Activation of p38MAPK by TGF-β in fetal rat hepatocytes requires radical oxygen production, but is dispensable for cell death

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Abstract We have previously found that transforming growth factor-β (TGF-β) induces an increase in radical oxygen species (ROS) production that mediates its apoptotic effects in fetal hepatocytes. In this paper we show that TGF-β activates p38 mitogen-activated protein kinase (p38MAPK) and ROS may be responsible for this activation. Activation of p38MAPK occurs late, coincident with the maximal production of ROS, it is inhibited by radical scavengers and it is accentuated by the presence of glutathione synthesis inhibitors. However, p38MAPK does not appear to be involved in any of the apoptotic events: loss of Bcl-x_L levels, cytochrome *c* release, cleavage of caspase substrates and loss of cell viability.

Key words: Transforming growth factor-β; Apoptosis; p38 mitogen-activated protein kinase; Hepatocyte; Radical oxygen species

1. Introduction

Transforming growth factor-β (TGF-β) plays an important role mediating hepatocyte apoptosis [1,2]. We have previously found that TGF-β induces an increase in radical oxygen species (ROS) production and a decrease in intracellular glutathione content [2,3], which precedes the loss of mitochondrial transmembrane potential ($\Delta\psi_m$), the release of cytochrome *c* and the activation of caspase-3 [4] in rat fetal hepatocytes. TGF-β treatment produces a decrease in both protein and mRNA levels of Bcl-x_L [4,5]. Presence of radical scavengers blocks the decrease in Bcl-x_L levels, $\Delta\psi_m$ collapse, cytochrome *c* release and activation of caspase-3 [4], which indicates that during the apoptosis mediated by TGF-β in fetal hepatocytes, ROS might be responsible for the decrease in Bcl-x_L mRNA levels and the mitochondrial-dependent cell death.

Members of the mitogen-activated protein (MAP) kinase family are involved in signal transduction of apoptosis as well as cell growth and differentiation [6]. c-Jun N-terminal kinases (JNKs) and p38MAP kinases (p38MAPKs) appear to be preferentially activated by cytotoxic stress (including oxidative stress) and by pro-inflammatory cytokines [6–8]. p38MAPKs have been implicated both as positive and negative regulators of cell survival [8] and different p38 isoforms seem to account for these opposite effects. In particular, p38MAPK activity plays a critical role in nitric oxide-mediated cell death in neurons by stimulating Bax translocation to the mitochondria [9] and contributes to the UV-induced apoptosis in keratinocytes by mediating the release of mitochondrial cytochrome *c* into the cytosol [10]. In rat fetal brown adipocytes, p38MAPK mediates tumor necrosis factor-α-induced growth inhibition and apoptosis [11]. Bone morphogenetic protein 2, a member of the TGF-β superfamily, induces apoptosis by activating TGF-activated kinase (TAK1) and subsequent phosphorylation of p38MAPK in mouse hybridoma MH60 cells [12]. In contrast, activation of p38MAPK protects primary neonatal rat cardiomyocytes from anisomycin-induced apoptosis [13] and activation of the transcription factor MEF2 by p38MAPK has been shown to be required for the survival of developing neurons [14].

p38MAPK pathway is activated by TGF-β in different cell types [15–19]. Although it appears to be established that p38MAPK could play a role in the TGF-β-induced chemotactic responses [15] and regulation of gene expression [16–19], nothing is known about its possible role in its apoptotic effects. In the current study we have used rat fetal hepatocytes in primary culture to determine how TGF-β1 activates p38MAPK and whether this activation is related or not to the oxidative stress and apoptosis induced by TGF-β1 in these cells.

2. Materials and methods

2.1. Isolation of fetal rat hepatocytes and culture

Hepatocytes from 20 day old fetal Wistar rats were isolated by collagenase disruption, plated in arginine free medium 199, supplemented with ornithine (200 μM), fetal calf serum (10%), penicillin (120 μg/ml) and streptomycin (100 μg/ml) and incubated as previously described [2].

2.2. Western blot analysis

To detect active p38MAPK, Western blot analysis was performed as previously described [11]. An anti-phospho-p38MAPK antibody from New England Biolabs, and an anti-p38MAPKα antibody from Santa Cruz were used. Bcl-x, Bid and albumin protein levels were analyzed as described [4], using an anti-Bcl-x polyclonal antibody from Santa Cruz, anti-Bid polyclonal antibody from R&D Systems and anti-albumin polyclonal antibody from Nordic Immunological Lab. To analyze cytochrome *c* release, mitochondria were isolated from cytosol by digitonin treatment as described [20]. Cytochrome *c*

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Abbreviations: MAPK, mitogen-activated protein kinase; TGF-β, transforming growth factor-β; ROS, radical oxygen species

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levels in the mitochondrial fraction were analyzed by Western blot [4] with a monoclonal antibody from Pharmingen.

2.3. MAPKAPK2 kinase assay

MAPKAPK2 kinase activity was assayed in immunocomplexes, prepared with an anti-MAPKAPK2 antibody from StressGen Biotechnologies Corp. and human recombinant Hsp27 protein (StressGen Biotechnologies Corp.) was used as substrate [11]. Phosphorylated Hsp27 was visualized by autoradiography.

2.4. Glutathione determination

Cellular glutathione was extracted and analyzed as described [2,3]. Using GSH as standard, glutathione content is expressed as pmol/μg protein and represented in figures as percentage with respect to control cells.

2.5. Analysis of caspase-3 and caspase-8 activities

Cells were lysed as previously described [5]. For caspase-3 activity, reaction mixture contained 20 mM HEPES pH 7.0, 10% glycerol, 2 mM dithiothreitol (DTT), 30 μg protein/condition and 20 μM Ac-DEVD-AMC substrate. Reaction mixture for caspase-8 activity contained 20 mM PIPES pH 7.2, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (w/v) CHAPS, 10% sucrose, 70 μg protein/condition and 22.6 μM Ac-IETD-AFC substrate. After 2 h of incubation in the dark, enzymatic activity was measured in a Microplate Fluorescence Reader FL600 (Bio-Tek). A unit of caspase activity is defined as the amount of active enzyme necessary to produce an increase of one arbitrary unit in the luminescence spectrophotometer after 2 h. Protein concentration of cell lysates was determined by the Bio-Rad protein assay kit.

2.6. Cytotoxicity assay

Cells were washed twice with phosphate-buffered saline and the remaining viable adherent cells were stained with crystal violet as described [3]. Results are expressed as percentage of absorbance with respect to control, untreated cells.

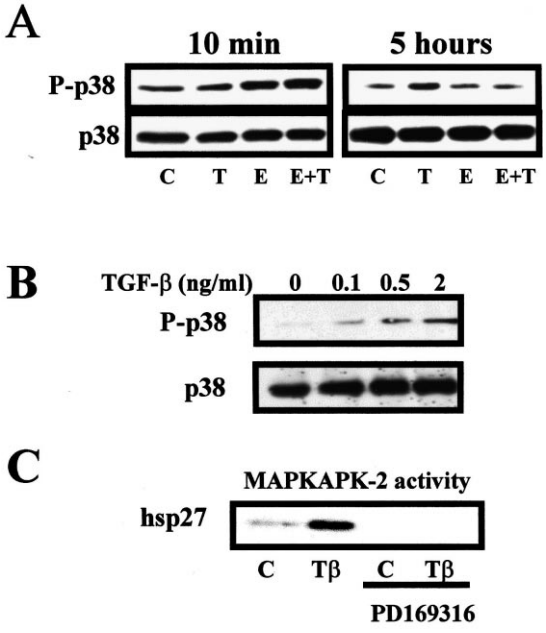

Fig. 1. TGF-β activates p38MAPK in fetal rat hepatocytes. A: Fetal hepatocytes were incubated for 10 min or 5 h in the absence (C) or in the presence of 2 ng/ml TGF-β (T), 20 ng/ml EGF (E) or both (E+T). After this time, proteins were extracted and analyzed by Western blot using antibodies against the active form (P-p38) and total form (p38). B: Dose–response effect of TGF-β on p38MAPK activation analyzed by Western blot after incubation for 5 h of fetal hepatocytes with different concentrations of TGF-β. C: MAPKAPK2 activity in immunocomplexes and inhibition by PD169316 (400 nM). PD169316 was added 1 h before TGF-β. In all cases, a representative experiment of three is shown.

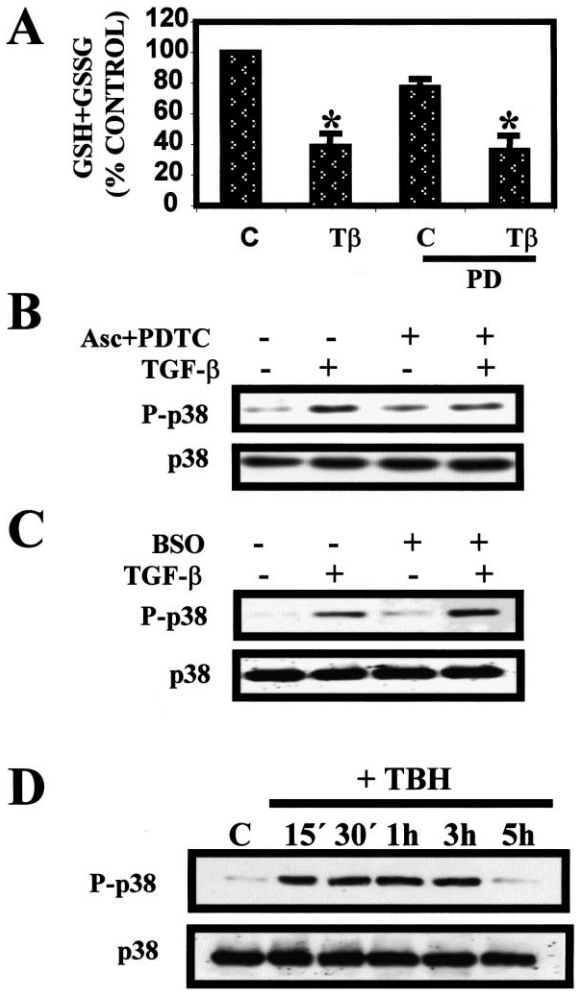

Fig. 2. Involvement of ROS in the activation of p38MAPK by TGF-β. A: Cells were treated for 8 h without or with 2 ng/ml TGF-β and without or with PD169316 (400 nM). After this time, cells were collected and cellular glutathione was analyzed. Results are expressed as % of control and are means ± S.E.M. of duplicate dishes from three independent experiments. Data were compared versus untreated cells by Student's test; $*P < 0.05$. B,C: Activation of p38MAPK was analyzed by Western blot with 30 μg protein from fetal hepatocytes incubated for 5 h without or with 2 ng/ml TGF-β. B: Effect of the presence of radical scavengers (1 mM ascorbate+50 μM PDTC). A representative experiment of four is shown. C: Effect of the presence of an inhibitor of glutathione synthesis (1 mM BSO). A representative experiment of two is shown. D: Effect of 0.25 mM TBH on p38MAPK activation in fetal hepatocytes. A representative experiment of two is shown.

3. Results

3.1. TGF-β activates p38MAPK in fetal rat hepatocytes

First, we studied whether TGF-β activates p38MAPK in fetal rat hepatocytes. We initially used high concentrations of this factor (2 ng/ml) to observe maximal effects. p38MAPK is activated after incubation of fetal hepatocytes with TGF-β for 5 h, but not at shorter times (Fig. 1A). We have previously described that epidermal growth factor (EGF) is a potent mitogen in fetal hepatocytes, which counteracts the apoptotic response of TGF-β [21]. EGF activated p38MAPK at short times (10–30 min) either in the absence or in the presence of TGF-β. However, this activation was transient, since at longer times, EGF had not any effect. Interestingly,

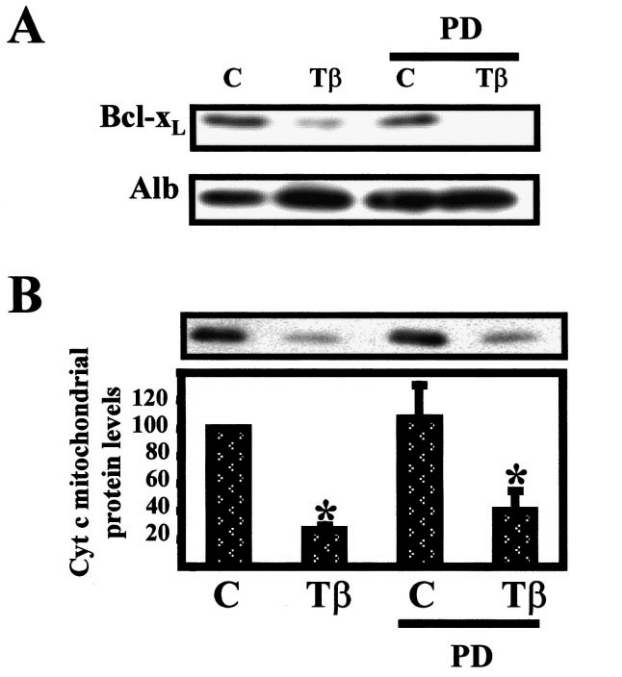

Fig. 3. Role of p38MAPK on Bcl-x_L down-regulation and cytochrome *c* release induced by TGF-β in fetal hepatocytes. A: After 15 h incubation of fetal hepatocytes without or with 2 ng/ml TGF-β and without or with PD169316 (400 nM), proteins were extracted and Bcl-x_L levels were analyzed by Western blot. Albumin content was analyzed as control. A representative experiment of three is shown. B: Cells were incubated for 15 h under the same conditions described in A. After this time, mitochondria were separated from cytosol and cytochrome *c* content was analyzed by Western blot. Bars represent the densitometric analysis of three independent experiments and are expressed as % control (mean ± S.E.M.). Data were compared versus untreated cells by Student's test; $^*P < 0.05$.

EGF blocked the p38MAPK activation induced by TGF-β at 5 h, which could suggest that p38MAPK is not activated under conditions where the apoptotic program is impaired.

Activation of p38MAPK was dose-dependent, reaching a maximum at 2 ng/ml TGF-β (Fig. 1B). This dose–response correlates with the oxidative stress process and apoptosis induced by this factor [2,4], but not with its ability to inhibit growth, which occurs at lower concentrations [22].

Finally, we confirmed these results in a MAPKAPK2 assay. MAPKAPK2, one of the p38MAPK downstream targets, was also activated by TGF-β at 5 h. This activation was blocked by pre-treatment with the specific p38MAPK inhibitors PD169316 (Fig. 1C) and SB203580 (results not shown). Since PD169316 did not inhibit other kinases, whereas SB203580 also inhibited JNK (results not shown), we selected PD169316 to be used in all the experiments described below.

3.2. Involvement of ROS in the activation of p38MAPK by TGF-β

Since TGF-β induces ROS production and glutathione depletion, which occurs after 3–6 h incubation [2–4], we decided to examine whether p38MAPK is involved in this oxidative stress. Fig. 2A shows that the presence of PD169316 had no significant effect on the decrease of glutathione levels induced by TGF-β. Therefore, p38MAPK is not necessary for the oxidative stress induced by TGF-β in fetal hepatocytes. We next decided to study whether the increase in ROS could be responsible for the activation of p38MAPK. We have reported that TGF-β-induced increase in peroxide content can be blocked by the presence of radical scavengers, such as pyrrolidine carbodithoic acid (PDTC) and ascorbic acid (ASC), which are also able to impair cell death [2,4]. Fig. 2B shows that the presence of PDTC+ASC prevented the activation of p38MAPK by TGF-β. Furthermore, in the presence of a prooxidant, such as BSO (DL-buthionine-(*S*,*R*)-sulfoximine), an inhibitor of glutathione synthesis that we previously used to decrease glutathione content in fetal hepatocytes [3], the activation of p38MAPK was more accentuated (Fig. 2C). These results indicate that the oxidative stress induced by TGF-β could be ruling the activation of p38MAPK. We next examined whether exogenous peroxide (*tert*-butyl-hydroperoxide, TBH) would be able to activate p38MAPK. As it is shown in Fig. 2D, 0.25 mM TBH alone produced an activation of p38MAPK. These results indicate that ROS production could be responsible for the activation of p38MAPK by TGF-β in fetal hepatocytes.

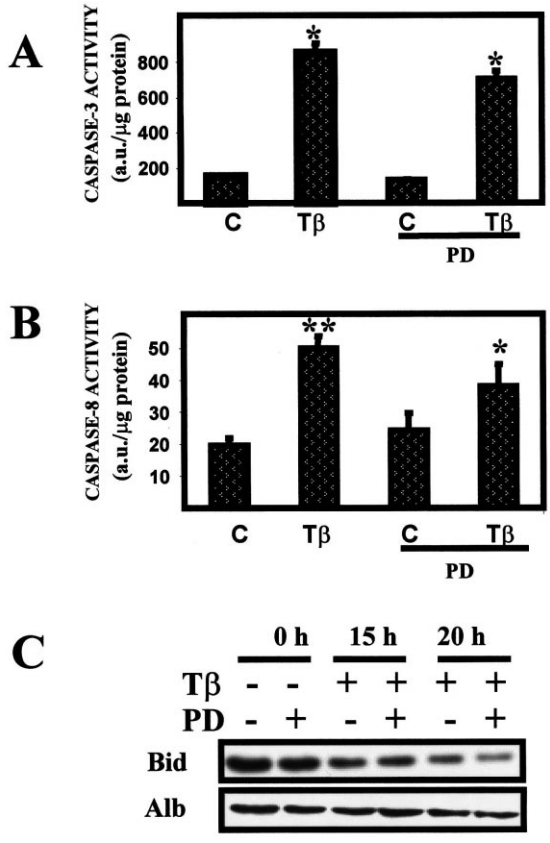

Fig. 4. Role of p38MAPK on TGF-β-induced caspase activation. A,B: Fetal hepatocytes were treated for 15 h without or with 2 ng/ml TGF-β and without or with PD169316 (400 nM), cells were lysed and caspase-3 (A) and caspase-8 (B) assayed. Results are expressed as units/μg protein and are mean ± S.E.M. of three independent experiments with duplicate dishes. Data were compared versus untreated cells by Student's test; $^*P < 0.01$; $^{**}P < 0.001$. C: Cells were incubated for 15 h or 20 h under the same conditions described in A. After this time, total proteins were extracted and the levels of Bid analyzed by Western blot. Albumin content was assayed as control. A representative experiment of three is shown.

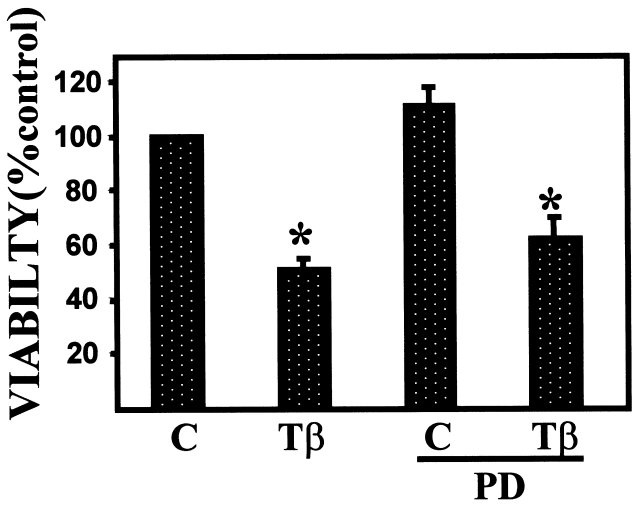

Fig. 5. p38MAPK is dispensable for TGF-β-induced cell death. After 24 h incubation of fetal hepatocytes without or with 2 ng/ml TGF-β and without or with PD169316 (400 nM), cell viability was analyzed. Results are expressed as % of control, untreated cells. The means ± S.E.M. of triplicate dishes from five independent experiments are shown. Data were compared versus untreated cells by Student's test; $*P < 0.001$.

3.3. p38MAPK is dispensable for the regulation of Bcl-x_L levels and cytochrome c release induced by TGF-β

We next decided to analyze whether p38MAPK is involved in the regulation of Bcl-x_L expression. Fig. 3A shows that Bcl-x_L levels decreased in response to TGF-β, regardless the presence of the p38MAPK inhibitor. To know if p38MAPK could mediate other processes, independently on Bcl-x_L, which activate cytochrome *c* release from mitochondria, we next studied the cytochrome *c* mitochondrial content. As shown in Fig. 3B, the p38MAPK inhibitor did not affect the mitochondrial depletion of cytochrome *c* induced by TGF-β in fetal hepatocytes.

3.4. p38MAPK is dispensable for caspase activation and loss of cell viability induced by TGF-β

Finally, we analyzed whether p38MAPK is involved in the activation of caspase-8 and/or caspase-3 by a mitochondria-independent mechanism. TGF-β activated caspase-3 (4-fold control values) and caspase-8 (2.5-fold control values) (Fig. 4). The presence of PD169316 slightly attenuated the activation in both cases, with a higher effect on caspase-8. In order to know the relevance of this effect on the cleavage of caspase-8 substrates, we analyzed the levels of Bid, which has been described as an important substrate of caspase-8 [23,24]. Bid levels considerably decreased after incubation with TGF-β, regardless the presence of the p38MAPK inhibitor (Fig. 4C).

In agreement with the results presented in Figs. 3 and 4, p38MAPK inhibition was not able to rescue fetal hepatocytes from the cell death induced by TGF-β (Fig. 5).

4. Discussion

The TGF-β family of polypeptide factors is capable of regulating several cellular processes, including proliferation, lineage determination, differentiation, motility, adhesion and death [25]. Some of the TGF-β actions may be mediated by oxidative stress. This cytokine activates an H_2O_2-generating NADH oxidase [26] and down-regulates the expression of antioxidative enzymes [27], which increase ROS intracellular content [2,27–30]. ROS appear to be involved in the apoptosis mediated by this factor [2–4,29] and in some of its transcriptional effects [28,30]. However, the mechanisms by which ROS mediate these effects are not completely understood.

The p38 subfamily of MAPKs was first associated with stress responses but it can mediate different cellular functions, such as growth, differentiation and apoptosis [6–8]. Results presented in this paper indicate that TGF-β activates p38MAPK (Fig. 1) and ROS may be responsible for this activation (Fig. 2). Firstly, activation of p38MAPK occurs late, coincident with the maximal production of ROS [2,4]. Secondly, the presence of radical scavengers (such as ASC and PDTC) blocks this activation and the presence of glutathione synthesis inhibitors, such as BSO, accentuates the effect. Finally, the incubation of fetal hepatocytes in the presence of TBH alone produces an early activation of p38MAPK. Since ROS are responsible for the down-regulation of Bcl-x_L expression, mitochondrial collapse and cell death induced by TGF-β in fetal hepatocytes [2–4], it seemed attractive to hypothesize that p38MAPK would mediate the response to ROS in terms of apoptosis. However, we have not observed any involvement of p38MAPK on any of the mitochondrial-dependent apoptotic events (Fig. 3B). Furthermore, although the p38MAPK inhibitor slightly attenuates caspase-8 activity, neither the cleavage of Bid, nor the loss of cell viability is affected by the p38MAPK inhibitor (Figs. 4 and 5).

p38MAPKs have been proposed as regulators of apoptosis [9–14]. However, it is worthy to note that, although there are more and more evidences for the involvement of oxidative stress in the activation of p38MAPKs [31–33], it is not so clear that these kinases mediate the cytotoxicity induced by ROS. Treatment of HeLa cells with H_2O_2 resulted in sustained activation of p38MAPK, but inhibition of this pathway had no effect on cell survival [31]; activation of p38MAPK by chromium(VI) is mediated through oxidative stress but does not affect cytotoxicity [34]; and in U937 human lymphoid cells a low dose of H_2O_2 rapidly causes p38MAPK cascade activation, causing mitotic arrest, but lacking apoptosis induction. Zhuang et al. have recently proposed in human leukemia cells that the involvement of p38MAPK in the apoptosis induced by oxidative stress is dependent on the ROS produced [35]. Thus, although both singlet oxygen and hydrogen peroxide selectively stimulate the phosphorylation of p38, this MAPK mediates Bid cleavage and caspase-3 activation during the apoptosis induced by singlet oxygen but not by hydrogen peroxide [35].

Which could be the role of this ROS-mediated p38MAPK activation? It has recently been described that p38MAPK plays a critical role in arresting growth of hepatocytes in near-term fetal rats [36]. However, the possible role of p38MAPK mediating growth arrest by TGF-β in fetal hepatocytes appears improbable because p38MAPK is not activated by low concentrations of the cytokine (Fig. 1), enough to arrest growth but not to induce ROS production and apoptosis [22]. Furthermore, results in our laboratory indicate that TGF-β continues arresting growth of fetal hepatocytes in the presence of the p38MAPK inhibitor (results not shown). It has also been suggested that p38MAPK could play a role in the TGF-β-induced chemotactic responses [15] and regulation of gene expression [16–19]. Since ROS appear to be also in-

volved in some of these transcriptional effects [28,30], we could speculate that p38MAPK might mediate ROS regulation of gene expression by TGF-β. Further work will be necessary to confirm this hypothesis.

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References

[1] Oberhammer, F.A., Pavelk, A.M., Sharma, S., Tiefenbacher, R., Purchio, A.F., Bursch, W. and Schulte-Hermann, R. (1992) Proc. Natl. Acad. Sci. USA 89, 5408–5412.
[2] Sánchez, A., Álvarez, A.M., Benito, M. and Fabregat, I. (1996) J. Biol. Chem. 271, 7416–7422.
[3] Sánchez, A., Álvarez, A.M., Benito, M. and Fabregat, I. (1997) Hepatology 26, 935–943.
[4] Herrera, B., Álvarez, A.M., Sánchez, A., Fernández, M., Roncero, C., Benito, M. and Fabregat, I. (2001) FASEB J. 15, 741–751.
[5] Fabregat, I., Herrera, B., Fernández, M., Álvarez, A.M., Sánchez, A., Roncero, C., Ventura, J.J., Valverde, A.M. and Benito, M. (2000) Hepatology 32, 528–535.
[6] Chang, L. and Karin, M. (2001) Nature 410, 37–40.
[7] Rincon, M., Flavell, R.A. and Davis, R.A. (2000) Free Radic. Biol. Med. 28, 1328–1337.
[8] Nebreda, A.R. and Porras, A. (2000) Trends Biochem. Sci. 25, 257–260.
[9] Ghatan, S., Larner, S., Kinoshita, Y., Hetman, M., Patel, L., Xia, Z., Youle, R.J. and Morrison, R.S. (2000) J. Cell Biol. 150, 335–347.
[10] Assefa, Z., Vantiegem, A., Garmyn, M., Declercq, W., Van denabeele, P., Van denheede, J.R., Bouillon, R., Merlevede, W. and Agostini, P. (2000) J. Biol. Chem. 275, 21416–21421.
[11] Valladares, A., Álvarez, A.M., Ventura, J.J., Roncero, C., Benito, M. and Porras, A. (2000) Endocrinology 141, 4383–4395.
[12] Kimura, N., Matsuo, R., Shibuya, H., Nakashima, K. and Taga, T. (2000) J. Biol. Chem. 275, 17647–17652.
[13] Zechner, D., Craig, R., Hanford, D.S., McDonough, P.M., Sabadini, R.A. and Glembotski, C.C. (1998) J. Biol. Chem. 273, 8232–8239.
[14] Mao, Z., Bonni, A., Xia, F., Nadal-Vicens, M. and Greenberg, M.E. (1999) Science 286, 785–790.
[15] Hannigan, M., Zhan, L., Ai, Y. and Huang, C.-K. (1998) Biochem. Biophys. Res. Commun. 246, 55–58.
[16] Hanafusa, H., Ninomiya-Tsuji, J., Masuyama, N., Nishita, M., Fujisawa, J., Shibuya, H., Matsumoto, K. and Nishida, E. (1999) J. Biol. Chem. 274, 27161–27167.
[17] Ravanti, L., Hakkinen, L., Larjava, H., Saarialbo-Kere, U., Foschi, M., Han, J. and Kahari, V.M. (1999) J. Biol. Chem. 274, 37292–37300.
[18] Johansson, N., Ala-aho, R., Uitto, V., Grenman, R., Fusenig, N.E., Lopez-Otin, C. and Kahai, V.M. (2000) J. Cell Sci. 113, 227–235.
[19] Wagers, A.J. and Kansan, G.S. (2000) J. Immunol. 165, 5011–5016.
[20] Piqué, M., Barragan, M., Dalmau, M., Bellosillo, B., Pons, G. and Gil, J. (2000) FEBS Lett. 480, 193–196.
[21] Fabregat, I., Sánchez, A., Álvarez, A.M., Nakamura, T. and Benito, M. (1996) FEBS Lett. 384, 14–18.
[22] De Juan, C., Benito, M., Álvarez, A. and Fabregat, I. (1992) Exp. Cell Res. 202, 495–500.
[23] Luo, X., Budihardio, I., Zou, H., Slaughter, C. and Wang, X. (1998) Cell 94, 481–490.
[24] Li, H., Zhu, H., Xu, C. and Yuan, J. (1998) Cell 94, 491–501.
[25] Massague, J. and Chen, Y.G. (2000) Genes Dev. 14, 627–644.
[26] Thannickal, V.J. and Fanburg, B.L. (1996) J. Biol. Chem. 270, 30334–30338.
[27] Kayanoki, Y., Fujii, J., Suzuki, K., Kawata, S., Matsuzawa, Y. and Taniguchi, N. (1994) J. Biol. Chem. 269, 15488–15492.
[28] Ohba, M., Shibanuma, M., Kuroki, T. and Nose, K. (1994) J. Cell. Biol. 126, 1079–1088.
[29] Lafon, C., Mathieu, C., Guerrin, M., Pierre, O., Vidal, S. and Valette, A. (1996) Cell Growth Differ. 7, 1095–1104.
[30] García-Trevijano, E.R., Iraburu, M.J., Fontana, L., Domínguez-Rosales, J.A., Auster, A., Covarrubias-Pinedo, A. and Rojkind, M. (1999) Hepatology 29, 960–970.
[31] Wang, X., Martindale, J.L., Liu, Y. and Holbrook, N.J. (1998) Biochem. J. 333, 291–300.
[32] Gabbita, S.P., Robinson, K.A., Stewart, C.A., Floyd, R.A. and Hensley, K. (2000) Arch. Biochem. Biophys. 376, 1–13.
[33] Kurata, S. (2000) J. Biol. Chem. 275, 23413–23416.
[34] Chuang, S.M., Liou, G.Y. and Yang, J.L. (2000) Carcinogenesis 21, 1491–1500.
[35] Zhuang, S., Demirs, J.T. and Kochevar, I.E. (2000) J. Biol. Chem. 275, 25939–25948.
[36] Award, M.M., Enslen, H., Boylan, J.M., Davis, R.J. and Grupusso, P.A. (2000) J. Biol. Chem. 275, 38716–38721.