The MAP kinase cascades are activated during post-ischemic liver reperfusion

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Abstract We have investigated the involvement of MAP kinase cascades in the response of the liver to post-ischemic reperfusion. Both JNKs and ERKs are activated but the duration and magnitude of the increase in their activities appear to be different. JNK activation is more marked but shorter than that of ERKs. The increase observed in the phosphotyrosine content of the 52 kDa Shc protein, accompanied by an increased amount of co-immunoprecipitated Grb2, and the activation of Raf-1 kinase provide evidence of the involvement of a Ras–Raf-dependent pathway, with a time course that is similar to that of ERK activation. The treatment of rats with IL-1 receptor antagonist modified all of the described effects, suggesting that IL-1 plays a role in the response of the liver to reperfusion.

Key words: Liver; Post-ischemic reperfusion; MAPK cascade; Signal transduction

1. Introduction

Mitogen-activated protein kinases (MAPKs) are Ser/Thr kinases that convert extracellular stimuli to intracellular signals controlling gene expression [1]. Extracellularly regulated kinases (ERKs), the best studied members of the MAPK family, are rapidly activated upon stimulation of cell surface receptors and play a central role in proliferation and differentiation [2]. The most recently identified members of the MAPK family [3], the c-Jun NH_2 terminal kinases (JNKs), are induced by a variety of stimuli including heat shock, ultraviolet radiation, protein synthesis inhibitors and inflammatory cytokines such as IL-1 and TNF [3–5]. Both ERKs and JNKs phosphorylate and activate nuclear proteins [6]. ERKs regulate Elk1 [7], which controls the expression of c-*fos* and other genes [8], and JNKs phosphorylate the amino-terminal transactivating domain of Jun and ATF2, increasing their transcriptional activity [9].

Liver ischemia can cause severe cell damage without leading to cell death if not too prolonged [10]. On the other hand, reperfusion can induce oxidative stress [11], the release of proinflammatory cytokines by Kupffer cells [12,13] and various biochemical processes [14] including the reprogramming of gene expression with a rapid induction of c-*fos*, c-*jun* and stress genes [15]. As for the signaling pathways controlling these transcriptional events we demonstrated that PKC activity is reduced for many hours after post-ischemic reperfusion [16]. In the present study, based on the regulatory role of the MAPK cascades in gene expression, the activation of MAPKs upon heat shock of the liver in vivo [17] and the similarities in gene reprogramming during heat shock and post-ischemic reperfusion of liver [18], we investigated the possible involvement of JNK and ERKs in the liver's response to reperfusion.

2. Materials and methods

2.1. Materials

MEK1-[K97A], anti-MAPK R2, and anti-Shc polyclonal antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Anti-Raf-1 (C-12) and anti-JNK1 polyclonal antibodies, GST-Jun (aa 1–79) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Grb2 and anti-phosphotyrosine (RC 20) monoclonal antibodies were from Transduction Laboratories (Lexington, KY, USA). Human recombinant IL-1 receptor antagonist (IL-1 RA), expressed in *Bacillus subtilis*, of 98% purity, 1×10^6 IU/mg, was a kind gift of Diana Boraschi, Centro Ricerche Dompé, L'Aquila (Italy). Myelin basic protein (MBP) and all other reagents were from Sigma Chem. Co. (St. Louis, MO, USA).

2.2. Animals and treatment

Male Wistar rats of 250 g, housed and handled as prescribed by EC regulations, were used throughout and were subjected to partial liver ischemia of 1 h duration as described [10]. After different times of reperfusion the rats were killed, the livers were rapidly excised and deep frozen. IL-1 RA, diluted with sterile saline, was injected intraperitoneally at total doses ranging from 100 μg to 400 μg per 100 g body weight, given in two equal portions; the first 30 min before ischemia, the second at the removal of the clamp.

2.3. JNK immunocomplex kinase assay

Livers were lysed in RIPA buffer containing: 0.15 M NaCl, 50 mM Tris pH 7.4, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 2 μg/ml aprotinin, 40 μg/ml bestatin, 0.5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.7 μg/ml pepstatin A, 20 mM β-glycerophosphate, 50 μM sodium orthovanadate, 10 mM *p*-nitrophenol phosphate (PNPP), which makes it possible to obtain highly reproducible results minimizing loss of enzyme activity [19]. 300 μg of proteins of lysates were incubated with 5 μg of anti-JNK1 antibody or with 5 μl of preimmune serum for 1 h at 4°C and adsorbed to protein A Sepharose. After several washes, immunoprecipitates were incubated for 20 min at 30°C in 50 μl of reaction mixture containing: 20 mM HEPES pH 7.4, 20 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 mM PNPP, 1 mM dithiothreitol (DTT), 50 μM sodium orthovanadate, 20 μM ATP, 10 μCi [γ-^{32}P]ATP and 2 μg of GST-Jun containing Ser-63 and -73, specific phosphorylation sites for JNK [4]. Kinase reactions were stopped with Laemmli's buffer [20] and the samples were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The radioactivity incorporated into GST-Jun was detected by autoradiography and evaluated by densitometric analysis.

2.4. ERK immunocomplex kinase assay

300 μg of cytosolic proteins were subjected to immunoprecipitation with 4 μg of anti-MAPK R2 antibody or 5 μl of preimmune serum and the immunoprecipitates were assayed for their capacity to phosphorylate the MBP in the presence of [γ-^{32}P]ATP as previously described [21]. MBP phosphorylation, detected by SDS-PAGE and autoradiography, was evaluated by densitometric analysis.

2.5. Immunoprecipitation of Shc proteins

Immune complexes were obtained as described [17]. Briefly, 1 mg of proteins of lysates were incubated with 3 μg of polyclonal anti-Shc

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antibody for 5 h at 4°C and adsorbed to protein A Sepharose. Immunoprecipitates were extensively washed and resuspended in Laemmli's buffer [20] for successive immunoblot analysis.

2.6. Raf-1 immune complex kinase assay

Livers were lysed in RIPA buffer containing: 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, 1 mM aprotinin, 20 μM leupeptin, 5 mM sodium orthovanadate and 50 mM NaF, which allows an excellent solubilization of the Raf-1 protein and prevents co-immunoprecipitation of associated kinases [22]. 500 μg of proteins of lysates were incubated with 1.5 μg of anti-Raf-1 antibody or with 5 μl of preimmune serum for 3 h at 4°C and adsorbed to protein A Sepharose. After several washes, immunoprecipitates were incubated for 20 min at 25°C in 50 μl of kinase buffer containing: 30 mM HEPES pH 7.4, 7 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM DTT, 15 μM ATP, 20 μCi of [γ-^{32}P]ATP and 1 μg of MEK1-[K97A] as specific substrate. Kinase reactions were stopped with electrophoresis buffer and the MEK1 phosphorylation detected, by SDS-PAGE and autoradiography, was evaluated by densitometric analysis.

2.7. Procedure controls

Optimal conditions for immunoblotting and immunoprecipitation reactions had been defined by preliminary experiments in which different amounts of proteins were probed with different amounts of antibodies. Further, the experiments of immunoprecipitation were occasionally accompanied by assays of reprecipitation of the supernatants with the same antibody to verify that the immunoprecipitation was complete. The specificity of the immunoprecipitates and of the kinase reactions was always evaluated by verifying that preimmune serum did not precipitate proteins reacting with specific antibodies or phosphorylating specific substrates. All phosphorylation reactions were studied within their linear range determined by pilot experiments of time course performed in the described conditions.

2.8. Immunoblot analysis

SDS-PAGE and Western blotting were performed as described earlier [21]. Blots were developed using the enhanced chemiluminescent method.

2.9. Other procedures

The protein content was estimated by Bradford's method [23] using BSA as standard. Quantitative measurements of immunoreactive or radioactive bands was performed by densitometric analysis using Image Master software (Pharmacia, Milwaukee, WI, USA). Means ±S.E.M. of volumetric measurements (OD×mm^2) of at least three separate experiments were compared with Student's *t*-test.

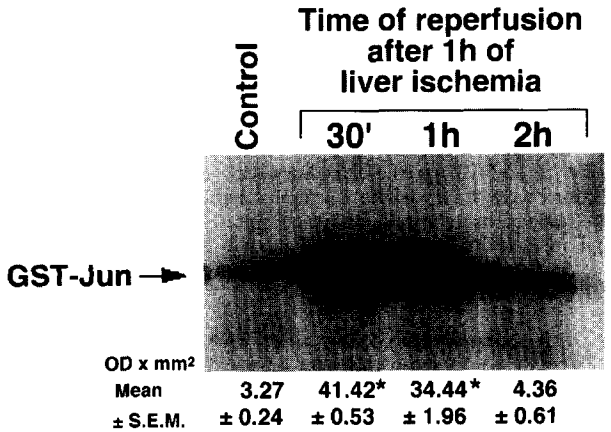

Fig. 1. Time-dependent effect of post-ischemic liver reperfusion on JNK activity. Detection of phosphorylated GST-Jun was done by resolving whole immune complex kinase assays by SDS-PAGE and autoradiography. The autoradiograph is representative of three separate experiments. Along the bottom, the values of quantitative analysis are reported. *$P < 0.01$ vs. control.

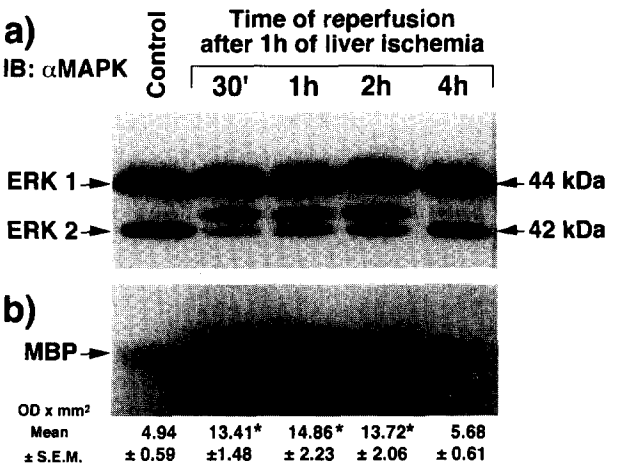

Fig. 2. Time-dependent effect of post-ischemic liver reperfusion on ERKs. a: Immunoblot analysis of liver extract proteins with anti-MAPK antibody representative of three separate experiments. b: Autoradiograph of SDS-PAGE of the ERK immune complex kinase assays. Along the bottom, the values of quantitative analysis of MBP phosphorylation of three separate experiments are reported. *$P < 0.05$ vs. control.

3. Results

The effects of post-ischemic liver reperfusion were compared with observations made in normal rats, since sham operations and ischemia did not modify parameters under study.

3.1. JNK activity during post-ischemic liver reperfusion

Reperfusion caused a remarkable significant activation of JNK-1. GST-Jun phosphorylation by immunoprecipitated JNK became respectively 12 and 10 times the control value at 30 min and 1 h of reperfusion; the effect disappeared after 2 h (Fig. 1).

3.2. ERK activity during post-ischemic liver reperfusion

We first analyzed the electrophoretic behavior of ERKs by immunoblotting experiments since their activation results in mobility shifts of the enzymes [24]. Anti-MAPK R2 antibodies correctly detected two proteins of 44 and 42 kDa, corresponding to ERK 1 and ERK 2 proteins in liver extracts from control animals. After ischemia, the restoration of blood supply caused a decrease in the electrophoretic mobility of a part of both isoenzymes that was already evident after 30 min, was maintained for 1 and 2 h, and disappeared after 4 h (Fig. 2a).

We also investigated the enzyme activity by immune complex kinase assay. The autoradiograph in Fig. 2b shows the MBP phosphorylation obtained in a typical experiment. Densitometric analysis of three different experiments showed that the amount of ^{32}P incorporated into MBP was significantly increased after 30 min of reperfusion, reaching values that were 3 times higher than the controls. The effect was maintained at the same level after 1 and 2 h of reperfusion, returning to basal level at 4 h.

3.3. Shc protein involvement during post-ischemic liver reperfusion

By immunoblot analysis of Shc immunoprecipitates, we found that anti-Shc antibodies recognized the 66, 52 and 46 kDa isoforms of Shc proteins in the livers of control rats, and

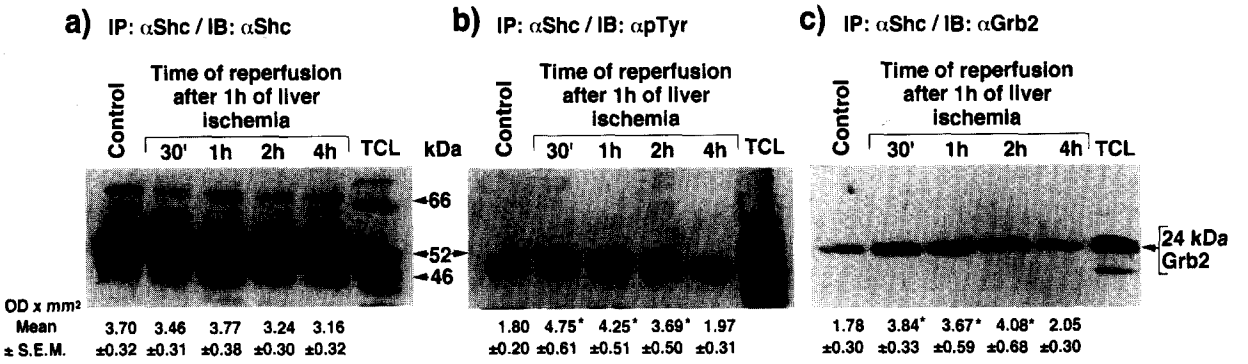

Fig. 3. Time-dependent effect of post-ischemic liver reperfusion on (a) the pattern of immunoprecipitated Shc proteins, (b) the tyrosine phosphorylation of immunoprecipitated Shc proteins and (c) the amount of co-immunoprecipitated Grb2. TCL = total cellular lysate. Quantitative analysis of three separate experiments is reported along the bottom; the values in (a) refer to the 52 Shc protein. $^*P < 0.05$ vs. control.

the reperfusion did not modify their amount (Fig. 3a). After the stripping of the anti-Shc antibodies, the same blots were probed with anti-phosphotyrosine antibodies (Fig. 3b). In control animals the 52 kDa Shc protein had a basal level of phosphotyrosine content which significantly increased 2-fold after 30 min of reperfusion. This effect was maintained for 2 h and disappeared after 4 h. Tyrosine phosphorylation never involved the 66 and 46 kDa isoforms of Shc proteins. Finally by probing the same blots with anti-Grb2 antibodies (Fig. 3c) we detected that the amount of Grb2 precipitated with Shc proteins was 2-fold the normal value after 30 min of reperfusion. This significant effect was maintained until 2 h and disappeared at 4 h of reperfusion.

3.4. Effect of post-ischemic liver reperfusion on Raf-1

We examined Raf-1 electrophoretic mobility by immunoblot experiments (Fig. 4a). In total liver extracts from control animals, a polyclonal anti-Raf-1 antibody detected a 74 kDa protein, as reported for c-Raf-1 [25]. After 30 min of reperfusion, Raf-1 showed a shift in its electrophoretic mobility which was maintained for 1 and 2 h, and disappeared after 4 h of reperfusion. Raf-1 kinase activity was studied by immunocomplex kinase assay and the autoradiograph in Fig. 4b shows the MEK1 phosphorylation obtained in a typical experiment. We found that Raf-1 activity increased at 30 min, reaching significant values after 1 and 2 h of reperfusion, when it was 2-fold the control value. MEK1 phosphorylation returned to basal level after 4 h.

3.5. Effects of IL-1 receptor antagonist treatment

To assess the possible role of IL-1 in the induction of the MAPK cascades during post-ischemic reperfusion, we pretreated the rats with IL-1 RA [26], which can prevent the binding of IL-1α and IL-1β to the cells [27]. All observations were made after 30 min of reperfusion. IL-1 RA at a dose of 400 μg/100 g b.wt. reduced the increase in Jun phosphorylation induced by reperfusion by 50%, whereas the doses of 100, 200, and 300 μg/100 g b.wt. had no effect (Fig. 5a). The study of the electrophoretic behavior of ERKs showed that IL-1 RA at a dose of 400 μg/100 g b.wt. decreased by 50% the extent of the ERK 2 mobility shift due to reperfusion; lower doses did not have any effect (Fig. 5b). Finally the increase in phosphotyrosine content of the 52 kDa Shc protein upon reperfusion was reduced by about 40% when the IL-1 RA dose was 400 μg/100g b.wt. (Fig. 5c). A comparable decrease in the amount of Grb2 co-immunoprecipitated with Shc proteins was detected (Fig. 5d). Lower doses did not have any effect.

4. Discussion

The present results show that post-ischemic liver reperfusion rapidly activates both JNKs and ERKs. Although the separate activation of JNK and ERK is associated with stress and growth signals, respectively, the concomitant induction of both enzymes has also been frequently observed [28,29]. In our experimental model, although the time necessary for the activation of the two enzymes seemed to be the same, duration and magnitude of the increase in enzymatic activities appeared to be different, since the activation of JNKs was more marked but shorter than that of ERKs. It has been demonstrated that the duration of JNK and ERK activation may be responsible for the quality of cell responses. Persistent

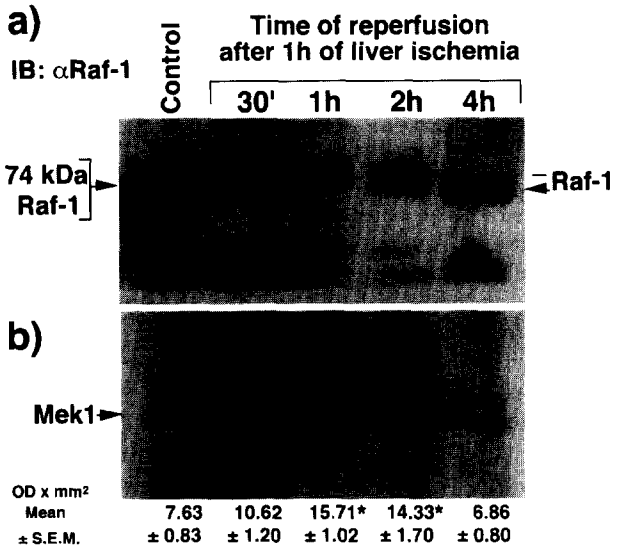

Fig. 4. Time-dependent effect of post-ischemic liver reperfusion on Raf-1 kinase. a: Immunoblot analysis of liver extract proteins with anti-Raf-1 antibody, representative of three separate experiments. The dashed arrow indicates the electrophoretic mobility shift of Raf-1. b: Autoradiograph of SDS-PAGE of the Raf-1 immune complex kinase assays. Along the bottom, the values of quantitative analysis of Mek1 phosphorylation in three separate experiments are reported. $^*P < 0.05$ vs. control.

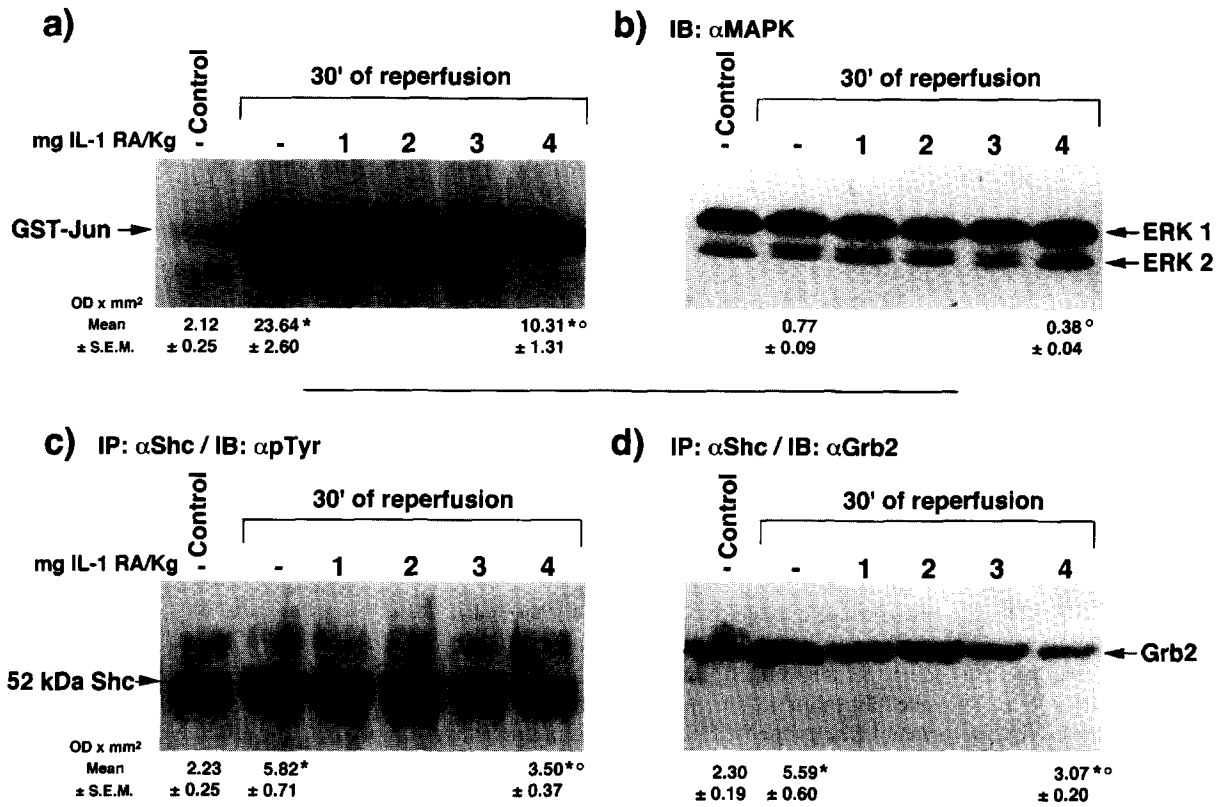

Fig. 5. Effects of different doses of IL-1 RA on the response of the liver to reperfusion. a: GST-Jun phosphorylation by JNK immunocomplex kinase assay. b: Immunoblot analysis of ERKs from liver extract proteins. c and d: Immunoblot analysis of Shc immunoprecipitates with anti-phosphotyrosine and anti-Grb2 antibodies, respectively. Along the bottom of each panel the results of densitometric analysis of three different experiments are reported, limited to the samples showing a significant effect of IL-1 RA. The values in (b) refer to the ERK 2 shift. $^{*}P < 0.05$ vs. control, $^{\circ}P < 0.05$ vs. reperfusion with no IL-1 RA.

JNK activation has been described in Jurkat T cells after apoptosis-inducing treatment [30], whereas transient activation is universal in JNK responses to stress [31]. In PC12 cells, transient ERK activation is associated with a proliferative response, while persistent activation leads to a differentiative response [32]. The behavior of JNK and ERK activity in our experimental model is consistent with a condition of cell repair without proliferation or apoptosis. JNK and ERK activation is currently believed to involve two separate but interconnected pathways [1]: MEKK1, which lies on the JNK pathway, may be an effector of Ras [33], which lies on the ERK pathway; likewise, ceramide-activated protein kinase (CAPK), which is mostly involved in JNK activation [34], may phosphorylate Raf and lead to ERK activation [35]. Furthermore, Ras could be a regulatory factor for both kinases, as it is located upstream of Raf and, according to some observations, also upstream of Rho/Rac [36], whose activation leads to activation of JNKs [37]. Therefore, Ras-dependent as well as Ras-independent pathways may exist for ERKs and JNKs, and a Ras-independent–Raf-dependent pathway may lead to ERK activation. Ras may be activated as a consequence of the tyrosine phosphorylation of Shc proteins: these phosphotyrosine residues, acting as recognition sites for binding of the Grb2-Sos complex [38], lead to Ras activation triggering the GDP-GTP exchange reaction [39]. We found that the tyrosine phosphorylation of Shc proteins, functional to the recruitment of Grb2, occurs during post-ischemic liver reperfusion suggesting that Ras is induced. Although we cannot exclude the possibility that Ras may be involved in the activation of both JNKs and ERKs, the observation that Ras activating events and Raf-1 kinase are still operating after 2 h of reperfusion (when ERKs but not JNKs are activated) suggests that a Ras–Raf-dependent pathway primarily controls the ERK cascade.

Our results showing that IL-1 RA treatment lessens the effects of liver reperfusion suggest that IL-1 plays a pleiotropic role in the activation of the MAPK cascades, since it modulates JNKs, ERKs, and Shc tyrosine phosphorylation. Finally, the partial effects of IL-1 RA treatment on liver responses to reperfusion are consistent with the release of TNF-α and IL-6 (in addition to IL-1) by Kupffer cells observed during post-ischemic liver reperfusion [12], and with the possible intervention of other pathophysiological factors such as ATP and substrate depletion.

As a whole, our results indicate that liver reperfusion after non-necrogenic ischemia rapidly triggers the MAPK cascades involving both JNKs and ERKs by a mechanism that is mediated, at least in part, by IL-1. As many important substrates for these enzymes are transcription factors, we propose that JNKs and ERKs play a role in the genomic response of the liver to reperfusion; the interactions between them and between their transcriptional factor targets might be an important modulatory mechanism allowing reperfusion-specific gene expression.

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