Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 1785–1795
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Role of mechanical and redox stress in activation of mitogen-activated protein kinases in primary cultured rat hepatocytes

Sang K. Kim [a,b], Kimberley J. Woodcroft [a,1], Soo Jin Oh [b], Mohamed A. Abdelmegeed [a], Raymond F. Novak [a,*]

[a] *Institute of Environmental Health Sciences, Wayne State University, 2727 Second Avenue, Room 4000, Detroit, MI 48201, USA*
[b] *College of Pharmacy and Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764, South Korea*

Received 9 May 2005; accepted 22 July 2005

Abstract

Mechanical stress is known to activate signaling cascades, including mitogen-activated protein kinase (MAPK) pathways. Although mechanical stress has been implicated in hepatic cirrhosis and liver regeneration following hepatectomy, the signaling pathway(s) that may be activated in hepatocytes in response to mechanical stress have not been determined. Using primary cultured rat hepatocytes to examine cellular signaling in response to mechanical stress associated with medium change, we observed that the phosphorylation status of extracellular signal-regulated kinase 1/2 (ERK1/2), Jun N-terminal kinase and p38 MAPK, but not Akt, was altered. MAPK activation, especially ERK1/2, was rapidly increased within 5 min, followed by a subsequent decrease to below basal levels between 30 min and 1 h following medium change. MAPK/ERK kinase (MEK1/2) phosphorylation followed the same pattern. The phosphorylation of Raf-1 in response to medium change was also consistent with Raf-1 serving as an upstream regulator of MEK1/2-ERK1/2 signaling. Phosphorylation of ERK1/2 was increased by mechanical stress alone, suggesting that mechanical stress may be primarily responsible for ERK1/2 activation in response to medium change. Medium change produced a marked decline in oxidized glutathione and malondialdehyde levels, and the antioxidant *N*-acetyl-L-cysteine decreased basal ERK1/2 phosphorylation, suggesting a role for oxidative stress in maintaining basal ERK1/2 phosphorylation in cultured hepatocytes. These data suggest that medium change results in immediate activation of the MAPK signaling pathway due to mechanical stress, followed by a subsequent inactivation of MAPK signaling due to a reduction in oxidative stress levels. These processes may be associated with alteration of hepatic hemodynamic circulation observed in hepatic diseases and in liver transplantation.

Keywords: Mechanical stress; Oxidative stress; Signaling pathways; ERK1/2; Hepatocytes

1. Introduction

Hepatic hemodynamic circulation, resulting in mechanical stress, may be altered in pathophysiological conditions, such as hepatic fibrosis and cirrhosis, and in hepatic surgery, including experimental partial hepatectomy and liver transplantation [1,2]. It has long been recognized that patients with hepatic cirrhosis exhibit hyperdynamic circulation syndrome characterized by high cardiac output and reduced peripherial vascular resistance [3], which contributes to venous portal hypertension [1], an indictor of mechanical stress in the liver. Schoen et al. [4] have suggested that hemodynamic change-induced mechanical stress and the subsequent release of nitric oxide may trigger the liver regeneration cascade in a partially hepatectomized liver. These results raise the possibility that mechanical stress contributes to both liver pathogenesis and regeneration.

Endothelial cells lining the vascular wall are constantly under the influence of hemodynamic forces that induce two major types of mechanical stress. Mechanical strain or stretch occurs as the radius or the length of a blood vessel

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; MEK, MAPK/ERK kinase; GSH, total glutathione; GSSG, GSH disulfide; NAC, *N*-acetyl-L-cysteine; EGF, epidermal growth factor; MDA, malondialdehyde; TOSC, total oxy-radical scavenging capacity; HBSS, Hanks' balanced salt solution

* Corresponding author. Tel.: +1 313 577 0100; fax: +1 313 577 0082.
E-mail address: r.novak@wayne.edu (R.F. Novak).
[1] Present address: Biostatistics and Research Epidemiology, Henry Ford Health System, Detroit, MI 48202, USA.

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.07.019

increases. A second type of stress is termed fluid shear stress, which is caused by the flow of blood over the endothelial cells. The nature and magnitude of mechanical stress plays an important role in long-term maintenance of the structure and function of the blood vessel [5]. The regulation by mechanical stress in endothelial cells is mediated by the activation of mechanosensitive signaling pathways that include the mitogen-activated protein kinase (MAPK) family, which consist of the extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs), p38 MAPKs and ERK5. However, which signaling pathway(s) may be activated in response to mechanical stress in hepatocytes has not been addressed.

MAPK signaling cascades, which are regulated by phosphorylation and dephosphorylation on serine and/or threonine residues, regulate important biologic activities [6,7]. Each MAPK is a member of a three-protein kinase cascade; a MAPK kinase kinase phosphorylates a MAPK kinase that subsequently phosphorylates the MAPK [8]. The most extensively investigated instance of MAPK activation involves signal transduction by receptor tyrosine kinases, which activate MAPK cascades in response to growth factors and insulin [9]. Of the various MAPKs, the ERK1 (p44 MAPK) and ERK2 (p42MAPK) subfamily was the first to be characterized [10]. In response to growth factors, the basic ERK signaling cascade following activation of the receptors includes the G-protein Ras, Raf-1 (MAPK kinase kinase), MEK1/2 (MAPK kinase) and ERK1/2 (MAPK) [11]. Signals relayed by MAPKs regulate a wide variety of cellular processes, including cell growth and proliferation, tumorigenesis, inflammation, immune response, apoptosis and embryonic development, which depend on the type of stimulus and cell context [7,12].

ERK1/2, a proline-directed serine/threonine kinase at the end of the ERK signaling cascade, can also be activated in response to stress signals including UV irradiation, mechanical stress and oxidative stress [5,8,13]. JNK and p38 MAPK, stress-activated protein kinases, are slightly activated by insulin or growth factors and strongly activated by endogenous and exogenous stress stimuli, including cytokines and G-protein-coupled receptor agonists, UV irradiation, heat- or cold-shock, osmotic stress, mechanical stress and oxidative stress [5,12–14]. In contrast to the detailed understanding of MAPK signaling pathways activated in response to growth factors, much less is known about the stimulation of MAPK cascades in response to cellular stress.

These studies were conducted to test the hypothesis that medium change, and the associated mechanical stress, results in an alteration of basal phosphorylation of MAPKs in primary cultured rat hepatocytes. We report that mechanical stress due to medium change activates MAPKs, especially ERK1/2 and its upstream regulators Raf-1 and MEK1/2. We also provide data implicating a reduction of oxidative stress in the decreased phosphorylation of ERK1/2 following the initial increase in ERK1/2 phosphorylation in response to medium change.

2. Materials and methods

2.1. Materials

Modified Chee's medium and L-glutamine were obtained from Invitrogen (Carlsbad, CA). Insulin (Novolin R) was purchased from Novo-Nordisk (Princeton, NJ). Collagenase (type I) was purchased from Worthington Biochemicals (Freehold, NJ). Vitrogen (95–98% type I collagen, 2–5% type III collagen) was obtained from Cohesion Technologies (Santa Clara, CA). Antibodies against ERK1/2, JNK, p38 MAPK, Akt, MEK1/2, phospho-ERK1/2 (threonine-202, tyrosine-204), phospho-JNK (threonine-183, tyrosine-185), phospho-p38 MAPK (threonine-180, tyrosine-182), phospho-Akt (serine-473), phospho-MEK1/2 (serine-217/221), phospho-Raf-1 (serine-259) and phospho-Raf-1 (serine-338) were purchased from Cell Signaling Technology (Beverly, MA). Anti-Raf-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit antibody was obtained from Bio-Rad Laboratories (Hercules, CA). Enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). *N*-Acetyl-L-cysteine (NAC) and all other reagents were purchased from Sigma (St. Louis, MO).

2.2. Primary rat hepatocyte culture

Hepatocytes were isolated from the livers of male Sprague–Dawley rats (200–300 g) using collagenase perfusion as previously described [15]. Hepatocytes were plated onto dishes covalently coated with Vitrogen, and modified Chee's medium was fortified as described [15] and supplemented with 0.1 μM dexamethasone and 1 μM insulin. Cells were plated at a density of 3×10^6cells/60 mm dish and each culture tray had four dishes. Hepatocytes were incubated in a humidified 37 °C incubator with 95% air/5% CO_2 atmosphere. Four hours after plating, cells were washed with insulin-free medium several times. Medium was replaced with medium containing no insulin and changed every 24 h. Following medium change at 48 h after plating, cells were harvested after 5 min, 10 min, 30 min, 1 h, 3 h or 24 h. Immediately after each culture tray was removed from the incubator, medium was aspirated under vacuum, requiring ~2.5 s to remove the medium from each dish. Three mililiters of fresh medium pre-warmed up to 37 °C was added into each dish using an electronic pipette aid equipped with a 10 ml disposable pipette. In some experiments, fresh 25 °C medium was added. Fresh medium was added to dishes within 20 s after removing the existing medium. Time zero refers to hepatocytes harvested immediately before medium change. To produce mechanical stress, 60 mm dishes were shaken on a belly dancer at a rotational frequency of 40 rpm for 20 or 60 s. NAC (5 mM) was added 2 h prior to medium change. In separate experiments, medium was replaced with medium supplemented with insulin (10 nM) or epidermal growth factor (EGF) (50 ng/ml).

2.3. Immunoblot analysis

To determine the phosphorylation state of ERK1/2, JNK, p38 MAP kinase, Akt, MEK1/2 and Raf-1 cell lysates were prepared by scraping cells directly into 500 μl of Laemmli sample buffer. Cell lysates (10–15 μl) were separated by 10% gel electrophoresis and electrophoretically transferred to nitrocellulose. Blots were blocked for 1 h in 5% milk powder in Tris–HCl buffered saline containing 0.05% Tween 20 (TBS-T). Membranes were probed with the appropriate phospho-specific antibody (diluted 1:250–1:1000 in 5% bovine serum albumin in TBS-T) overnight at 4 °C followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1:2000 in 5% milk power in TBS-T). The results were normalized to total ERK1/2, JNK, p38 MAPK, Akt, MEK1/2 or Raf-1 by stripping the blots and re-probing with phosphorylation state-independent antibodies. Proteins were detected by enhanced chemiluminescence on Kodak X-OMAT film (Sigma Chemical Co.) and quantified by densitometry with a Molecular Dynamics scanning laser densitometer and ImageQuant analysis program (Amersham Biosciences, Piscataway, NJ).

2.4. GSH and GSSG level determination

To determine total glutathione (GSH) and GSH disulfide (GSSG) concentration, cells were washed twice in ice-cold PBS and then scraped into ice-cold 5% perchloric acid. The lysates were transferred into Eppendorf tubes and were clarified by centrifugation at 10,000 × *g* for 5 min at 4 °C. The supernatant was used for determination of GSH level and the pellets were used for protein determination. GSH and GSSG concentrations were determined using the enzymatic recycling method.

2.5. Malondialdehyde (MDA) level determination

To determine MDA levels, cells were lysed in 50 mM HEPES (pH 7.2), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100 and 1 mM $MnCl_2$. Cells were scraped into lysis buffer, and the lysates were transferred into Eppendorf tubes and passed through a 25-gauge needle. Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma–Aldrich). For preparation of samples, an aliquot of 30 μl of cell lysate was mixed with 0.3 ml of 0.2% thiobarbituric acid in 2 M sodium acetate buffer containing 1 mM diethylenetriaminepentaacetic acid, pH 3.5, and 3 μl of 5% butylated hydroxytoluene in 96% ethanol. The sample mixture was incubated at 95 °C for 45 min. After cooling at room temperature, the samples were centrifuged at 16,000 × *g* for 20 min and the supernatant used for HPLC analysis. HPLC equipment was from Shimadze: pump, model LC-10AT; system controller, model SCL-10A; injector, Rheodyne equipped with a 20-μl loop; RF-10A fluorescence detector. The MDA-thiobarbituric acid complex was separated using a 5 μm Symmetry C18 (4.6 mm × 150 mm) reversed phase column (Eka Chemicals, Bohus, Sweden). The mobile phase was composed of 45% methanol and 55% 50 mM sodium phosphate buffer, pH 7.0, passed through a 0.45 μm Millipore filter before use. The MDA-thiobarbituric acid complex was eluted at a flow-rate of 1.5 ml/min and monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm.

2.6. Total oxy-radical scavenging capacity (TOSC) assay

TOSC assay of Regoli and Winston [16] was used to evaluate antioxidant behavior of the medium. TOSC assay is based on the ethylene-yielding reaction of alpha-keto-gamma-methiolbutyric acid with peroxyl radicals and peroxynitrite. Peroxyl radicals were generated by thermal homolysis of 2,2′-azobis-amidinopropane at 35 °C. Peroxynitrite was produced by the decomposition of SIN-1. The assay conditions used were 0.1 mM alpha-keto-gamma-methiolbutyric acid and 20 mM 2,2′-azobis-amidinopropane or 0.07 mM SIN-1 (3-morpholinosyndnomine) in 100 mM potassium phosphate buffer, pH 7.4. To examine TOSC for fresh medium and medium conditioned by 1 day of culture with hepatocytes, both media were diluted 5-fold in distilled water and 0.1 ml of the diluent was added into each vial. The control reaction consisted of 0.1 ml of distilled water instead of diluted medium. Reactions were carried out in 12-ml rubber septum-sealed vials in a final reaction volume of 1 ml. Ethylene production was measured by GC analysis of 0.2-ml aliquots taken directly from the headspace of the reaction vials. Samples were monitored in sequence at 12-min intervals. Analyses were performed with a Hewlett-Packard (Series II 5890) gas chromatograph equipped with a 30 m capillary SPB-1 column (Supelco) and a flame ionization detector. The oven, injection, and detector temperatures were respectively, 60, 180, and 180 °C. Nitrogen was used as the carrier gas at a flow rate of 30 ml/min. TOSC values were quantified from the equation $TOSC = 100 - (SA/CA \times 100)$, where SA and CA were the integrated areas from the curve that best defined the experimental points during the reaction time course for sample and control reactions, respectively. A sample with no oxy-radical scavenging capacity receives a TOSC value of 0%, because it has the same area under the curve as the control reaction (SA/CA = 1). A sample that suppressed the ethylene formation entirely possesses an area under the curve of 0 (SA = 0), and thereby, a TOSC value of 100%. The specific TOSC was calculated by dividing the experimental TOSC by the volume of medium.

2.7. Statistical analysis

Significant differences between groups were determined by two-tailed Student's *t*-test or ANOVA followed by the

Newman–Keuls comparison test ($p < 0.05$). Statistical analysis was performed on triplicate cell lysates from a single hepatocyte preparation. Reproducibility of results was confirmed in two to four separate hepatocyte preparations.

3. Results

3.1. Effects of medium change on MAPK phosphorylation in primary cultured rat hepatocytes

In order to examine effects of medium change on MAPK activity, we monitored the phosphorylation of ERK1/2, JNK, p38 MAPK and Akt in hepatocytes over a 24-h period (Fig. 1). Fresh medium was added to dishes within 20 s after removing the existing medium. Control dishes received no fresh medium and no agitation. The phosphorylation of MAPKs was altered in a time-dependent manner after medium change. Changing the medium increased the phosphorylation of ERK1/2 (threonine-202, tyrosine-204) ~4-fold within 5 min and a similar increase was detected at 10 min (Fig. 1A). Interestingly, a marked decrease in ERK1/2 phosphorylation, to ~75–80% below basal levels, was observed at 30 min and 1 h following medium change. ERK1/2 phosphorylation returned to basal levels by 24 h. The phosphorylation of both JNK (threonine-183, tyrosine-185) and p38 MAPK (threonine-180, tyrosine-182) was also increased, followed by a decrease to below basal levels and subsequent return to basal levels, but these

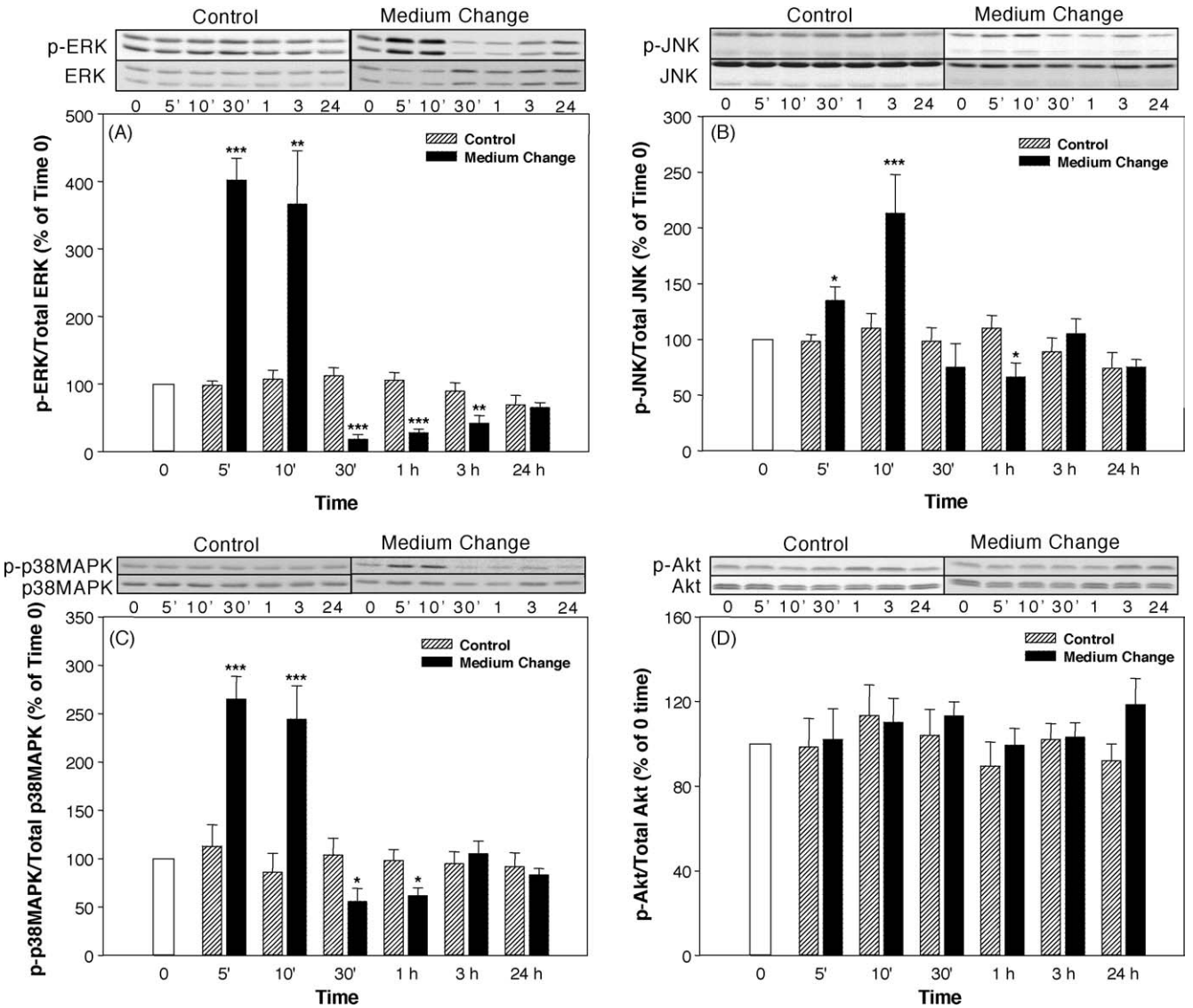

Fig. 1. Effect of medium change on phosphorylation of ERK1/2 (A), JNK (B), p38 MAPK (C), and Akt (D) in primary cultured rat hepatocytes. Following 48 h in culture in the absence of growth factors, conditioned medium was replaced with fresh medium and cells were harvested at the indicated times. Control cells were cultured without medium change. Time zero refers to hepatocytes harvested immediately before medium change. Data are mean ± S.D. of immunoblot band densities of three preparations of cell lysates. *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$, respectively, are significantly different than levels monitored in corresponding control hepatocytes.

effects appeared to be less robust relative to the effects on ERK1/2 (Fig. 1B and C). In contrast, medium change failed to affect the phosphorylation of Akt on serine 473 (Fig. 1D), suggesting that the phosphatidylinositol 3-kinase/Akt signaling cascade is not activated in response to stress resulting from medium change.

To determine whether MEK1/2 and Raf-1, upstream regulators of ERK1/2, were also activated in response to medium change, phosphorylation of these kinases was monitored after medium change in primary cultured rat hepatocytes (Fig. 2). Consistent with the pattern of ERK1/2 phosphorylation, phosphorylation of MEK1/2 (serine-217/221) was increased ~2.5-fold within 5 min, followed by a substantial decrease to below basal levels between 30 min and 1 h, with a return toward basal levels by 24 h (Fig. 2A). Raf-1 activation requires phosphorylation at serine 338 and dephosphorylation at serine 259 [17]. Thus, increased serine 338 phosphorylation is indicative of Raf-1 activation and increased serine 259 phosphorylation is indicative of Raf-1 inactivation. Medium change resulted in increased Raf-1 serine 338 phosphorylation and decreased serine 259 phosphorylation at 5 min, followed by a decrease in serine 338 phosphorylation below basal levels and an increase in serine 259 phosphorylation above basal levels 30 min and 1 h after medium change (Fig. 2B and C). Raf-1 phosphorylation at both sites returned to basal levels by 24 h. These results indicate that, consistent with the pattern of ERK and MEK phosphorylation, Raf-1 is rapidly activated in response to medium change followed by a decrease in activation by 30 min and a return to basal conditions by 24 h.

MAPKs can be activated in response to stress, including osmotic stress, temperature changes and mechanical stress [5,12,14,18]. During the time required to change medium, it is possible that the temperature in the tissue culture dish drops slightly and then rises again once fresh medium has been added. To examine the possible involvement of temperature changes in mediating ERK1/2 activation in response to medium change, existing medium was removed and fresh 37 °C medium added to dishes placed on a 37 °C heating block (Fig. 3A). Maintaining temperature failed to alter either the increase, or subsequent decrease below basal levels, in ERK1/2 phosphorylation induced by medium change (Fig. 3A). The effect of replacing medium with fresh medium at 25 °C was also examined. The ERK1/2 phosphorylation pattern in response to medium change with 25 °C medium was not different from that of 37 °C medium (data not shown). These results suggest that minor temperature changes during medium change do not contribute significantly to the alteration in ERK1/2 phosphorylation. To investigate whether mechanical stress might be involved in the medium change effects, ERK1/2 phosphorylation was monitored in hepatocytes after shaking on a belly dancer at a rotational frequency of 40 rpm for 20 or 60 s, with no change of medium (Fig. 3B). Mechanical stress for 20 s

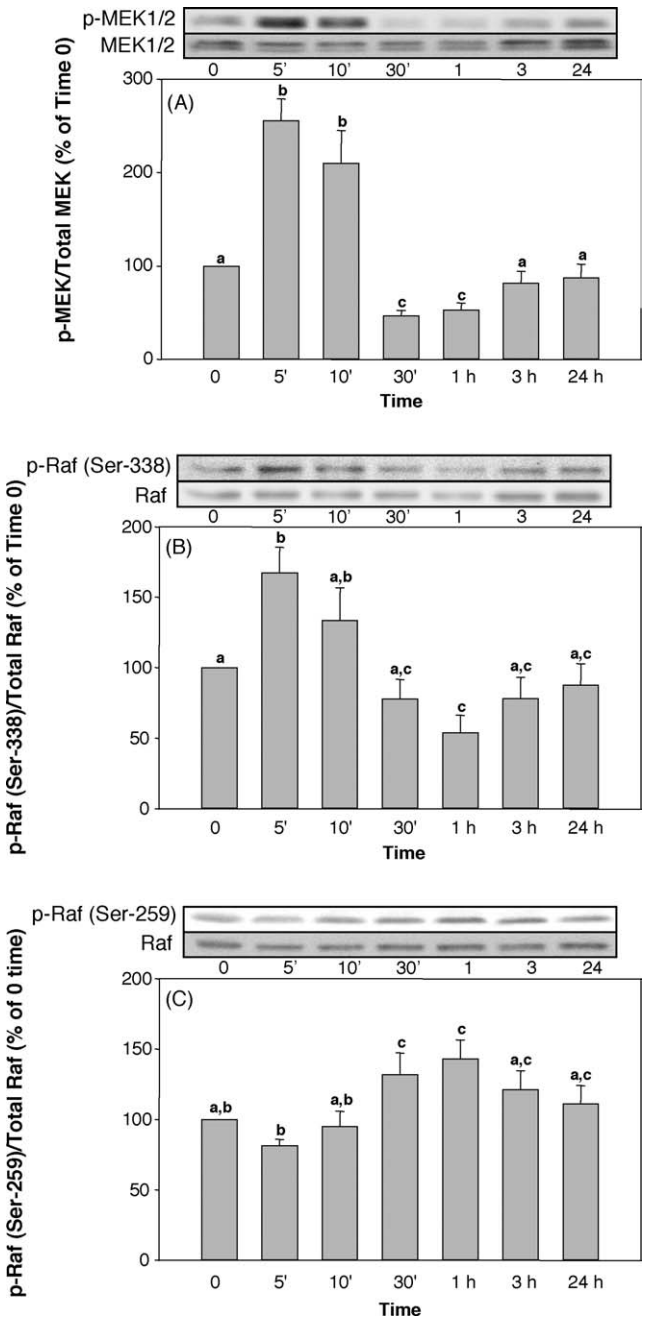

Fig. 2. Effect of medium change on MEK1/2 (A) and Raf-1 (B, C) phosphorylation in primary cultured rat hepatocytes. Following 48 h in culture in the absence of growth factors, conditioned medium was replaced with fresh medium and cells were harvested at the indicated times. Time zero refers to hepatocytes harvested immediately before medium change. Data are mean ± S.D. of immunoblot band densities of three preparations of cell lysates. Values with different letters are significantly different from each other, $p < 0.05$.

increased ERK1/2 phosphorylation ~2.3-fold at 5 and 10 min with a return to basal levels by 30 min. When hepatocytes were exposed to mechanical stress for 60 s, ERK1/2 phosphorylation was increased to ~3.1-fold at 5 min and this increase declined slightly after 10 min (to ~2-fold) with a return toward basal levels by 30 min (data not shown). In contrast to the pattern of ERK1/2 phosphorylation observed with medium change, the decreased

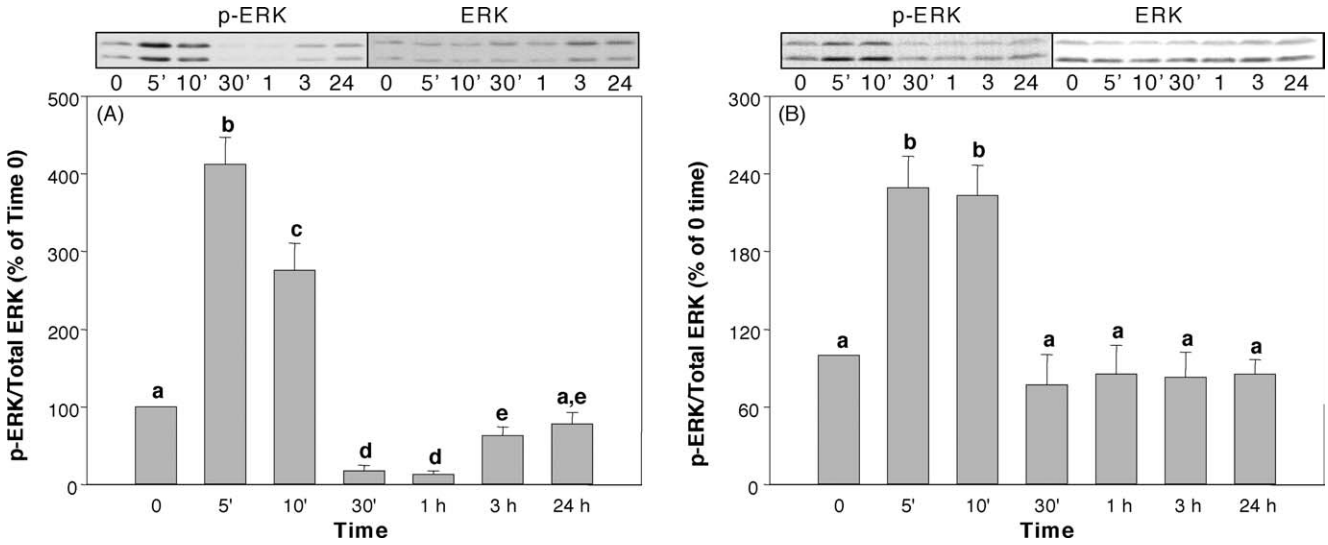

Fig. 3. Effect of temperature and mechanical stress associated with medium change on ERK1/2 phosphorylation in primary cultured rat hepatocytes. (A) conditioned medium was replaced with fresh medium in dishes placed on a 37 °C heating block; (B) culture dishes, without change of medium, were shaken at a rotational frequency of 40 rpm for 20 s. Time zero refers to hepatocytes harvested immediately before medium change or shaking. Data are mean ± S.D. of immunoblot band densities of three preparations of cell lysates. Values with different letters are significantly different from each other, $p < 0.05$.

phosphorylation of ERK1/2 below basal levels at 30 min and 1 h was not observed in response to mechanical stress alone. Mechanical stress also increased the phosphorylation of JNK ~2.4-fold at 10 min without the subsequent decrease in JNK phosphorylation (data not shown).

Both the components of Chee's medium, and mediators released from hepatocytes, such as reactive oxygen species, can affect cellular signaling pathways [13,19]. This raises the possibility that differences in components between fresh medium and medium conditioned by 1 day of culture with hepatocytes may play a role in alterations in MAPK phosphorylation induced by medium change. In order to test this possibility, we removed hepatocyte-conditioned medium and added it back to the same dishes (Fig. 4A), or replaced existing medium with Hanks' balanced salt solution (HBSS) (Fig. 4B). Re-addition of conditioned medium increased ERK1/2 phosphorylation ~3.6-fold within 5 min (Fig. 4A), comparable to ERK1/2 phosphorylation levels monitored in cells 5 min following addition of fresh medium (Fig. 1A). Replacing medium with HBSS at 37 °C also resulted in a ~6.7-fold increase in ERK1/2 phosphorylation within 5 min (Fig. 4B). These results suggest that differences in medium components between fresh medium and conditioned medium may not be primarily responsible for the activation of

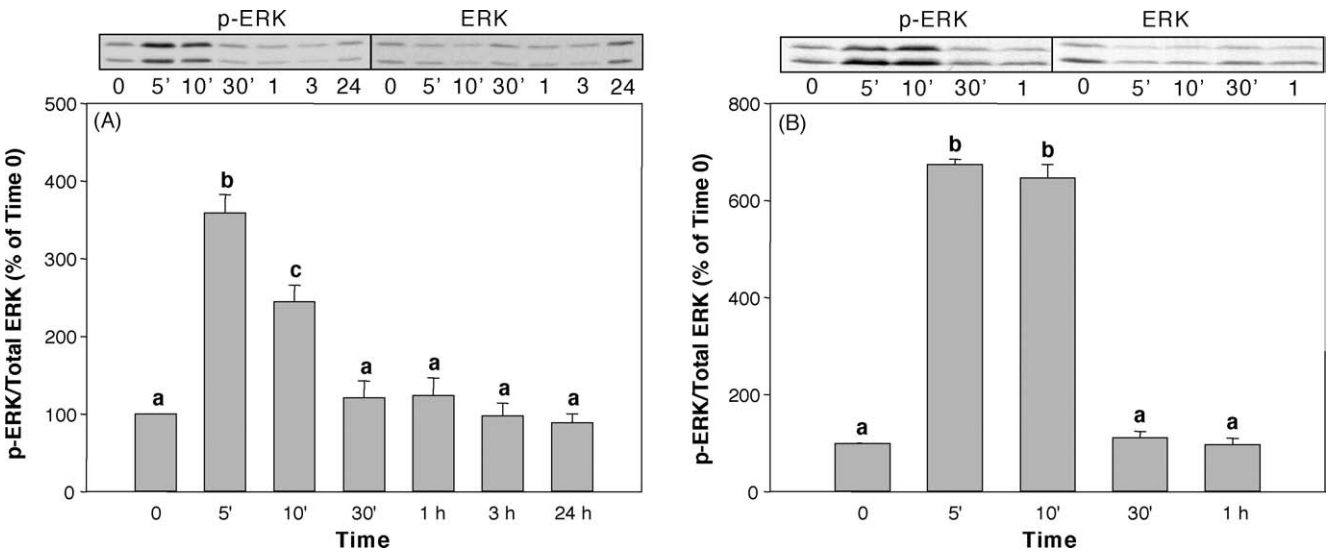

Fig. 4. Effect of addition of conditioned medium or HBSS on ERK1/2 phosphorylation in primary cultured rat hepatocytes. (A) conditioned medium was removed and added back to the same dish of hepatocytes; (B) conditioned medium was replaced with HBSS. Time zero refers to hepatocytes harvested immediately before medium change. Data are mean ± S.D. of immunoblot band densities of three preparations of cell lysates. Values with different letters are significantly different from each other, $p < 0.05$.

ERK1/2 following medium change. In contrast, both re-addition of conditioned medium and replacement with HBSS failed to cause the subsequent decrease in ERK1/2 phosphorylation below basal levels at 30 min and 1 h observed following medium change (Fig. 4A and B). These results suggest that addition of components in fresh medium are involved in mediating the decrease in ERK1/2 phosphorylation below basal levels in response to medium change.

3.2. Role of oxidative stress in MAPK activation during medium change

Existing evidence indicates that oxidative stress plays a critical role in regulation of MAPK signaling pathways [13,20]. To determine whether medium change results in an alteration of cellular redox state, total GSH and oxidized GSH (GSSG) were measured in hepatocytes 5 min, 10 min, 30 min, 1 h, 3 h and 24 h after medium change (Fig. 5A and B). Total GSH levels in hepatocytes were unaffected by medium change (Fig. 5A), whereas GSSG levels were markedly decreased (Fig. 5B). GSSG levels were decreased to ~85% of the initial level within 5 min of medium change, with a maximal decrease of 65% observed at 1 h (Fig. 5B). Consequently, the ratio of GSSG to total GSH, used as an indicator of redox state, decreased significantly after medium change. To determine whether medium change results in an alteration of lipid peroxidation, MDA levels were measured using HPLC equipped with a fluorescence detector (Fig. 5C). MDA levels were decreased to ~75% of the initial level within 5 min of medium change, which was maintained at least for 1 h. This decrease diminished after 3 h with a return toward basal levels by 24 h. These results indicate that spontaneous oxidative stress decreases as a result of medium change.

To determine whether a difference in the antioxidant capacity between fresh medium and hepatocyte-conditioned medium could account for the decrease in spontaneous oxidative stress, oxy-radical scavenging capacity toward peroxyl radicals and peroxynitrite was measured using TOSC assay (Fig. 6). Peroxyl radicals were generated by thermal homolysis of 2,2′-azobis-amidinopropane at 35 °C and peroxynitrite was produced by the decomposition of SIN-1. TOSC assay is based on the reaction between peroxyl radicals or peroxynitrite and alpha-keto-gamma-methiolbutyric acid, which is oxidized to ethylene [16]. Peroxyl radical-induced ethylene production was decreased to 49 or 61% by fresh medium or conditioned medium, respectively (Fig. 6A). Specific TOSC values (TOSC standardized to 1 μl of medium) of fresh medium or conditioned medium towards peroxyl radical were 2.56 ± 0.06 or 1.97 ± 0.02, respectively ($p < 0.001$, two-tailed Student's t-test). Addition of fresh medium or conditioned medium in reaction vials inhibited peroxynitrite-induced ethylene formation by 46 or 32%, respectively (Fig. 6B). Specific TOSC values of fresh medium or conditioned medium toward peroxynitrite were 2.29 ± 0.04 or 1.58 ± 0.05, respectively ($p < 0.001$, two-tailed Student's t-test). These results indicate that fresh medium has more antioxidant capacity than that of medium conditioned by 24 h of culture with hepatocytes.

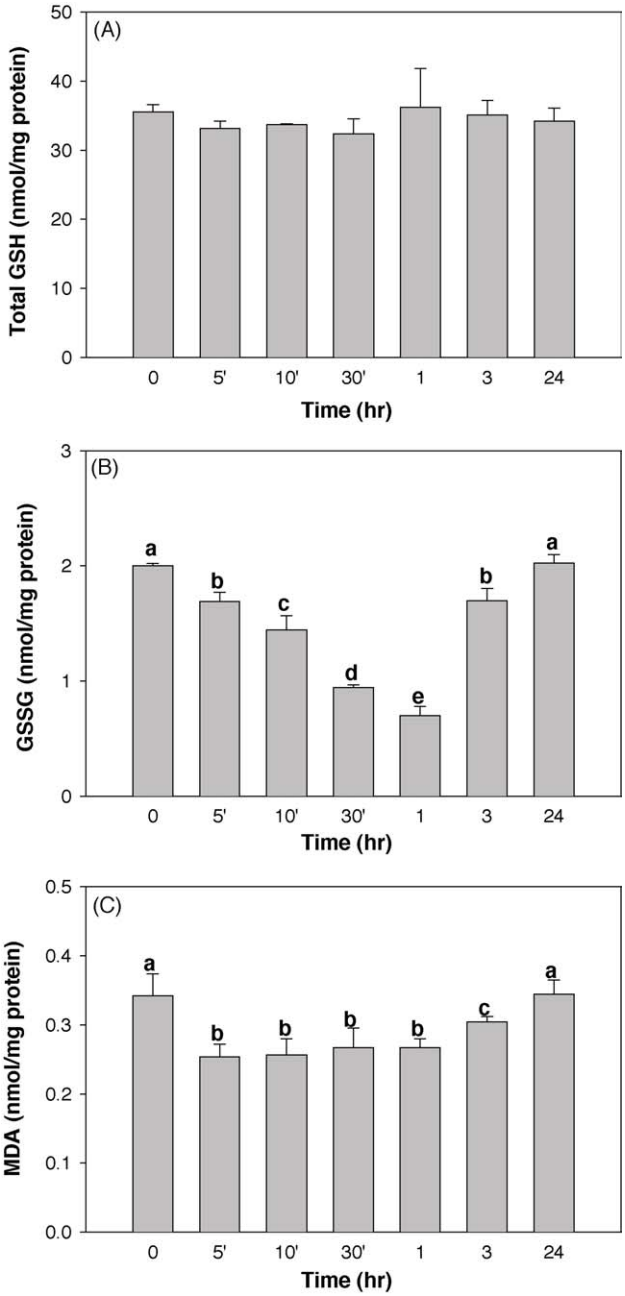

Fig. 5. Effect of medium change on total GSH (A), GSSG (B) and MDA levels in primary cultured rat hepatocytes. Cells were harvested at the indicated times following medium change. Time zero refers to hepatocytes harvested immediately before medium change. Data are mean ± S.D. of three to five preparations of cell lysates. Values with different letters are significantly different from each other, $p < 0.05$.

To examine the role of oxidative stress in the regulation of ERK1/2 phosphorylation in response to medium change, the effects of NAC, a water-soluble antioxidant, on ERK1/

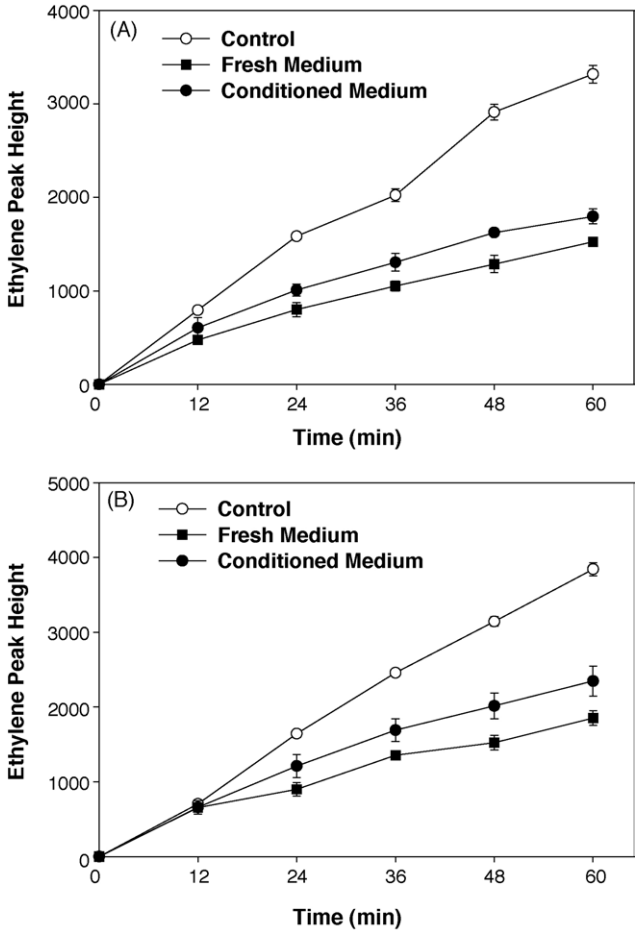

Fig. 6. Peroxyl radical (A) and peroxynitrite (B) scavenging time course for fresh medium and conditioned medium. Conditioned medium was obtained from hepatocytes cultured for 24 h with fresh medium. For the control reaction, water was added instead of medium. Data are mean ± S.D. of triplicate.

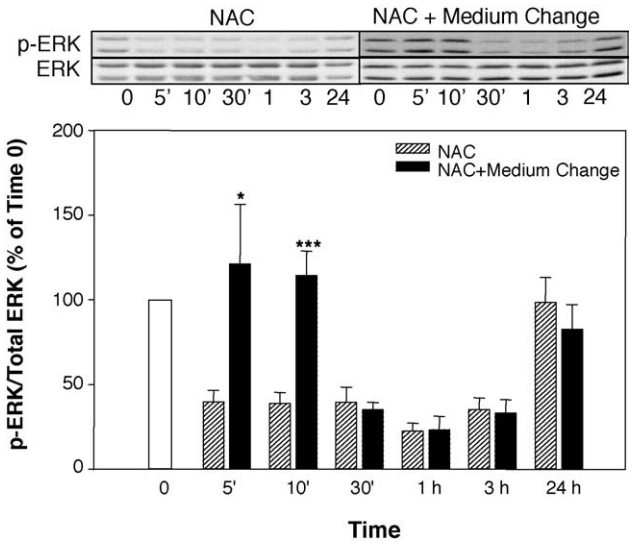

Fig. 7. Effect of NAC on ERK1/2 phosphorylation in response to medium change in primary cultured rat hepatocytes. Hepatocytes were treated with NAC (5 mM) alone or NAC for 2 h followed by replacement of medium with fresh medium containing NAC. Cells were harvested at the indicated times following medium change. Time zero refers to hepatocytes that were not treated with NAC and were harvested immediately before medium change. Data are mean ± S.D. of immunoblot band densities of three preparations of cell lysates. $*p < 0.05$ or $***p < 0.001$, respectively, are significantly different than levels monitored in corresponding hepatocytes treated with NAC alone.

2 phosphorylation were examined (Fig. 7). NAC was spiked into medium followed by rotation on a belly dancer at a rotational frequency of 40 rpm for 20 s. Two hours after addition of NAC, medium was replaced with fresh medium containing NAC. Control hepatocytes (NAC alone) did not receive fresh medium or any culture dish agitation. The addition of NAC alone, in the absence of medium change, resulted in decreased basal phosphorylation of ERK1/2, relative to the level monitored at time zero, at all time points except 24 h (Fig. 7). These data implicate oxidative stress in mediating the basal phosphorylation of ERK1/2 in primary cultured rat hepatocytes. Medium change in addition to NAC treatment increased ERK1/2 phosphorylation by ~3-fold at 5 and 10 min, relative to hepatocytes treated with NAC alone at identical time points (Fig. 7), suggesting that the medium change-induced activation of ERK1/2 does not involve oxidative stress. Medium change in addition to NAC treatment failed to result in a further decrease in ERK1/2 phosphorylation below the already decreased basal levels monitored in cells treated with NAC alone at 30 min and 1 h (Fig. 7). This is in contrast to the decrease in ERK1/2 phosphorylation below basal levels at 30 min and 1 h following medium change in the absence of NAC (Fig. 1A). One possible explanation for this is that NAC treatment maximally reduced spontaneous oxidative stress levels such that any decrease in oxidative stress levels resulting from medium change would be overshadowed by the effect of NAC. Thus, ERK1/2 phosphorylation may already be maximally decreased by NAC, thereby precluding any additional effect of medium change on ERK1/2 phosphorylation. Taken together, these results suggest that inhibition of spontaneous oxidative stress is responsible for the decrease in ERK1/2 phosphorylation below basal levels at 30 min and 1 h in response to medium change. Thus, the mechanism(s) involved in ERK1/2 activation and subsequent deactivation in response to medium change appear to be significantly different.

3.3. EGF- and insulin-mediated phosphorylation of ERK1/2

MAPKs play an important role in regulation of cell functions in response to growth factors [21,22], although the role of MAPKs appears to be dependent on cell context. However, there are inconsistencies in the literature as to whether particular MAPKs are activated in response to mechanical, oxidative, osmotic or heat stress. Moreover, there are conflicting reports on the activation of MAPKs in response to insulin in primary cultured rat hepatocytes [23–27]. Our results raise the possibility that the reason for

these discrepancies may be due to the effects of medium change on basal phosphorylation of MAPKs. In order to examine this possibility, the phosphorylation of ERK1/2 was monitored in primary cultured rat hepatocytes in response to medium change in the presence and absence of EGF (as a positive control) and insulin (Fig. 8). EGF treatment resulted in a substantial increase (6–40-fold) in the phosphorylation of ERK1/2, relative to corresponding control cells, at all time points examined (Fig. 8A). EGF also produced a minimum 7-fold increase in ERK1/2 phosphorylation, relative to time zero hepatocytes, at all time points (Fig. 8A). Insulin treatment increased ERK1/2 phosphorylation by ~2- to 3.5-fold, relative to corresponding control cells, at each time point examined (Fig. 8B). However, when insulin-mediated ERK1/2 phosphorylation was compared with that of time zero hepatocytes, no increase was evident at 30 min or 1 h (Fig. 8B).

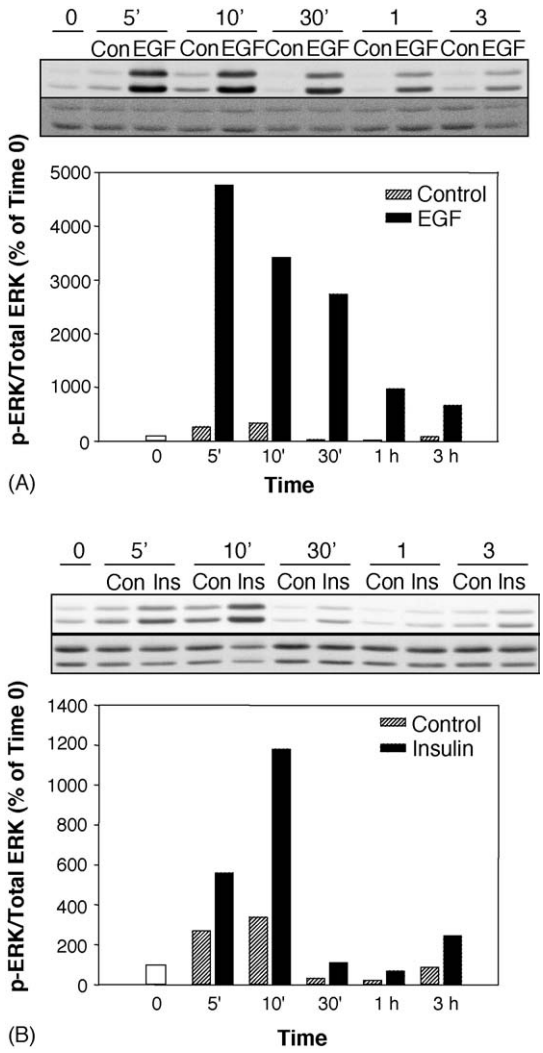

Fig. 8. Effect of EGF (A) or insulin (B) on ERK1/2 phosphorylation in primary cultured rat hepatocytes. Medium was replaced with fresh medium containing 50 ng/ml EGF or 10 nM insulin. Cells were harvested at the indicated times following medium change. Data represent immunoblot band densities of a single preparation of cell lysates.

4. Discussion

The major finding of this study is that medium change results in immediate activation of MAPK signaling pathways, followed by a subsequent inactivation of MAPK signaling, followed by a return to basal levels within 24 h. Our findings indicate that mechanical stress is responsible for the rapidly increased ERK1/2 phosphorylation in response to medium change, suggesting that mechanical stress can activate MAPKs in hepatocytes as it does in endothelial cells [5,28]. We also demonstrate that medium change results in subsequent inhibition of oxidative stress in hepatocytes, which appears to mediate the decrease in ERK1/2 phosphorylation below basal levels following the initial increase. These results suggest that basal MAPK phosphorylation may be regulated by the redox state of primary cultured rat hepatocytes, although the mechanism(s) responsible for regulation of redox state remains to be determined.

In the present study, addition of fresh medium resulted in a decrease in ERK1/2 phosphorylation below control values at 30 min and 1 h, following the rapidly increased phosphorylation of ERK1/2 at 5 and 10 min. In contrast, decreased phosphorylation of ERK1/2 was not observed when medium was replaced with conditioned medium or HBSS. These results suggest that addition of components in fresh medium may be involved in mediating the decrease in ERK1/2 phosphorylation induced by medium change. A number of studies have shown that redox state plays a role in the regulation of ERK1/2 activation [13,20]. To test the possible involvement of redox state in the decrease in ERK1/2 phosphorylation in response to medium change, total GSH, GSSG and MDA levels were determined. Interestingly, medium change resulted in markedly decreased GSSG and decreased MDA levels, but no change in total GSH levels was observed. These data suggest that the decrease in spontaneous oxidative stress resulting from addition of fresh medium may play a role in the decrease in ERK1/2 phosphorylation below basal levels observed 30 min and 1 h following medium change. Total antioxidant capacity of fresh medium toward peroxyl radicals and peroxynitrite was ~1.3- and ~1.5-fold greater, respectively, compared with conditioned medium. Although it remains to be determined how medium change regulates redox state in primary cultured rat hepatocytes, these data do demonstrate that fresh medium change reinforces antioxidant capacity. Moreover, treatment of hepatocytes with NAC decreased basal phosphorylation of ERK1/2. These results suggest that oxidative stress plays a critical role in regulation of ERK1/2 in hepatocytes cultured in the absence of hormones and growth factors as well as in hepatocytes in response to medium change.

This study shows for the first time that mechanical stress activates ERK1/2 in primary cultured rat hepatocytes. Evidence indicates that mechanical stresses, such as fluid

shear stress and circumferential stress, activate multiple signal transduction molecules, such as ERK1/2 [29], ERK5 [30], JNK [31,32] and p38 MAPK [33] in endothelial cells. Moreover, it has been suggested that the mechanical stress-induced activation of these signaling pathways may be involved in regulation of proliferation, morphological changes, and gene expression in endothelial cells [29,33–35]. It remains to be determined, however, whether MAPK activation induced by mechanical stress has an important role in regulation of hepatocyte function.

The hepatic sinusoids, specialized hepatic capillaries, are lined by a discontinuous layer of fenestrated sinusoidal endothelial cells, which have an important filtration role. The hepatic sinusoidal system contains double-barreled structures formed by the sinusoidal lumen and the space of Disse [36] and thus, hepatocytes are directly exposed to blood pressure through the sieve plates. Sato et al. [2] have suggested that hepatocytes as well as sinusoidal endothelial cells are responsible for hepatic blood pressure due to mechanical stress, the dragging frictional force created by blood flow. Mechanical stress can be increased in pathophysiological conditions, such as hepatic fibrosis and cirrhosis, and hepatic surgery including experimental partial hepatectomy and liver transplantation [1,2]. Schoen et al. [4] suggested that a hemodynamic change results in increased mechanical stress in partial hepatectomized liver leading to the subsequent release of nitric oxide, which can trigger the liver regeneration cascade. Significant reductions of albumin and urea synthesis have been observed in cultured rat hepatocytes in response to mechanical stress [37] and in regenerating rat liver after partial hepatectomy [38,39]. These reports, together with the present study, warrant further studies for determining the role of mechanical stress-induced ERK1/2 activation in liver regeneration.

The results of the present study show that the phosphorylation of MEK1/2 and Raf-1 is altered in hepatocytes in response to medium change in a manner that parallels the pattern of phosphorylation of ERK1/2. Medium change rapidly increased the phosphorylation of MEK1/2 within 5 min followed by a substantial decrease, below control values, at 30 min and 1 h. These results indicate that MEK1/2 serves as an upstream regulator of ERK1/2 in response to medium change, similar to the regulation of ERK1/2 activation in response to growth factors. Our data demonstrate that Raf-1 phosphorylation on serine-259 and serine-338 is regulated, in an opposing manner, in response to medium change in primary cultured rat hepatocytes. The regulation of Raf kinases is a complex process involving inter- and intra-molecular interactions, as well as phosphorylation of the regulatory and catalytic domains of the protein [17]. It is believed that serine-338 phosphorylation is a good indicator of Raf-1 activation, whereas serine-259 is phosphorylated in resting cells. Recent studies suggest that phosphorylation of serine-259 along with phosphorylation of serine-621 creates an auto-inhibited conformation state maintained by a 14-3-3 dimer [11]. The binding of Raf-1 to Ras and translocation to the plasma membrane can displace 14-3-3 from phosphoserine-259, which makes it accessible to dephosphorylation by protein phosphatase 2A [40]. Our results suggest that Raf-1 is regulated by both phosphorylation and dephosphorylation in hepatocytes in response to medium change.

In general, ERK1/2 are phosphorylated and activated within a few minutes in response to growth factors or stresses. In the present study, phosphorylation of ERK1/2 was also increased within 5 min following medium change or physical agitation of hepatocytes. We raised the question whether ERK1/2 activation in hepatocytes in response to the mechanical stress resulting from medium change might interfere with the determination of ERK1/2 activation in response to growth factors. Our results demonstrate that maximal ERK1/2 phosphorylation was substantially greater in response to the growth factor EGF than in response to medium change (∼20-fold versus ∼4-fold, respectively), and the time course of ERK1/2 phosphorylation differed in response to insulin relative to medium change (maximal at 10 min versus 5 min, respectively). Thus, ERK1/2 activation in response to growth factors can be differentiated from that in response to medium change. However, these results suggest that an identical time point control group is required to determine ERK1/2 phosphorylation in response to weak stimuli.

In summary, the results of the present study suggest that medium change results in immediate activation of the MAPK signaling pathway due to mechanical stress, followed by a subsequent inactivation of MAPK signaling due to a reduction in oxidative stress levels. Moreover, this study suggests that Raf-1 may be regulated in hepatocytes by both phosphorylation and dephosphorylation in response to medium change, and that Raf-1 serves as an upstream regulator of MEK1/2 and ERK1/2 in response to medium change. Future investigations defining both the upstream and downstream signaling events involved in mechanical stress- and oxidative stress-dependent regulation of MAPKs will advance our understanding of the regulation of hepatocyte function in physiological and pathological conditions.

Acknowledgements

This work was supported by National Institute of Health grant ES03656 (RFN), and by the Cell Culture and Imaging and Cytometry Facility Cores of EHS Center grant P30 ES06639 from the National Institute of Environmental Health Sciences.

References

[1] Bernardi M, Trevisani F. Systemic and regional hemodynamics in preascitic cirrhosis. J Hepatol 1997;27:588–91.

[2] Sato Y, Tsukada K, Hatakeyama K. Role of shear stress and immune responses in liver regeneration after a partial hepatectomy. Surg Today 1999;29:1–9.
[3] Kowalski JH, Abelmann WH. The cardiac output at rest in Laennec's cirrhosis. J Clin Invest 1953;32:1025–33.
[4] Schoen JM, Wang HH, Minuk GY, Lautt WW. Shear stress-induced nitric oxide release triggers the liver regeneration cascade. Nitric Oxide 2001;5:453–64.
[5] Traub O, Berk BC. Laminar shear stress: mechanisms by which endothelial cells transduce an atheroprotective force. Arterioscler Thromb Vasc Biol 1998;18:677–85.
[6] Chang L, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001;410(6824):37–40.
[7] Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. Annu Rev Immunol 2002;20:55–72.
[8] Dent P, Yacoub A, Fisher PB, Hagan MP, Grant S. MAPK pathways in radiation responses. Oncogene 2003;22:5885–96.
[9] van der Geer P, Hunter T, Lindberg RA. Receptor protein-tyrosine kinases and their signal transduction pathways. Annu Rev Cell Biol 1994;10:251–337.
[10] Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, et al. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 1991;65:663–75.
[11] Chong H, Vikis HG, Guan KL. Mechanisms of regulating the Raf kinase family. Cell Signal 2003;15:463–9.
[12] Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001;81:807–69.
[13] Torres M, Forman HJ. Redox signaling and the MAP kinase pathways. Biofactors 2003;17:287–96.
[14] Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J Exp Biol 2003;206:1107–15.
[15] Woodcroft KJ, Novak RF. Insulin differentially affects xenobiotic-enhanced, cytochrome P-450 (CYP)2E1, CYP2B, CYP3A, and CYP4A expression in primary cultured rat hepatocytes. J Pharmacol Exp Ther 1999;289:1121–7.
[16] Regoli F, Winston GW. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. Toxicol Appl Pharmacol 1999;156:96–105.
[17] Dhillon AS, Kolch W. Untying the regulation of the Raf-1 kinase. Arch Biochem Biophys 2002;404:3–9.
[18] Lindquist JM, Rehnmark S. Ambient temperature regulation of apoptosis in brown adipose tissue. Erk1/2 promotes norepinephrine-dependent cell survival. J Biol Chem 1998;273:30147–56.
[19] Lee YJ, Galoforo SS, Berns CM, Chen JC, Davis BH, Sim JE, et al. Glucose deprivation-induced cytotoxicity and alterations in mitogen-activated protein kinase activation are mediated by oxidative stress in multidrug-resistant human breast carcinoma cells. J Biol Chem 1998;273:5294–9.
[20] Owuor ED, Kong AN. Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol 2002;64:765–70.
[21] Taha C, Klip A. The insulin signaling pathway. J Membr Biol 1999;169:1–12.
[22] Coutant A, Rescan C, Gilot D, Loyer P, Guguen-Guillouzo C, Baffet G. PI3K-FRAP/mTOR pathway is critical for hepatocyte proliferation whereas MEK/ERK supports both proliferation and survival. Hepatology 2002;36:1079–88.
[23] Chen J, Ishac EJ, Dent P, Kunos G, Gao B. Effects of ethanol on mitogen-activated protein kinase and stress-activated protein kinase cascades in normal and regenerating liver. Biochem J 1998;334: 669–76.
[24] Peak M, Rochford JJ, Borthwick AC, Yeaman SJ, Agius L. Signalling pathways involved in the stimulation of glycogen synthesis by insulin in rat hepatocytes. Diabetologia 1998;41:16–25.
[25] Zhao AZ, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, Krebs EG, et al. Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. J Biol Chem 2000;275:11348–54.
[26] Carrillo JJ, Ibares B, Esteban-Gamboa A, Feliu JE. Involvement of both phosphatidylinositol 3-kinase and p44/p42 mitogen-activated protein kinase pathways in the short-term regulation of pyruvate kinase L by insulin. Endocrinology 2001;142:1057–64.
[27] Kim SK, Woodcroft KJ, Kim SG, Novak RF. Insulin and glucagon signaling in regulation of microsomal epoxide hydrolase expression in primary cultured rat hepatocytes. Drug Metab Dispos 2003;31:1260–8.
[28] Pearce MJ, McIntyre TM, Prescott SM, Zimmerman GA, Whatley RE. Shear stress activates cytosolic phospholipase A2 (cPLA2) and MAP kinase in human endothelial cells. Biochem Biophys Res Commun 1996;218:500–4.
[29] Schwachtgen JL, Houston P, Campbell C, Sukhatme V, Braddock M. Fluid shear stress activation of egr-1 transcription in cultured human endothelial and epithelial cells is mediated via the extracellular signal-related kinase 1/2 mitogen-activated protein kinase pathway. J Clin Invest 1998;101:2540–9.
[30] Yan C, Takahashi M, Okuda M, Lee JD, Berk BC. Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. J Biol Chem 1999;274:143–50.
[31] Go YM, Patel RP, Maland MC, Park H, Beckman JS, Darley-Usmar VM, et al. Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of c-Jun NH(2)-terminal kinase. Am J Physiol 1999;277:1647–53.
[32] Jo H, Sipos K, Go YM, Law R, Rong J, McDonald JM. Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in endothelial cells. Gi2- and Gbeta/gamma-dependent signaling pathways. J Biol Chem 1997;272:1395–401.
[33] Azuma N, Akasaka N, Kito H, Ikeda M, Gahtan V, Sasajima T, et al. Role of p38 MAP kinase in endothelial cell alignment induced by fluid shear stress. Am J Physiol Heart Circ Physiol 2001;280:H189–97.
[34] Illi B, Nanni S, Scopece A, Farsetti A, Biglioli P, Capogrossi MC, et al. Shear stress-mediated chromatin remodeling provides molecular basis for flow-dependent regulation of gene expression. Circ Res 2003;93:155–61.
[35] Ni CW, Hsieh HJ, Chao YJ, Wang DL. Shear flow attenuates serum-induced STAT3 activation in endothelial cells. J Biol Chem 2003;278:19702–8.
[36] Braet F, De Zanger R, Baekeland M, Crabbe E, Van Der Smissen P, Wisse E. Structure and dynamics of the fenestrae-associated cytoskeleton of rat liver sinusoidal endothelial cells. Hepatology 1995;21:180–9.
[37] Tilles AW, Baskaran H, Roy P, Yarmush ML, Toner M. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. Biotechnol Bioeng 2001;73:379–89.
[38] Moriyama M, Makiyama I, Shiota M, Uesugi K, Kannan Y, Ohta M, et al. Decreased ureagenesis from alanine, but not from ammonia and glutamine, in the perfused rat liver after partial hepatectomy. Hepatology 1996;23:1584–90.
[39] Kurumiya Y, Nozawa K, Sakaguchi K, Nagino M, Nimura Y, Yoshida S. Differential suppression of liver-specific genes in regenerating rat liver induced by extended hepatectomy. J Hepatol 2000;32:636–44.
[40] Kubicek M, Pacher M, Abraham D, Podar K, Eulitz M, Baccarini M. Dephosphorylation of Ser-259 regulates Raf-1 membrane association. J Biol Chem 2002;277:7913–9.