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ORIGINAL PAPER

Systematical analysis of impacts of heat stress on the proliferation, apoptosis and metabolism of mouse hepatocyte

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Abstract Heat stress will stimulate cells of living organisms to generate heat shock proteins (Hsps). In the mouse liver, impacts of heat stress on hepatocyte proliferation, apoptosis and metabolism have not been studied systematically at different temperatures. In this research, the test mice were heated to 40, 42, 44 and 46°C, respectively, for 20 min and recovered at room temperature for 8 h in normal feeding conditions; the control animals were kept at room temperature without heat stress. The expression levels of Hsp70, Pcna, Bax, Bcl2, cytochrome P450 1A2 (CYP1A2), CYP2E1 and analog of CYP3A4 (not reported in mouse before), the parameters reflecting stress strength, cell proliferation, apoptosis and metabolism, were detected by western blotting, immunohistochemistry and semi-quantitative RT-PCR in test and control mice. Haematoxylin–eosin (H&E) staining and TUNEL analysis were further used to study the impacts of heat stress at different temperatures on hepatocellular necrosis and apoptosis. Serum AST and ALT levels, the markers of liver injury, were measured after heat stress at different temperatures. The data show that Hsp70 expression was significantly increased when temperature increased ($P < 0.05$). At lower temperatures (40 or 42°C), expression of Pcna, CYP1A2 and analog of CYP3A4 were considerably increased ($P < 0.05$) while hepatocyte necrosis and apoptosis were not induced ($P > 0.05$). At higher temperatures (44 or 46°C), expression of Pcna was decreased while hepatocyte necrosis and apoptosis were induced ($P < 0.05$). Expressions of CYP1A2 and analog of CYP3A4 were decreased especially at 46°C ($P < 0.05$). Expression of CYP2E1 could not be detected to increase at 40°C but was at high levels at 42, 44 and 46°C ($P < 0.05$). Expressions of AST and ALT were not different between the test mice and control mice at 40°C while they were significantly higher in the test mice than those in the control mice at 42 ($P < 0.05$), 44 and 46°C ($P < 0.01$). In conclusion, heat stress at lower temperatures promotes hepatocyte proliferation and improves the metabolic efficiency in mouse liver while heat stress at higher temperatures inhibits hepatocyte proliferation, promotes hepatocyte apoptosis and induces hepatocyte necrosis. This may give a hint to understanding human liver injury in high temperatures. Moreover, it is the first time that the analog of CYP3A4 was detected in mouse hepatocellular cytoplasm. It is worthwhile to dissect its function in future work.

Keywords Heat stress · Cell proliferation · Apoptosis · Metabolism

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Introduction

The exposure to heat stress or other stressful stimuli activates a protective mechanism in cells of all living organisms, including a rapid, but transient induction of proteins called heat shock proteins (Hsps). These proteins serve to minimize

cellular damage produced by diverse environmental or physiological stressors [1]. Heat shock protein (HSP) induction, cell proliferation and apoptosis, and cell metabolism regulation are different distinct responses to heat stress. Heat stress can cause cell death if cellular defense mechanisms are insufficient to cope with the stress. This is particularly obvious when the temperature increases well above that of the normal environment and/or exposure time is prolonged. Heat stress and related illnesses are a major concern in the military, sports, and workers in hot environments. Heat stress results in physiologic responses of increased temperature, heart rate and sweating [2].

The liver, as a major site of metabolism and detoxification, is a system of choice in studies involving toxicoproteomics, metabolic disorder and stress effects due to various patho-biological processes [3]. Exploration of the underlying mechanism of the effect of heat stress on liver is a major concern to understanding the patho-physiology of heat stress-related illnesses. We wanted to systematically study the effects of heat stress at different temperature on proliferation, apoptosis and metabolism of hepatocytes in mice, which will help to explain the mechanism of effect of heat stress on the liver and the patho-physiology of heat stress-related illnesses.

Proliferating cell nuclear antigen (pcna) is a subunit of the mammalian DNA polymerase delta and is synthesized primarily during the S phase of the cell cycle [4]. Pcna is a relay or anchoring molecule that functions as a molecular integrator for proteins involved in the control of the cell cycle, DNA replication, DNA repair, and cell death [5, 6]. Pcna has been shown to be a good marker to distinguish proliferating cells [7, 8]. Cytochrome P450 (CYP) enzymes are a superfamily of *b*-type heme-containing proteins found in organisms from all domains of life [9]. They are one of the most important enzymes in the liver which are major catalysts in the oxidative transformation of a diversity of endogenous and exogenous compounds. Among them, CYP3A4, CYP1A2 and CYP2E1 are the most important CYPs in the liver of human. CYPlA2 principally participates in metabolizing chemicals and environmental toxins, whereas CYP3A4 is involved in metabolizing a large number of endogenous and exogenous compounds, and CYP2El exhibits polymorphism and is a toxicologically important enzyme.The activities of CYP450 and its isoforms can determine the response of a patient to drug therapy [9–11]. Pro-apoptotic (Bax) and anti-apoptotic (Bcl2) are closely associated with hepatocyte apoptosis. So these molecules were used to study the effects of heat stress on proliferation, apoptosis and metabolism of hepatocytes in mice.

Although heat stress and related illnesses have been studied [3], the effect of heat stress on the liver has not been systematically studied at a molecular level. The study presented here examined the altered gene and protein expressions in the liver of mice exposed to heat stress at different temperatures to observe the effects of heat stress on proliferation, apoptosis and metabolism of hepatocytes in the mice.

Materials and methods

Animals

BALB/c mice (approximately 6–8 weeks old, 22 ± 2 g) were purchased from the experimental animals center of Henan province and maintained in an air-conditioned animal room at 25°C with free access to water and food under 12 h light/dark cycles. All animals were allowed to adapt to the environment for 1 week before the experiment and fed on laboratory chow. All protocols conformed to National Institute of Health (NIH) guidelines and all animals received care in compliance with the Principles of Laboratory Animal Care.

Heat stress experiments

Our previous work showed that the maximum Hsp70 induction occurred at 8 h post-heat stress treatment at 40°C for 20 min. The mice were randomly divided into five groups. Every group had six mice including three males and three females. The whole bodies of mice in groups one to four were respectively heated to 40, 42, 44 and 46°C for 20 min with 60% relative humidity in a ventilated and humidified chamber. The animals were then allowed to recover at room temperature for 8 h in normal feeding conditions. The control (unstressed) animals in group five were kept at room temperature and sacrificed along with their counterparts. Livers were collected and rinsed in phosphate-buffered saline (PBS) and then immediately frozen in liquid nitrogen or fixed overnight in 10% neutral buffered formalin for subsequent analysis.

Western blotting

Protein samples of 70 μg from the mice in different groups were adjusted to the composition of the electrophoresis sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 5% beta-mercaptoethanol, 2% SDS, 0.1% bromphenol blue) and boiled for 5 min prior to analysis. SDS-PAGE (10% polyacrylamide gels) in 1 mm slab gel was performed as described by Sambrook and Russell [12]. The proteins were transferred from the gel to the nitrocellulose membranes. Then, the membrane was probed with a monoclonal antibody to mouse Hsp70, Pcna, Bcl2, Bax (Santa Cruz) or a polyclonal antibody to mouse CYP1A2 or CYP2E1

(Santa Cruz), and the signal was detected by horseradish peroxidase detection system using DAB (Sigma). CYP3A4 is generally the most abundant CYP form in the human liver; however, it has not been reported that CYP3A4 exists in the liver of mouse [13]. We wanted to detect whether there is an analog of CYP3A4 in the liver of mouse and study the effects of heat stress on the analog of CYP3A4 in the hepatocytes of mouse. So western blotting was also performed using a rabbit polyclonal antibody to human CYP3A4 (Santa Cruz; product number: sc-53850) using the method mentioned above. Protein bands were quantified with Gel Pro Analyzer software 4.0 (Media Cybernetics, Bethesda, MD, USA) and the intensities of the bands were normalized against beta-actin. Every experiment was repeated three times.

Semi-quantitative RT-PCR

To determine the effects of heat stress on proliferation, apoptosis and metabolism of hepatocytes at gene level, semi-quantitative RT-PCR was conducted. The specific oligonucleotide primers are shown in Table 1. RT-PCR were performed and repeated at least three times on cDNA. Briefly, cDNA was synthesized from 1 μg of RNA in the presence of ribonuclease inhibitor (BBI, Toronto, Canada), dNTPs, Oligo(dT) 18 primers, and RevertAidTM M-Mulv reverse transcriptase (Fermentas, MD, USA) in a total of 25 μL reaction mix. RT-PCR was performed using a TaKaRa mRNA Selective PCR kit (TaKaRa, Japan). The PCR products were visualized by ultraviolet illumination after electrophoresis for 30 min at 100 V through 1.5% agarose gels (COSMO BIO, Tokyo, Japan) and staining in Trisborate/EDTA buffer containing 0.5 mg/ml of ethidium bromide. The signal intensity of the bands with the expected size was measured using Gel Pro Analyzer software 4.0 software. The intensities of the bands were normalized against *beta-actin*. Experiments in the absence of reverse transcriptase were conducted as negative controls.

Necrosis and apoptosis analysis

Samples of liver were fixed in 10% formaldehyde for 24 h and then dehydrated and embedded in paraffin. Six-micrometer-thick sections were cut from each paraffin-embedded tissue and stained with H&E to evaluate the degree of necrosis in the liver of mice exposed to heat stress at different temperature and in the control mice. Apoptosis in the hepatocytes of mice exposed to heat stress at different temperature was further analyzed using a commercial kit (GENMED, Shanghai, China) based on the TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) of apoptotic cells. Sections were examined microscopically for specific staining and photographs were taken using a digital image-capture system (Olympus, Tokyo, Japan).

Immunohistochemistry detection

Six-micrometer-thick sections were cut from each paraffin-embedded tissue as prepared above. The sections of liver from the mice exposed to heat stress at different temperatures and the control mice were immunostained with a monoclonal antibody to mouse Hsp70, Pcna, a polyclonal antibody to mouse CYP1A2 or CYP2E1, and a polyclonal antibody to human CYP3A4 (dilution 1:300) as described

Table 1 Oligonucleotide primers used in RT-PCR

Gene name	GenBank Accession No.	Forward primer	Reverse primer	Expected size (bp)
Heat shock protein 70 (Hsp70)	NM_031165	5′-AAATCATCAGCTGGCTGGATAAGAA	5′-CATGCCACCTGCACTCTGGTA	256
Proliferating cell nuclear antigen (Pcna)	NM_011045	5′-GTAGTTGTCGCTGTAGGC	5′-GTTGCTCCACATCTAAGTC	492
B-cell leukemia/ lymphoma 2 (Bcl2)	NM_177410	5′-CCGCCTCTTCACCTTTCA	5′-GGTTATCATACCCTGTTCTC	348
Bcl2-associated X protein (Bax)	NM_007527	5′-CCAGGATGCGTCCACCAA	5′-AAAGTAGAAGAGGGCAACCAC	196
Cytochrome P450, family 1, subfamily a, polypeptide 2 (Cyp1a2)	NM_009993	5′-CATCGGCTCCACTCCTGT	5′-GCATCTCCTCGCTCTTCC	413
Cytochrome P450, family 2, subfamily e, polypeptide 1 (Cyp2e1)	NM_021282	5′-CCACCCTCCTCCTCGTAT	5′-CTTGACAGCCTTGTAGCC	218
Beta-actin (Actb)	NM_007393	5′-CTGTCCCTGTATGCCTCTG	5′-ATGTCACGCACGATTTCC	218

by Xu et al. [8]. The signal was detected using the Polink-2 plus polymer HRP detection system (Zhongshan, Beijing, China) using DAB. A negative control was carried out on each slide by omitting the primary antibody. Sections were examined microscopically for specific staining and photographs were taken using a digital image-capture system (Olympus).

Determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities

Serum AST and ALT levels in the heat-stressed mice and control mice were determined with a commercial assay kit (Nanjing Jiancheng Biological Technology, China). Enzyme activities were expressed as an international unit per liter (IU/L).

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Comparisons between groups were performed using independent Student's *t* test and SPSS software 13.0.

Results

Western blot of Hsp70, Pcna, Bax, Bcl2, CYP1A2, CYP2E1 and analog of CYP3A4 expression in the liver of heat-stressed mice and control mice

The expression of Hsp70, Pcna, Bcl2, Bax, CYP1A2, CYP2E1 and analog of CYP3A4 were analyzed by western blot analysis (Fig. 1). As shown in Fig. 1b, Hsp70 level was considerably increased with increasing temperature. The level of Hsp70 at 40°C heat stress was 2.07-fold of the control ($P < 0.05$). The maximal increase was noticed at 46°C heat stress, when the level of Hsp70 was 5.26-fold higher than the controls ($P < 0.01$). Figure 1c shows that heat stress significantly induced the expression of Pcna at 40°C ($P < 0.05$) and 42°C ($P < 0.01$). The expression of Pcna was down-regulated after heat stress at 44 and 46°C when compared with unstressed controls ($P < 0.05$). Bax expression showed no obvious difference after 40 and 42°C heat stress ($P > 0.05$), but significantly increased at 44 and 46°C after heat shock treatment when compared with controls ($P < 0.01$) (Fig. 1d). Bcl2 expression was notably increased after heat exposure at 40°C ($P < 0.05$), but significantly decreased after heat stress at 42, 44 and 46°C when compared with the controls ($P < 0.05$) (Fig. 1e). Figure 1f shows that heat stress significantly induced the expression of CYP1A2 after heat stress at 40, 42 ($P < 0.01$) and 44°C ($P < 0.05$) compared with the controls, but CYP1A2 expression at 40°C was much higher than that at 42 and 44°C after heat stress ($P < 0.05$). Heat stress considerably inhibited the expression of CYP1A2 after heat shock treatment at 46°C compared with the controls ($P < 0.05$). CYP2E1 expression was significantly up-regulated after 42 ($P < 0.05$), 44 and 46°C ($P < 0.01$) heat stress, but showed no obvious difference after 40°C heat stress ($P > 0.05$) in comparison with the controls (Fig. 1g). In addition, the analog of CYP3A4 was found to exist in the hepatocytes of mice (Fig. 1a). The analog of CYP3A4 expression was significantly up-regulated after 40°C heat stress ($P < 0.01$) and 42°C heat stress ($P < 0.05$), but significantly decreased after heat stress at 46°C when compared with the controls ($P < 0.05$) (Fig. 1h).

The effect of heat stress on Hsp70, Pcna, Bax, Bcl2, CYP1A2 and CYP2E1 genes expression in the liver of mice

In order to assess the effect of heat stress on the mRNA expression of *Hsp70, Pcna, Bax, Bcl2, CYP1A2* and *CYP2E1*, semi-quantitative RT-PCR was performed (Fig. 2). Figure 2b shows that heat treatment caused a great increase in *Hsp70* mRNA with the temperature increasing in comparison with the controls (40 and 42°C, $P < 0.05$; 44 and 46°C, $P < 0.01$), which was consistent with the results of protein level. The expression of *Pcna* mRNA was rapidly and strongly increased after heat stress at 40 and 42°C ($P < 0.01$) and considerably decreased after heat stress at 44 and 46°C ($P < 0.05$) compared with the controls (Fig. 2c). Heat stress had a different effect on the expression of *Bax* and *Bcl2* mRNA. Low temperature of heat stress such as 40 and 42°C could induce the expression of *Bcl2* mRNA ($P < 0.05$), while high temperature of heat stress such as 44 and 46°C could induce the expression of *Bax* mRNA ($P < 0.01$) when compared with the control mice (Fig. 2d, e). Figure 2f indicates that 40, 42 and 44°C heat treatment could significantly induce *CYP1A2* mRNA expression ($P < 0.01$), but 46°C heat treatment inhibited *CYP1A2* mRNA expression ($P < 0.05$) compared with the controls. *CYP2E1* mRNA expression was strongly increased after 42, 44 and 46°C heat stress ($P < 0.05$), but 40°C heat stress did not significantly affect the *CYP2E1* mRNA expression ($P > 0.05$) in comparison with the control mice (Fig. 2g). These results nearly accorded with the results of western blot.

Necrosis examination by HE staining

Figure 3 shows that 40 and 42°C heat shock did not significantly induce hepatocyte necrosis ($P > 0.05$) while 44 ($P < 0.05$) and 46°C ($P < 0.01$) heat shock considerably induced hepatocyte necrosis in the mice when compared with the controls.

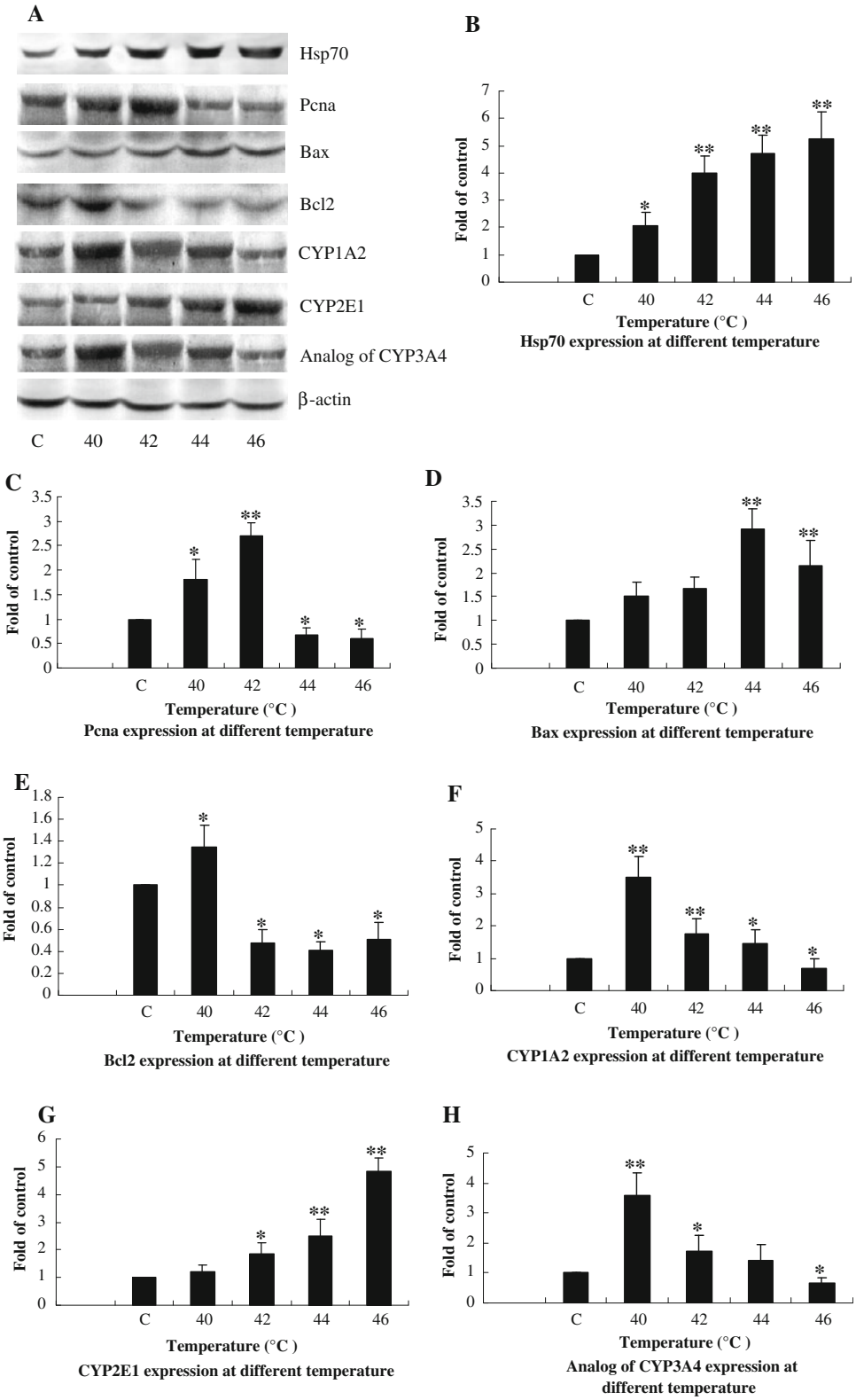

Fig. 1 **a** Western blots of Hsp70, Pcna, Bax, Bcl2, CYP1A2, CYP2E1 and analog of CYP3A4 in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative scan obtained by PhosphorImaging is shown. *C* unstressed control mice. Data obtained after quantification of immunoreactive bands by Gel Pro Analyzer software 4.0. The *values* represent the mean ± SEM from individual animals presented on the above blot. The intensities of the bands were normalized against the loading control of beta-actin. Fold increase of Hsp70 (**b**), Pcna (**c**), Bax (**d**), Bcl2 (**e**), CYP1A2 (**f**), CYP2E1 (**g**) and analog of CYP3A4 (**h**) in the liver of mice after heat stress at different temperature was compared with the control (the liver of unstressed mice). Significance: $*P < 0.05$, $**P < 0.01$ compared with controls

TUNEL detection

TUNEL detection showed that heat stress of high temperature such as 44 and 46°C significantly induced the hepatocytes apoptosis and extensive DNA fragmentation appeared in the liver of mice compared with the control mice ($P < 0.01$) (Fig. 4d–f). Only a few apoptotic cells appeared in the liver of unstressed control mice and 40°C

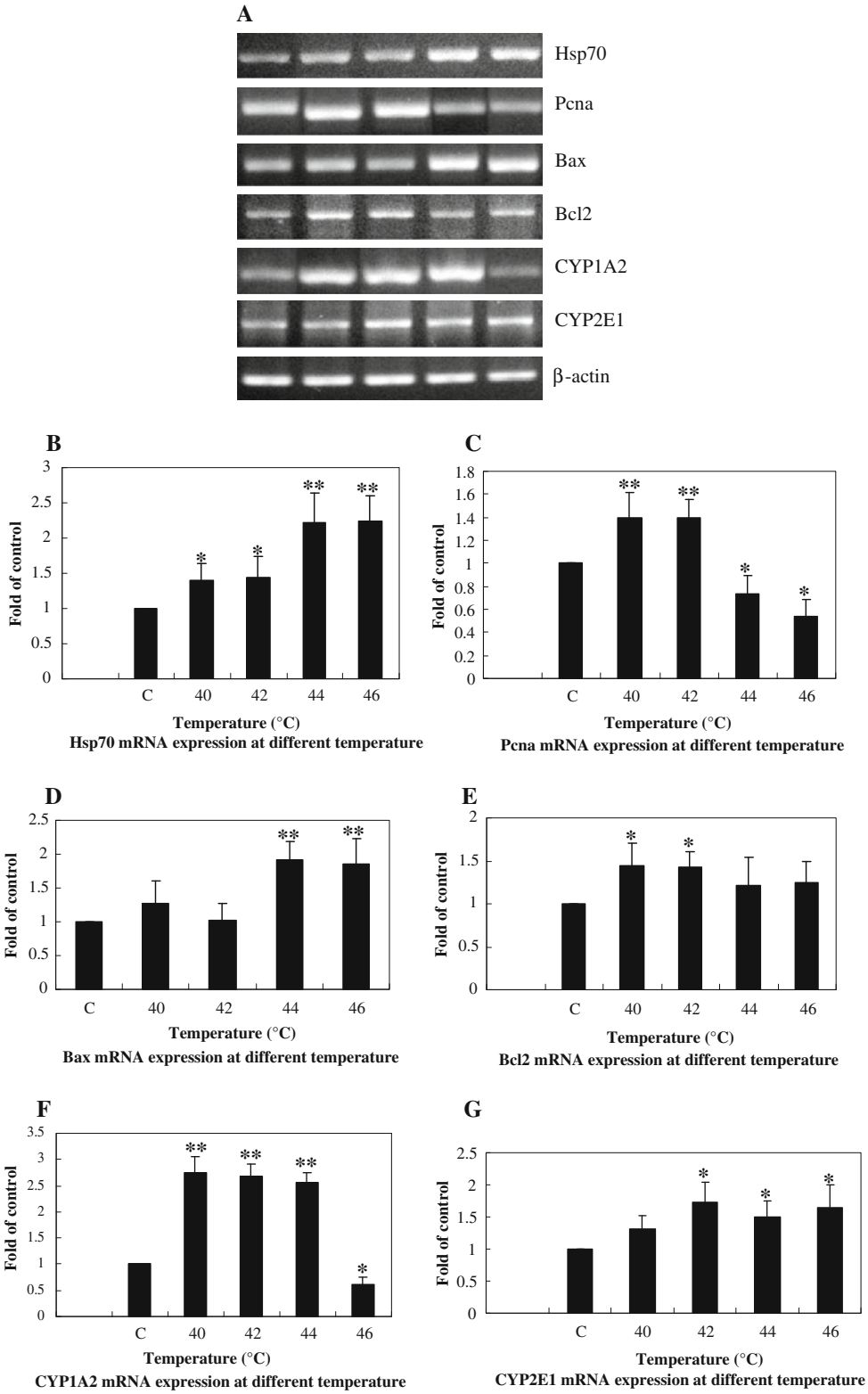

Fig. 2 **a** Semi-quantitative RT-PCR of Hsp70, Pcna, Bax, Bcl2, CYP1A2 and CYP2E1 in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative scan obtained by PhosphorImaging is shown. *C* unstressed control mice. Data obtained after quantification of amplification bands by Gel Pro Analyzer software 4.0. The values represent the mean ± SEM from individual animals presented on the above image. The intensities of the bands were normalized against the loading control of beta-actin. Fold increase of Hsp70 (**b**), Pcna (**c**), Bax (**d**), Bcl2 (**e**), CYP1A2 (**f**) and CYP2E1 (**g**) in the liver of mice after heat stress at different temperature was compared with the controls (the liver of unstressed mice). Significance: $*P < 0.05$, $**P < 0.01$ compared with controls

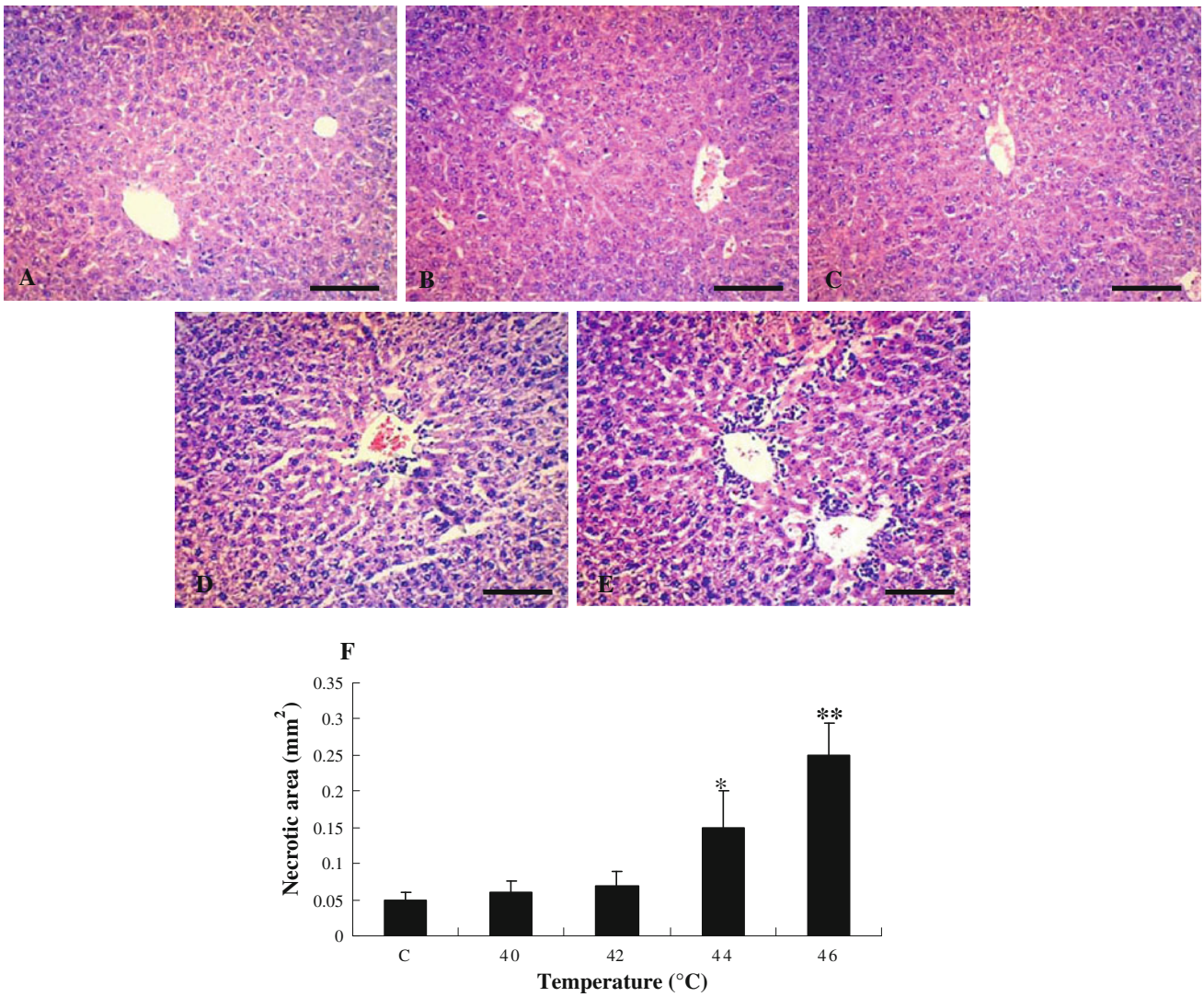

Fig. 3 Histologic examination of liver injury in the control mice (**a**) and the mice exposed to 40 (**b**), 42 (**c**), 44 (**d**), and 46°C (**e**) whole body heat stress by H&E staining. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. *C* unstressed controls. **f** Necrotic areas. Representative findings from at least 10-mm^2 tissue sections were counted for each mouse. Significance: $*P < 0.05$, $**P < 0.01$ when compared with controls. *Scale bar* 50 μm

and 42°C stressed mice (Fig. 4a–c, f), which is consistent with the normal cell cycle events.

Detection of Hsp70, Pcna, CYP1A2, CYP2E1 and analog of CYP3A4 expression in the liver of mice after heat shock treatment by immunohistochemistry

Figure 5 shows that Hsp70 was mostly expressed in the cytoplasm of hepatocytes of mice. The expression of Hsp70 was significantly increased in the hepatocytes of mice with increasing heat stress temperature when compared with the control mice ($P < 0.01$). The results were consistent with the western blot results (Fig. 1b). Pcna was mainly expressed in the nucleus of hepatocytes and was found significantly increased at 40 ($P < 0.05$) and 42°C ($P < 0.01$) heat stress but tremendously decreased at 44 and 46°C ($P < 0.05$) heat stress in the hepatocytes of mice (Fig. 6) The results were also consistent with the western blot results (Fig. 1c). CYP1A2 and CYP2E1 were mainly expressed in the cytoplasm of hepatocytes (Figs. 7 and 8). Heat stress of low temperatures such as 40 and 42°C could considerably induce the expression of CYP1A2 ($P < 0.01$) while heat stress of high temperature such as 46°C significantly inhibited the expression of CYP1A2 ($P < 0.05$) in the liver of mice (Fig. 7). CYP2E1 was significantly up-regulated in the hepatocytes of mice with the heat stress temperature increasing from 42 to 46°C compared with the control mice ($P < 0.01$), but heat stress of 40°C did not significantly induce CYP2E1 expression ($P > 0.05$) (Fig. 8). Figure 9 proves that analog of CYP3A4 really exists in the hepatocellular cytoplasm of mice. The analog

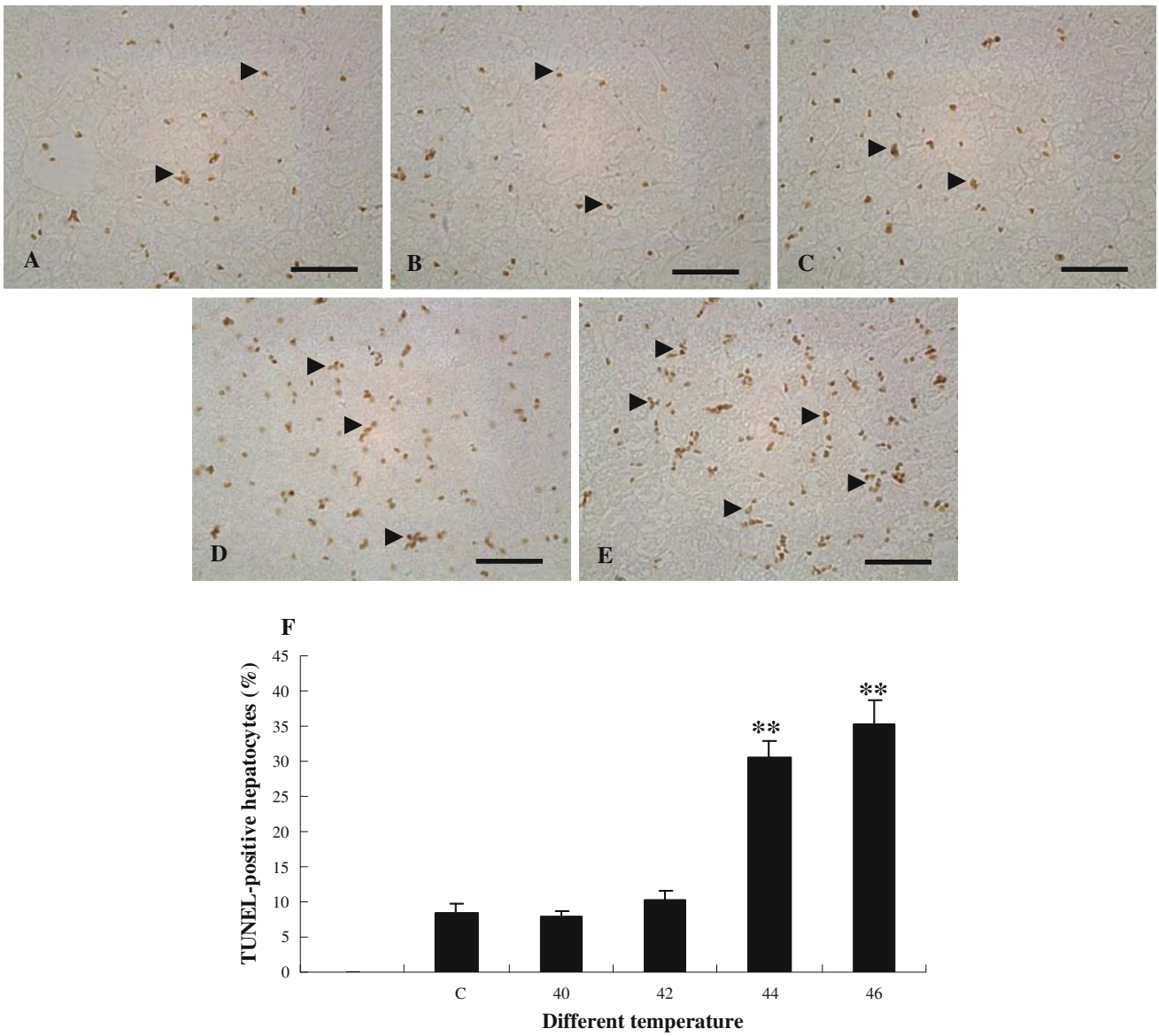

Fig. 4 Photomicrographs of results of TUNEL assay of liver sections prepared from the control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. The *arrowheads* indicate the position of the apoptotic cells in the liver of unstressed control mice (**a**), 40°C stressed mice (**b**), 42°C stressed mice (**c**), 44°C stressed mice (**d**) and 46°C stressed mice (**e**). **f** Percentages of TUNEL-positive cells among total hepatocytes; at least 12-mm^2 tissue sections were counted for each mouse. *C* unstressed controls. Significance: $^{**}P < 0.01$ compared with controls. *Scale bar* 50 μm

expression of CYP3A4 was up-regulated after heat stress at 40 and 42°C ($P < 0.01$) but down-regulated after heat stress at 46°C ($P < 0.05$).

Alterations of serum AST and ALT level in the mice after heat stress at different temperature

Serum AST and ALT activities were used as markers of liver injury. Figure 10 shows the changes of serum AST and ALT levels in the mice after heat stress at different temperature. There were no significant differences between 40°C heat-stressed mice and the control mice in the levels of AST and ALT ($P > 0.05$). But the AST and ALT levels of 42°C ($P < 0.05$), 44 and 46°C ($P < 0.01$) heat-stressed mice were significantly higher than those in the control mice.

Discussion

This study examined the effect of heat stress on the expression of genes and proteins related to proliferation, apoptosis and metabolism in the hepatocytes of mice.

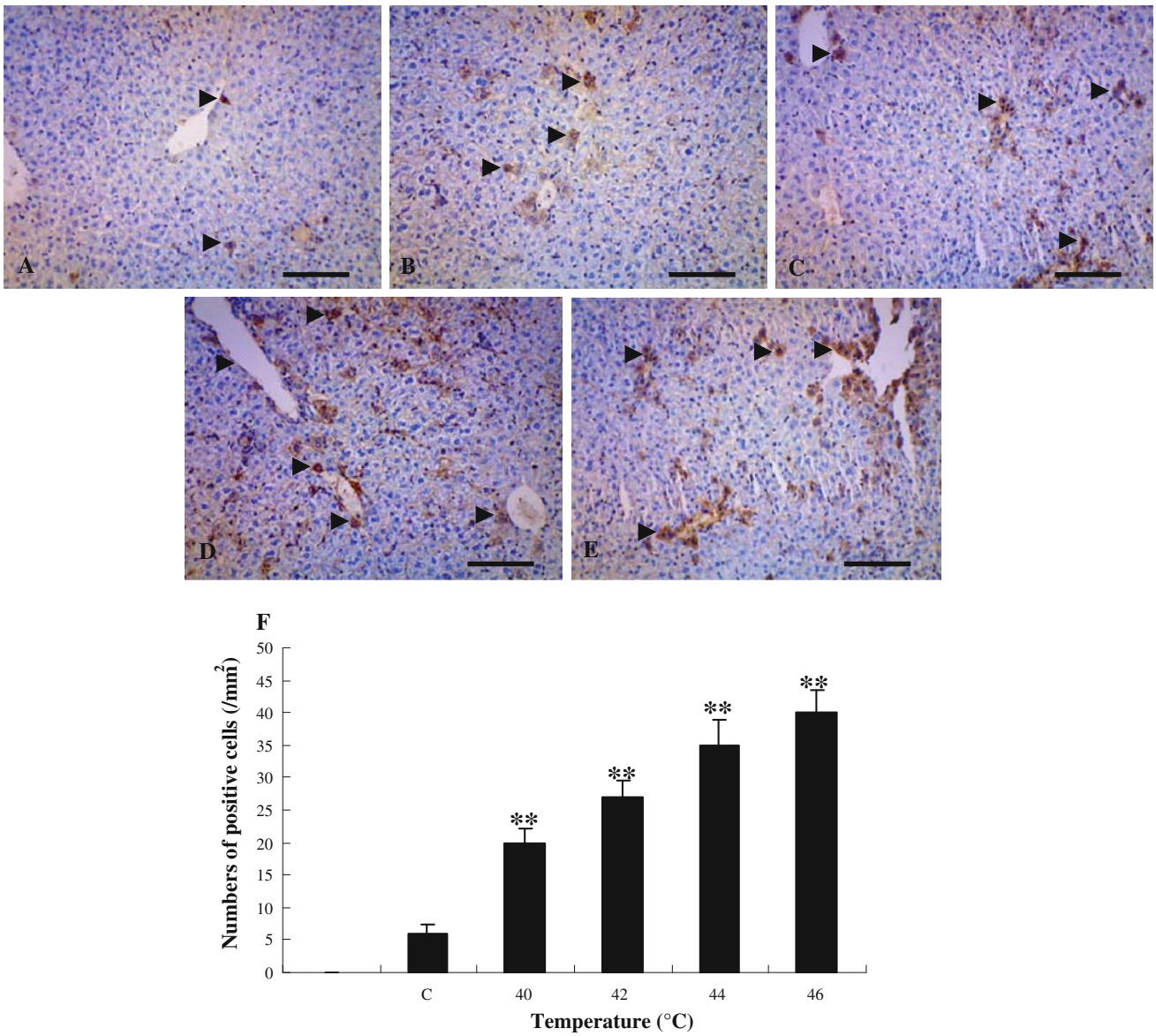

Fig. 5 Photomicrographs of immunohistochemical staining of Hsp70 in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. The *arrowheads* indicate the Hsp70 positive cells in the liver of unstressed control mice (**a**), 40°C stressed mice (**b**), 42°C stressed mice (**c**), 44°C stressed mice (**d**) and 46°C stressed mice (**e**). **f** Numbers of $Hsp70^{+}$ cells in the control mice and mice after heat stress at different temperatures; at least 12-mm^2 tissue sections were counted for each mouse. *C* unstressed controls. Significance: $^{**}P < 0.01$ compared with controls. *Scale bar* 50 μm

Our results showed that Hsp70 level was considerably increased with increasing temperature (Figs. 1, 2 and 5). The up-regulated expression of Hsp70 may function in a molecular chaperone role in the recovery of cells from stress or improve the cell's viability against other kinds of cellular stress [14]. Our results further indicated that heat stress of low temperatures, such as 40 and 42°C, significantly induced the expression of Pcna while heat stress of high temperatures, such as 44 and 46°C, considerably inhibited Pcna expression ($P < 0.05$) (Figs. 1, 2 and 6). Pcna is a subunit of the mammalian DNA polymerase delta and is synthesized primarily during the S phase of the cell cycle [4]. Pcna is a relay or anchoring molecule that functions as a molecular integrator for proteins involved in the control of the cell cycle, DNA replication, DNA repair, and cell death [5, 6]. Pcna has been shown to be a good marker to distinguish proliferating cells [7, 8]. Our results explained that heat stress of low temperature helped to promote the proliferation of hepatocytes while heat stress of high temperature could inhibit the proliferation of hepatocytes in the mice. Heat stress of low temperatures, such as 40 and 42°C, significantly induced Hsp70

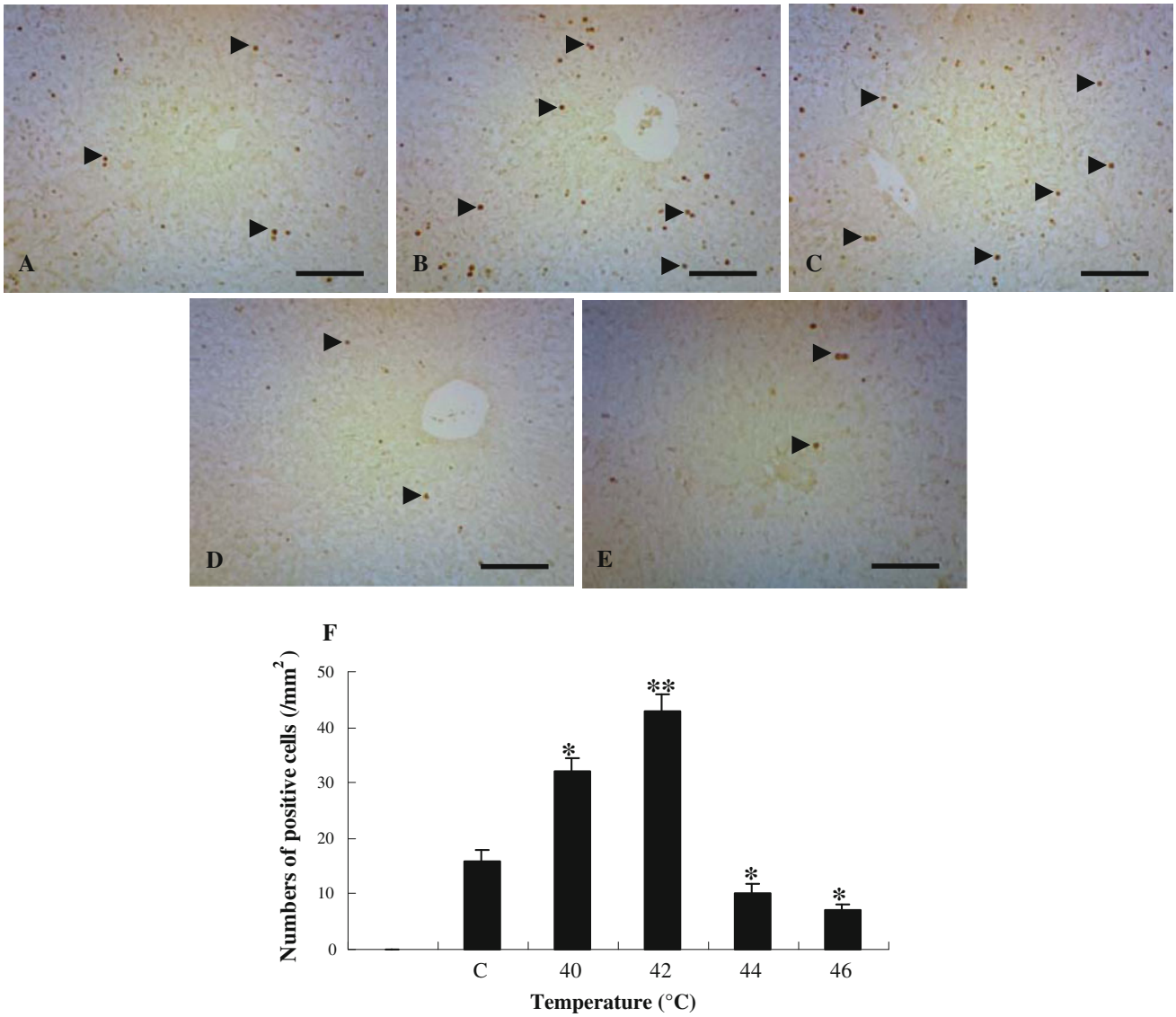

Fig. 6 Photomicrographs of immunohistochemical staining of Pcna in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. The *arrowheads* indicate the Pcna positive cells in the liver of unstressed control mice (**a**), 40°C stressed mice (**b**), 42°C stressed mice (**c**), 44°C stressed mice (**d**) and 46°C stressed mice (**e**). **f** Numbers of $Pcna^{+}$ cells in the control mice and mice after heat stress at different temperatures; at least 12-mm^2 tissue sections were counted for each mouse. *C* unstressed controls. Significance: $*P < 0.05$, $**P < 0.01$ when compared with controls. *Scale bar* 50 μm

expression. Hsp70 may serve the folding and translocation of proteins of the mitogen-activated signal cascade, particularly with the Src kinase, with tyrosine receptor kinases, with Raf and the MAP-kinase activating kinase (MEK), and activate the signaling pathway of promoting the proliferation of hepatocytes, which could help to promote the proliferation of hepatocytes [15]. Although heat stress of high temperatures, such as 44 and 46°C, could induce more Hsp70 expression, cellular defense mechanisms are insufficient to cope with the excessive stress and hepatocytes induced necrosis, which inhibited the proliferation of hepatocytes. We presumed that high temperatures may induce liver injury by inhibiting the proliferation of hepatocytes in the workers who work in hot environments.

Our results also indicated that heat stress of high temperatures, such as 44 and 46°C, significantly induced the hepatocytes apoptosis while low temperatures, such as 40 and 42°C, did not affect the hepatocytes apoptosis in the mice (Fig. 4). Bax is a pro-apoptotic factor while Bcl2 is an anti-apoptotic factor [16]. We think that heat stress of high temperature could induce enough Hsp70 expression, which helped to up-regulate Bax expression, down-regulate Bcl2 expression and induce hepatocytes apoptosis. In addition,

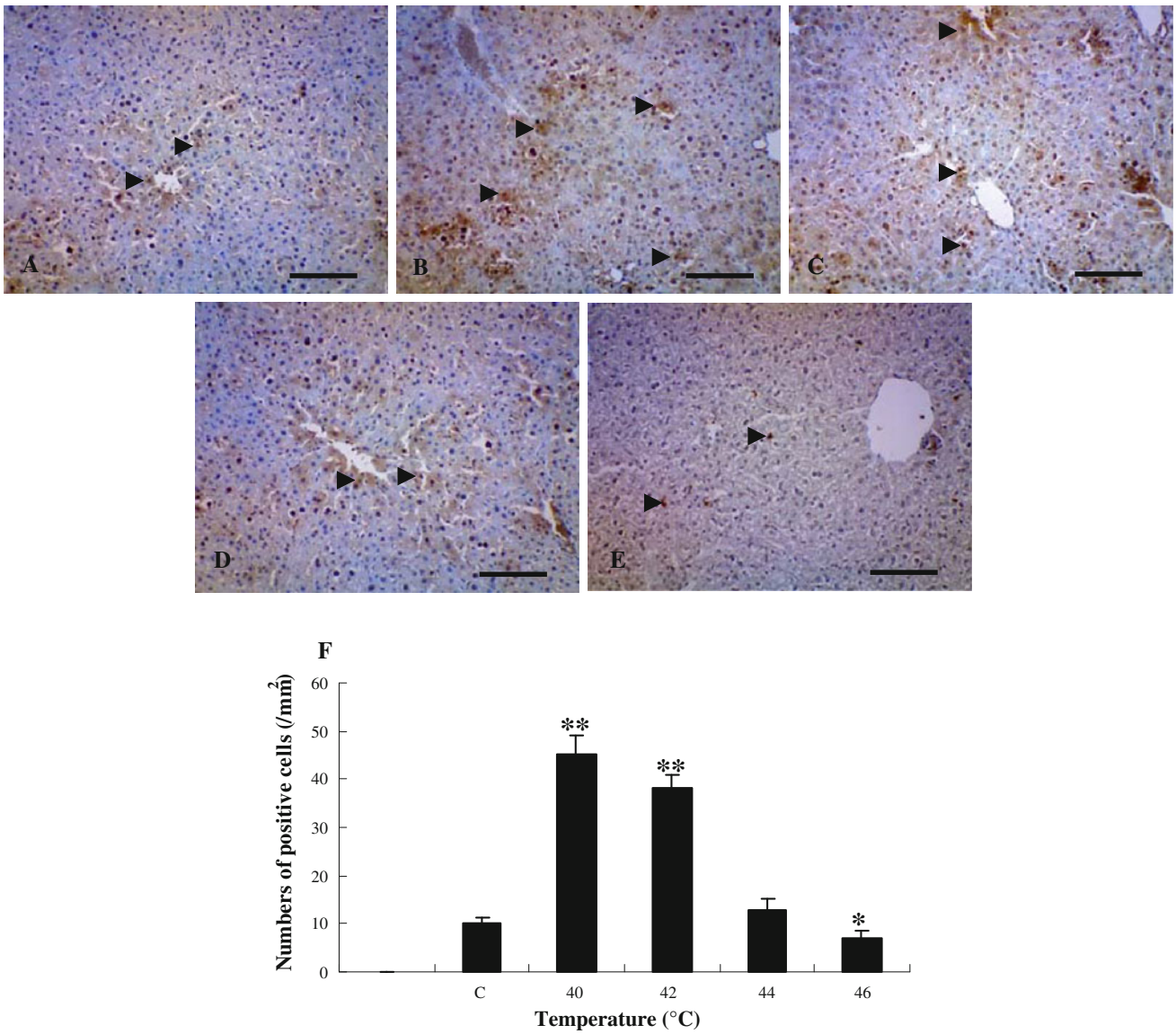

Fig. 7 Photomicrographs of immunohistochemical staining of CYP1A2 in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. The *arrowheads* indicate the CYP1A2 positive cells in the liver of unstressed control mice (**a**), 40°C stressed mice (**b**), 42°C stressed mice (**c**), 44°C stressed mice (**d**) and 46°C stressed mice (**e**). **f** Numbers of CYP1A2^{+} cells in the control mice and mice after heat stress at different temperatures; at least 12-mm^2 tissue sections were counted for each mouse. *C* unstressed controls. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with controls. *Scale bar* 50 μm

HE staining shows that 44 ($P < 0.05$) and 46°C ($P < 0.01$) heat shock considerably induced hepatocyte necrosis in the mice when compared with the controls (Fig. 3). These results suggested that high temperatures could also induce liver injury by inducing the apoptosis and necrosis of hepatocytes in the workers who work in hot environments.

Our results showed that a number of genes encoding cytochrome P450 enzymes were differentially expressed in the liver of mice after heat stress at different temperatures. Heat stress of low temperatures (40, 42°C) significantly induced the expression of CYP1A2 while extreme high temperature (46°C) could inhibit CYP1A2 expression in the mice. CYP1A2 is one of the major CYPs in human liver (approximately 13%) and metabolizes a variety of clinically important drugs. This enzyme also metabolizes several important endogenous compounds including steroids, retinols, melatonin, uroporphyrinogen and arachidonic acid. Like many of other CYPs, CYP1A2 is subject to induction and inhibition by a number of compounds [17]. Our results demonstrated that proper heat stress with low temperature could induce CYP1A2 expression to improve the metabolism function in the liver of mice. High

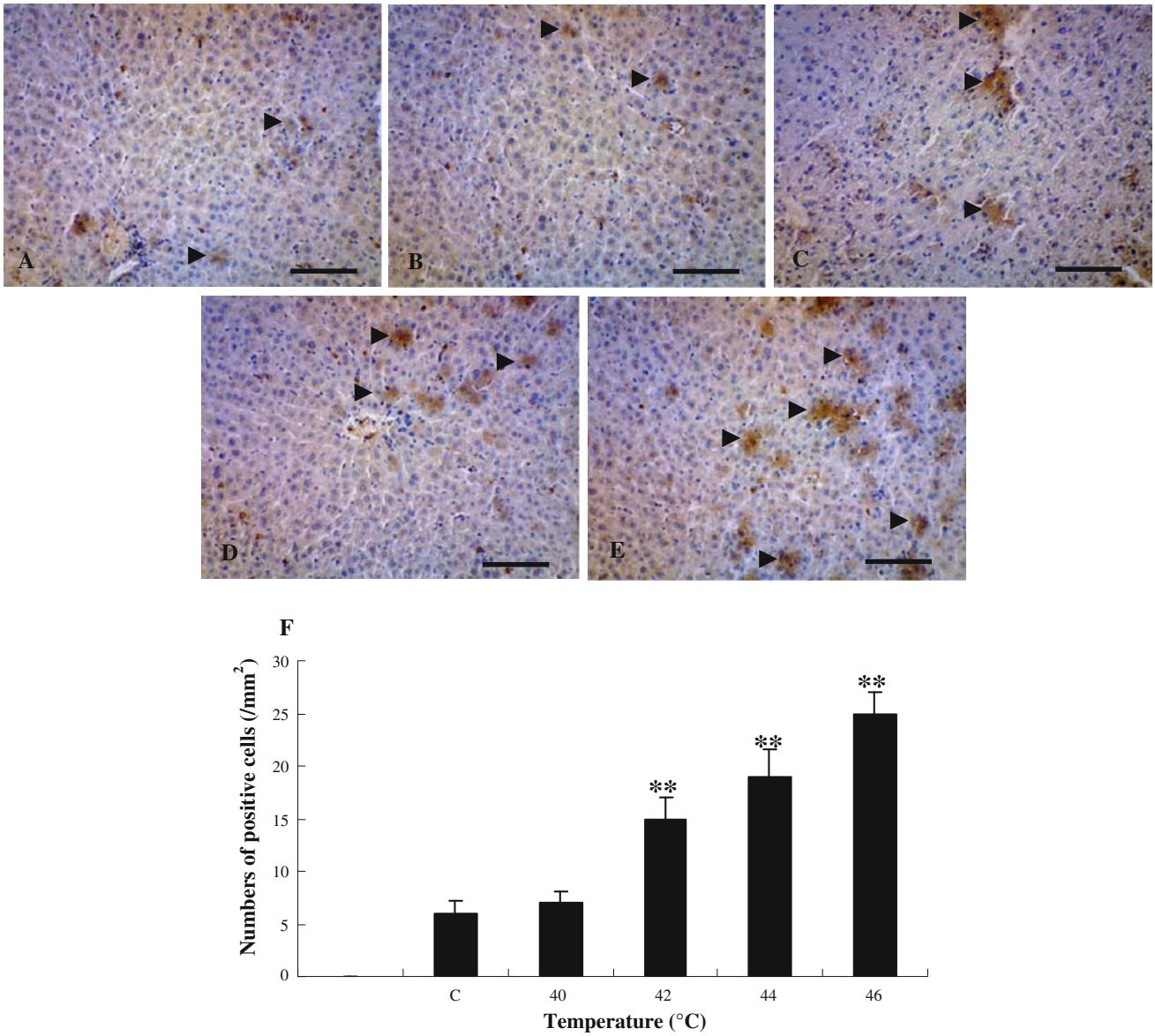

Fig. 8 Photomicrographs of immunohistochemical staining of CYP2E1 in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. The *arrowheads* indicate the CYP2E1 positive cells in the liver of unstressed control mice (**a**), 40°C stressed mice (**b**), 42°C stressed mice (**c**), 44°C stressed mice (**d**) and 46°C stressed mice (**e**). **f** Numbers of $CYP2E1^+$ cells in the control mice and mice after heat stress at different temperatures; at least 12 mm^2 tissue sections were counted for each mouse. *C* unstressed controls. Significance: $^{**}P < 0.01$ compared with controls. *Scale bar* 50 μm

temperature stress may inhibit CYP1A2 expression to affect the metabolism function of liver in the workers who work in hot environments.

Our results showed that CYP2E1 was differently expressed with the temperature increasing in the liver of mice. CYP2E1, a microsomal enzyme involved in xenobiotic metabolism and generation of oxidative stress, has been implicated in promoting liver injury [18]. Wong et al. [19] has demonstrated that CYP2E1 plays a major role in CCl_4 toxicity based on a previous study with Cyp2e1-null mice. Dey and Cederbaum [20] reported that induction of CYP2E1 promoted liver injury in obese mice. Dey and Kumar [18] think that changes in regulation of CYP2E1 under hyperglycemic conditions are tightly linked with increased oxidative stress and injury in liver. In our study, heat stress of high temperatures, such as 44 and 46°Cs significantly induced CYP2E1 expression ($P < 0.01$) in company with increasing AST and ALT levels ($P < 0.01$) (Figs. 1 and 10). Low temperature stress, such as 40°C, did not affect CYP2E1 expression and the levels of AST and ALT ($P > 0.05$) (Figs. 1 and 10), which did not induce liver injury in the mice. So we presume that CYP2E1 may

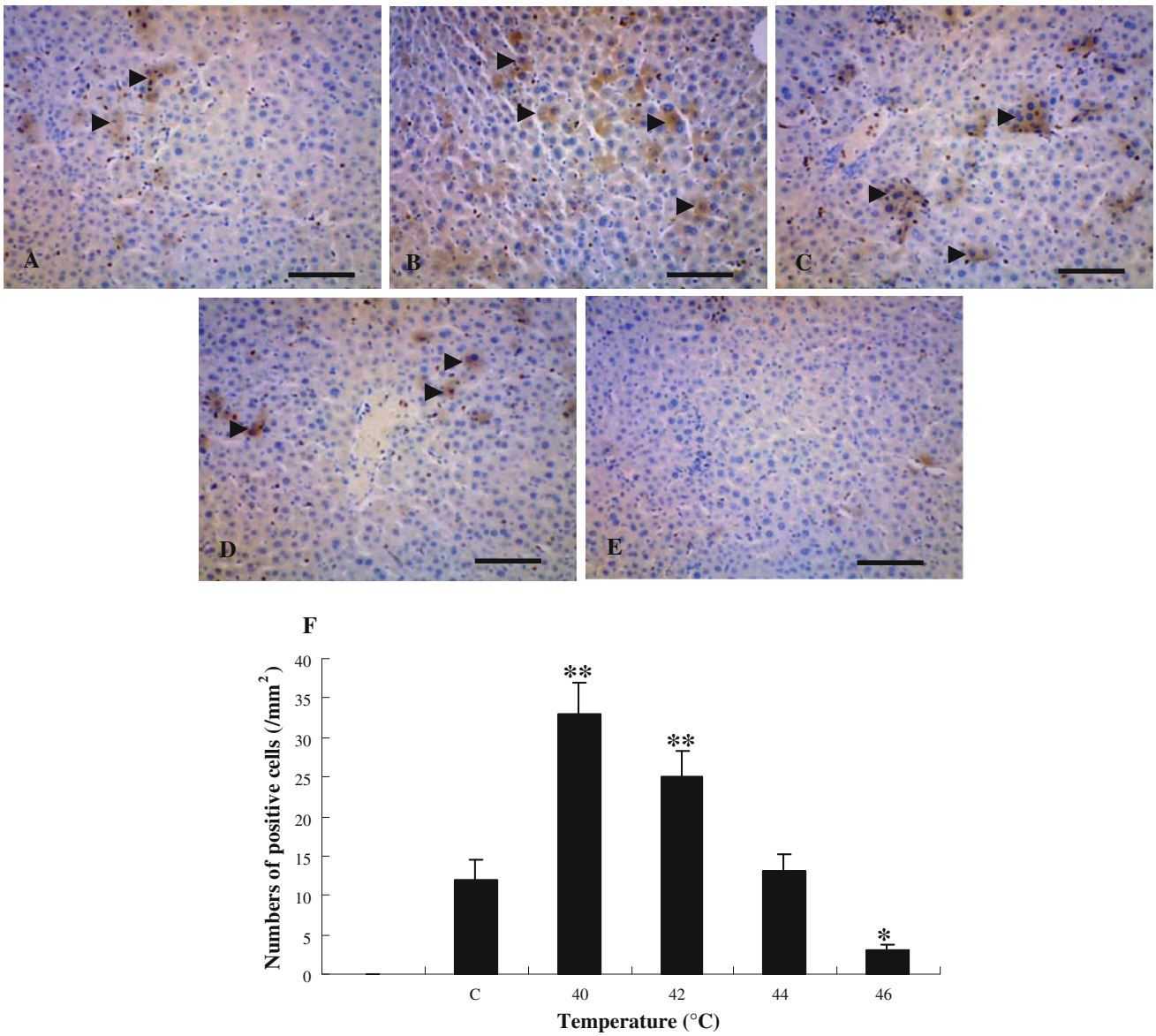

Fig. 9 Photomicrographs of immunohistochemical staining of the analog of CYP3A4 in the liver of control mice and the mice exposed to 40, 42, 44, and 46°C whole body heat stress. The experiment was repeated three times and the representative pictures obtained by a digital image-capture system are shown. The arrowheads indicate the analog of CYP3A4 positive cells in the liver of unstressed control mice (**a**), 40°C stressed mice (**b**), 42°C stressed mice (**c**), 44°C stressed mice (**d**) and 46°C stressed mice (**e**). **f** Numbers of the analog of CYP3A4^{+} cells in the control mice and mice after heat stress at different temperatures; at least 12 mm^2 tissue sections were counted for each mouse. *C* unstressed controls. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with controls. *Scale bar* 50 μm

be an important marker for liver injury in many conditions. In addition, high temperature stress may promote liver injury by inducing CYP2E1 expression in the workers who work in hot environments.

CYP3A4 is the most abundantly expressed drug-metabolizing P450 enzyme in human liver and contributes to the metabolism of a large number of drugs in use today. CYP3A4 is constitutively expressed in adult hepatocytes but it can also be transcriptionally induced by a variety of structurally diverse xenochemicals. CYP3A4 strongly contributes to the important variability in the therapeutic and toxic effects of drugs owing to the major role it plays in xenobiotic metabolism and the large intra- and inter-individual variability to which it is subjected [21]. But it has not been reported that CYP3A4 exists in the liver of mice. So we wanted to detect whether there is an analog of CYP3A4 in the liver of mice and study the effects of heat stress on the analog of CYP3A4 expression in the hepatocytes of mice. Western blot was also performed using a rabbit polyclonal antibody to human CYP3A4. Our results proved that there was an analog of CYP3A4 in the liver of mice (Figs. 1 and 9). The analog expression of CYP3A4

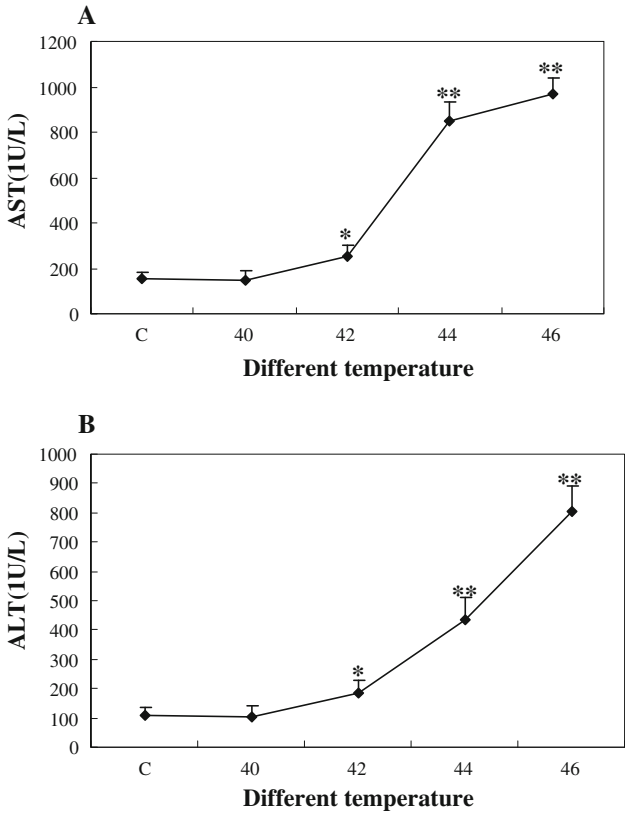

Fig. 10 Serum levels of AST (**a**) and ALT (**b**) were monitored in the control mice and mice exposed to heat stress at different temperatures. Values are expressed as the mean ± SEM for three animals. *AST* aspartate aminotransferase; *ALT* alanine aminotransferase. *C* unstressed controls. Significance: $*P < 0.05$, $**P < 0.01$ when compared with controls

was up-regulated after heat stress at 40 ($P < 0.01$) and 42°C ($P < 0.05$) but down-regulated after heat stress at 46°C ($P < 0.05$) (Figs. 1 and 9), which indicated that heat stress of low temperature helped to induce the analog of CYP3A4 expression, but high temperature inhibited the analog of CYP3A4 expression. This is possibly another reason why high temperature induces liver damage in the workers who work in hot environments.

The expression of CYP1A2 and analog of CYP3A4 was increased after heat stress at low temperatures but decreased after heat stress at high temperatures, while CYP2E1 expression was considerably increased after heat stress at high temperatures in the liver of mice. Our results suggest that CYP1A2, analog of CYP3A4, and CYP2E1 are all temperature-sensitive enzymes. CYP1A2 and analog of CYP3A4 may play different roles than CYP2E1 in the liver of mice at high temperature heat stress. The increasing of CYP2E1 expression promoted liver injury while the decreasing of CYP1A2 and analog of CYP3A4 expression further induced liver injury. In addition, there are some discrepancies between the results of protein expression profile and that of mRNA in Bcl2, CYP1A2 and CYP2E1 analyses because changes at the mRNA level do not always result in changes in protein expression in certain time periods for post-transcriptional processing and post-translation modification. But the whole trend is consistent for the results of protein expression profile and those of mRNA in Bcl2, CYP1A2 and CYP2E1 analyses.

In conclusion, our results suggested that heat stress of low temperatures could promote hepatocyte proliferation and improve the metabolism function of liver while heat stress of high temperatures could inhibit hepatocyte proliferation, promote hepatocyte apoptosis and induce hepatocyte necrosis, which may be the important reasons for liver injury of workers in high temperature environments of.

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