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D. Crenesse
A. Schmid-Alliana
J. Hornoy
B. Rossi
J. Gugenheim

Hypoxia-reoxygenation differentially stimulates stress-activated protein kinases in primary-cultured rat hepatocytes

D. Crenesse (✉)
Laboratoire de Physiologie,
Faculté de Médecine,
Université de Nice-Sophia Antipolis,
06107 Nice Cedex 2, France

A. Schmid-Alliana · B. Rossi
Unité INSERM U364
Faculté de Médecine
Université de Nice-Sophia Antipolis
06107 Nice Cedex 2, France

J. Hornoy · J. Gugenheim
Laboratoire de Recherche Chirurgicale,
Faculté de Médecine,
Université de Nice-Sophia Antipolis,
06107 Nice Cedex 2, France

Abstract Organ injury after ischemia and reperfusion (I/R) remains one of the most important limiting factors in liver surgery and transplantation. Oxygen-free radical (OFR) generation is considered a major cause of this damage. JNK_1/$SAPK_1$, a member of MAPK family, regulates cell adaptation to stressful conditions. The aim of this study was to determine if hypoxia-reoxygenation (H/R) can activate JNK_1/$SAPK_1$ and if OFR are involved in this activation. Primary cultured rat hepatocytes isolated from other liver cells and blood flow were submitted to warm and cold H/R phases mimicking surgical and transplant conditions. JNK_1/$SAPK_1$ was activated by both warm and cold H/R. Deferoxamine (1 m*M*), di-phenyleneiodonium (50 μ*M*) and N-acetylcysteine (10 m*M*) significantly inhibited this kinase activation.

Key words Hypoxia-reoxygenation · JNK_1/$SAPK_1$ · Rat · Hepatocytes

Introduction

Organ injury after ischemia and reperfusion (I/R) remains one of the most important limiting factors in liver surgery and transplantation. The liver is quite sensitive to ischemia, owing to its high metabolism, and morbidity and mortality rates increase rapidly with lengthening of the ischemic interval [4]. Oxygen-free radical (OFR) generation is considered a major cause of liver I/R injury [2]. It has been reported that I/R can modulate mitogen-activated protein kinases (MAPK) in hepatocytes [1, 10]. MAPKs are divided into two major subfamilies, the extracellular signal-regulated kinases (ERKs), which are generally stimulated by growth factors, and the stress-activated protein kinases (SAPKs), which are targets of environmental stress and cytokines. SAPKs seem to be associated with the induction of apoptotic cell death [12], and include JNK (also known as $SAPK_1$), p38 (also known as $SAPK_2$), and $SAPK_3$, and more recently identified $SAPK_4$ [6]. This present study was undertaken to determine if hypoxia and reoxygenation (H/R) activates JNK_1/$SAPK_1$, and if OFR are involved in this activation. Primary cultured rat hepatocytes isolated from other liver cells and blood flow were submitted to warm and cold H/R phases mimicking surgical and transplant conditions.

Materials and methods

Animal

Male Wistar rats, weighing 250–300 g, were purchased from Charles River Co. (Saint-Aubin-Lès-Elbeuf, Fance). Rats were treated in compliance with French rules on animal handling.

Hepatocyte isolation and culture

Hepatocytes were obtained according to methods previously described [5]. Cell viability was estimated to be superior to 90% by Trypan blue exclusion. Cells were then adjusted to a density of 0.5 10^6 cells/ml and cultured in dishes in the presence of 95% air and 5% CO_2. After 16 h of culture, the hepatocytes were used for experiments.

Experimental protocol

Culture plates were maintained at a selected temperature in an hermetic bag in which the PO_2 was adjusted to 50 ± 10 mm Hg by

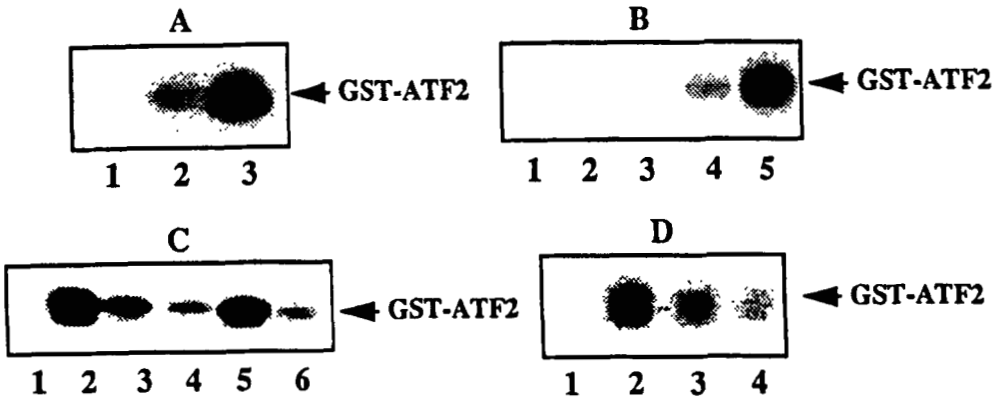

Fig. 1 $JNK_1/SAPK_1$ activation by hypoxia-reoxygenation (H/R) and action of oxygen-free radical (OFR) scavengers. **A** warm stress: hepatocytes were incubated at 37 °C, for 2 h under air (lane 1), 1 h under N_2 (lane 2), 1 h under N_2 followed by 1 h under air (lane 3). **B** cold stress: hepatocytes were incubated for 24 h at 4 °C under N_2 (lane 3), followed by either 5 min (lane 4) or 1 h (lane 5) under air at 37 °C. Control hepatocytes were incubated for 24 h under air at 37 °C (lane 1) or at 4 °C (lane 2). **C** ORF scavengers reduced $JNK_1/SAPK_1$ activation induced by warm H/R (lane 2): N-acetylcysteine, 10 m*M* (lane 3), Di-Phenyleneiodonium, 50 μ*M* (lane 4), deferoxamine, 1 m*M* (lane 6). N-tert-butyl-α-phenylnitrone, 100 μ*M* (lane 5) had no effect. Control group: 2 h under air (lane 1). **D** ORF scavenger reduced $JNK_1/SAPK_1$ activation induced by cold ischaemia followed by warm reoxygenation. H/R induced $JNK_1/SAPK_1$ activation (lane 2), whereas sole cold H had no effect (lane 1). Deferoxamine, 1 m*M*, reduced this response when it was used during all H/R (lane 4), and even during the last 4 h of cold H and warm R. For each condition, the figure shows a representative result from four independent experiments

N_2 supply, and was maintained constant. Culture media were nitrogen saturated. A sample of each medium was left under the same conditions and immediatly analyzed (blood gas analyzer Corning 2504) at the end of each experiment.

Warm hypoxia stress

Hepatocytes were transferred into Leibovitz medium (L15, Eurobio). Three groups were studied. Group 1 (control) corresponded to hepatocytes maintained at 37 °C under ambiant air for 2 h. In group 2 hepatocytes were maintained under hypoxic atmosphere for 1 h at 37 °C. Group 3 corresponded to conditions of group 1 followed by 1 h under ambiant air at 37 °C.

Cold hypoxia stress

Hepatocytes were transferred into L15 medium. Five groups were studied. Groups 1 and 2 (control) corresponded to cells preserved for 24 h under ambiant air at 4 °C or 37 °C, respectively. In group 3, cells were preserved at 4 °C for 24 h under hypoxic atmosphere. Group 4 corresponded to the conditions of group 3, followed by 5 min exposure to ambiant air at 37 °C. Group 5 cells treated as described for group 4, except that they were exposed for 60 min to ambiant air at 37 °C. When used, OFR scavengers were added to culture media 18 h before the stress (N-acetylcystein; NAC; 10 m*M*; N-tert-butyl-α-phenylnitrone; BP; 100 μ*M*) or only at the begining of the stress (di-phenyleneiodonium; DPI; 50 μ*M*: Deferoxamine; DFO; 1 m*M*).

Kinase assays

Cultured hepatocytes were submitted to the different stresses and lysed as described previously [9]. JNK_1 immune precipitates from cell lysates were incubated with [γ^{32}P]ATP and GST-ATF2, and then fractionated by SDS-PAGE followed by electroblotting as previously reported. The blot was exposed to X-ray film to reveal phosphorylation of GST-ATF2, then developed with anti-JNK_1 to measure total JNK_1 in precipitates.

Results

Stimulation of JNK activity by H/R

Among the different SAPKs (data not shown), only JNK_1 was activated. In primary cultured hepatocytes, warm hypoxia induced significant JNK_1 activation (Fig. 1A, lane 2), but to a lesser extent compared to that one observed after H/R (Fig. 1A, lane 3). In control hepatocytes, JNK_1 was not activated (Fig. 1A, lane 1). Cold hypoxia lasting 24 h failed to stimulate JNK_1 activity (Fig. 1B, lane 2), but reoxygenation induced JNK_1 activation after 1 h (Fig. 1B, lane 5). This response was marked after the 5th min of reoxygenation (Fig. 1B, lane 4). JNK_1 was not activated in control hepatocytes after 24 h at 37 °C (Fig. 1B, lane 1) or at 4 °C (Fig. 1B, lane 2).

OFR scavenger effects on JNK_1 activation

During warm H/R, DPI and DFO significantly reduced JNK_1 activation (68% and 72%, Fig. 1C, lanes 4 and 6, respectively). NAC moderately decreased JNK_1 activity (50%, Fig. 1C, lane 3) and BP had not effect (Fig. 1C, lane 5). DFO was also tested in cold H/R. It significantly reduced JNK_1 activation when it was used during all cold-hypoxic and warm-oxygenated phases (89%,

Fig. 1D, lane 4), as well as the last 4 h of cold hypoxia and all warm reoxygenation time (44%, Fig. 1D, lane 3).

Discussion

It is well recognized that ischemia and subsequent reperfusion induces substantial damage to organs leading to surgical or graft failures. OFR are significantly generated during these phases and are involved in cell injury. JNK_1, a member of MAPK family, regulates adaptation to stressful conditions. Some studies performed on isolated-perfused livers or in vivo have shown that I/R stimulates JNK_1 activation [1]. However, these stress conditions also stimulate TNFα, which is known to activate JNK_1. The aim of this study was to analyze the role of H/R and the involvement of OFR on JNK_1 stimulation in hepatocytes isolated from other liver cells and blood flow.

During warm hypoxia, mimicking surgical conditions, JNK_1 was slightly stimulated, while it was significantly increased following reoxygenation. In our experimental conditions, DPI, a mitochondrial and microsomal NADPH oxidase inhibitor, and DFO, an inhibitor of iron-catalyzed hydroxyl radical formation, significantly reduced JNK_1 activation. NAC, a hepatic glutathione precursor, had a slightly lesser inhibitory effect than other inhibitors tested. Rauen et al. [7] have shown that the hypoxia time necessary for increasing the formation of OFR is about 4 h in cultured hepatocytes. Our study suggested that OFR appeared after 1 h of reoxygenation following 1 h of warm hypoxia.

During cold hypoxia, mimicking preservation conditions, JNK_1 was not stimulated. The cold-induced slowing down of cellular metabolism could explain this difference. Following warm reoxygenation, mimicking the reperfusion phase, JNK_1 stimulation was induced quickly (from the 5th min). Our results differed from those of Bradham et al. [1]. Indeed, they have reported a high basal JNK_1 activity that could be consistent with JNK_1 activation by hepatocyte plating. We observed the same result when cells were preserved in University of Wisconsin (UW) solution (unpublished results). Conversely, when hepatocytes were preserved in culture medium, the JNK_1 level was not increased. Some UW components could induce an artifact. Thereby, the increase in reoxygenation-induced JNK_1 activation was more important in our results. Under our cold conditions, DFO also significantly inhibited JNK_1 activation.

Modulation of JNK_1 activation might present a new way to prevent the consequences of I/R of the liver. According to these results, a rational antioxidant therapy might offer considerable improvement in organ quality. Besides, Cerwenta et al. [3] have shown a positive effect of antioxidant treatment with a multivitamin infusion on postischemic liver function parameters in patients undergoing major liver surgery. NAC could be regularly used because it presents many other beneficial characteristics [8, 11] without any dangerous side effect.

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