 1.6-fold, $P < 0.01$ vs. hypoxia control), suggesting that ROS are directly responsible for the changes in mitochondrial Ca^{2+}.

Intracellular signaling, ROS production and mitochondrial Ca^{2+}

Reactive oxygen species production is subject to the regulation by upstream components as best understood for the activation of NADPH-dependent oxidases by the small G protein Rac [24]. But also MAPK pathways may control mitochondrial ROS production as demonstrated e.g. for RAF [8], MKK6 [25,26] or PKA [27]. To gain further insight into a possible role of intracellular signaling in limiting mitochondrial Ca^{2+} and ROS during hypoxia and reoxygenation, we tested the effect of inhibiting the MAPKs ERK and p38, for which a link to mitochondrial ROS production has been established previously [25,26]. We first determined the concentrations required for complete inhibition of the ERK-activating upstream kinase MEK or of p38 following stimulation with the potent activators serum or sorbitol. Effective concentrations were as follows: ERK: 10 μM, p38: 10 μM (data not shown). We then tested the effects of small molecular weight inhibitors of p38 (SB203580), and MEK (UO126) on ROS production and Ca^{2+} levels. As shown in Fig. 2 inhibition of p38 and to a lesser degree MEK or the presence of the anti-oxidant *N*-acetyl cysteine (NAC) resulted in reduced ROS and Ca^{2+} levels ($P < 0.05$ vs. hypoxia control). SB203580 also significantly prevented HR-induced death of HL-1 cells ($P < 0.05$ vs. hypoxia control), while inhibition of MEK failed to show a significant effect (Fig. 3). This may be because of its less pronounced ability to prevent the accumulation of mitochondrial ROS and Ca^{2+} (Fig. 2).

Discussion

Data presented here suggest that the activation of intracellular signaling pathways constitutes an early event during IR, which may precede changes in the second messengers ROS and Ca^{2+}. While strategies to limit oxidative stress during organ storage are pursued [3–6] our data, which so far are limited to the *in vitro* setting of HR, suggest that targeting intracellular signaling may be a better strategy as it prevents subsequent ROS/Ca^{2+} effects and eventually also could interfere with inflammatory responses occurring subsequently[28]. Testing the effects of targeting signaling cascades on the course of IRI has become feasible through the advances in the development of small molecular-weight inhibitors for a variety of signaling proteins [7,15].

In our analysis, we focused on the evolutionarily highly conserved MAPKs signaling pathways both, in the *in vivo* setting of ischemia/reperfusion as well as under *in vitro* conditions of hypoxia/reoxygenation using cardiomyocytes. The reason to also use cardiomyocytes was to directly assess the effects of hypoxia/reoxygenation in cells, which constitute the primary targets. In addition, these cells provide a test system, which may be more amenable to analysis and *in vitro* testing in future work. As shown in Fig. 1, the comparison of the different approaches

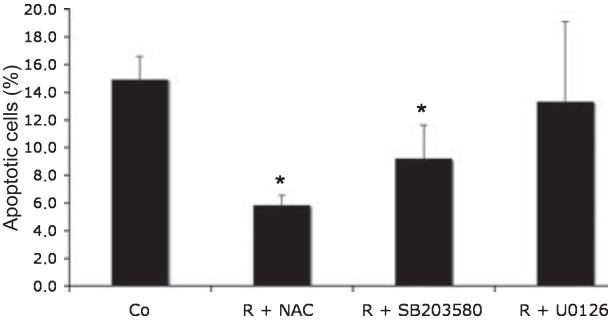

Figure 3 *N*-acetyl-cysteine (NAC) and SB203580 attenuate apoptosis occurring under hypoxia and reoxygenation. HL-1cells undergoing a hypoxia/reoxygenation protocol as described in the legend to Fig. 2 were either left untreated or cultured in the presence of 10 mM of NAC or 10 μM of SB203580. Fraction of apoptotic cells were determined after propidium iodide (PI) staining using FACS. Cells with subdiploid DNA content were labeled apoptotic. (10 mM NAC: 60.9% decrease vs. control; 10 μM SB203580: 38.4% decrease vs. control, $*P < 0.05$). Data are expressed as mean ± SEM ($n = 6$).

revealed a high degree of overlap in the activation/inactivation patterns of MAPKs. This suggested that changes in oxygen and nutrient levels *per se* were sufficient to trigger MAPK-signaling and deregulation of mitochondrial Ca^{2+} and ROS homeostasis. MAPKs have been implicated in the regulation of cell survival through *in vitro* and *in vivo* studies. A clearly protective function has been seen in the case of signaling via RAF-MEK-ERK [29,30]. The members of the p38 and JNK stress kinases primarily were thought to function in the response to cellular stress, as components of pro-death pathways. However, more recently, evidence has also been provided for a mitogenic and even survival function of these proteins [13]. The discrepancies may be rooted in differences in experimental approaches, timing, organ systems or animal strains. They may also reflect differences in signaling intensity or the activity/inactivity of additional signaling pathways.

Based on published evidence, mitochondrial changes figure prominently in the control of cardiomyocyte survival under IR [2]. These mitochondrial alterations provide an early and sensitive readout system to detect damaging effects and thus are helpful in identifying possible targets for therapeutic interference to prevent IRI. Following the characterization of the activation pattern of intracellular MAPK signaling after IR and hypoxia/reoxygenation, respectively, the main question was how these changes are related to cellular damages associated with IRI. Along these lines we were particularly interested in analysing the effects of MAPK activation on mitochondrial ROS and Ca^{2+} as they are directly linked to cell death under these conditions [2]. We observed in our cells an increase in both, ROS and Ca^{2+}, at the mitochondria when undergoing hypoxia/reoxygenation. Most notably, the use of the anti-oxidant NAC during hypoxia/reoxygenation decreased mitochondrial Ca^{2+} levels (Fig. 2c) and also prevented HL-1 cell death (Fig. 3), suggesting that mitochondrial accumulation of ROS functions as a critical cell-death trigger.

Both, MEK/ERK and p38, have been implicated in the control of mitochondrial ROS levels before [8,25,26]. Transgenic mice expressing wild type MKK6, an essential upstream activator of p38 kinases in the heart have been generated and the animals have been analysed for their response to IR [25]. Strikingly, MKK6 protected these hearts, most likely through direct effects on mitochondrial ROS production [26]. Overexpression was sufficient to cause hyperactivation of p38, through mechanisms, which are not entirely clear. While not directly tested, this implies that p38 is essential for mediating this protective effect. However, these findings differ from our own observation, that inhibiting p38 was linked to a reduction in ROS production and mitochondrial Ca^{2+} conditions closely associated with the induction of cardiomyocyte death. The differences may lie in the fact that in MKK6 transgenic mice, p38 is *a priori* active, which differs from the situation analysed in this article, when p38 activity goes from almost undetectable levels to a significant increase in activity, before returning to a basal level. Constitutive p38 activity present in MKK6-transgenic mice may exert a preconditioning function, which could explain subsequent protective effects. Additionally, differences may exist in the experimental setup and in the activities of additional signaling pathways under these conditions. Signaling by the ERK1,2 upstream components MEK1,2, RAF or SRC usually has been associated with decreased ROS levels under normal growth conditions [8]. Despite this we observed that ROS production following MEK inhibition was decreased in our experimental settings. The reason for this is currently unclear but goes along with observation of a possible pro-apoptotic function of this pathway under certain circumstances [31]. We currently lack any insight, how signaling proteins may control mitochondrial ROS levels. Possibilities to be considered include effects on the anti-oxidant systems but also direct effects on their production have been suggested [32,33].

Taken together, our data identify a critical role for p38 and ERK signaling under conditions of hypoxia and reoxygenation. As the disturbance of mitochondrial Ca^{2+} and ROS homeostasis features critically in triggering cell death, it is not surprising that survival and death pathways directly target the turnover of these molecules. This control may be achieved through effects on the production of ROS but also by increasing cellular anti-oxidant capacity. Given the fact that p38 is also involved in the control of inflammatory responses [28], targeting p38 may provide a vital strategy for preventing various processes, which jointly contribute to IRI.

Authorship

RS, PG, TK, RO, TR, MM, RO, FB, MIA carried out the experimental work; MH, AVKo, and AVKu provided protocols and analyses of ROS and/or Ca^{2+}; GB, SS, RÖ, RM established the animal experimental model used in this study and contributed to the critical analyses of the data. JT designed the study, coordinated the experiments and wrote the paper.

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