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Trichostatin A Enhances Gap Junctional Intercellular Communication in Primary Cultures of Adult Rat Hepatocytes

Mathieu Vinken,*[,1,2] Tom Henkens,* Tamara Vanhaecke,* Peggy Papeleu,* Albert Geerts,† Elke Van Rossen,† James Kevin Chipman,‡ Paolo Meda,§ and Vera Rogiers*

*Department of Toxicology, Vrije Universiteit Brussel, B-1090 Brussels, Belgium; †Department of Cell Biology, Vrije Universiteit Brussel, B-1090 Brussels, Belgium; ‡School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; and §Department of Cell Physiology and Metabolism, CMU Université de Genève, CH-1211 Genève 4, Switzerland

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The effects of histone deacetylase inhibitor Trichostatin A (TSA) on connexin (Cx) expression and gap junctional intercellular communication (GJIC) were investigated in primary cultures of adult rat hepatocytes. GJIC was monitored by using the scrape-loading/dye transfer method. Immunoblotting and immunocytochemistry were used to investigate Cx protein levels and localization. Cx gene expression was studied by means of quantitative reverse transcriptase–polymerase chain reaction. TSA increased Cx32 protein levels and affected negatively the Cx26 protein levels. The latter was preferentially located in the cytosol of cultured cells. TSA also promoted the appearance of Cx43 in the nuclear compartment of primary cultured hepatocytes. Overall, this resulted in enhanced GJIC activity. It is important to note that the time of onset of TSA treatment was crucial for the extent of its outcome and that the effects of TSA on Cx protein levels occurred independently of transcriptional changes. TSA differentially affects Cx proteins in primary rat hepatocyte cultures, suggesting distinct regulation and/or distinct roles of the different Cx species in the control of hepatic homeostasis. TSA enhances GJIC between primary cultured rat hepatocytes, an interesting finding supporting its use to further optimize liver-based *in vitro* models for pharmacotoxicological purposes.

Key Words: **HDAC inhibitor; TSA; connexin; GJIC; primary hepatocyte culture.**

Long-term cultivation of primary hepatocytes is largely impaired by the progressive loss of the hepatocellular-specific phenotype at both the functional and morphological level (Papeleu *et al.*, 2002). A major hallmark of functional deterioration in primary hepatocyte cultures includes the decreased capacity to communicate via gap junctions (Stoehr and Isom, 2003). Gap junctional intercellular communication (GJIC) involves the direct exchange of small (< 1 kDa) and hydrophilic molecules, mainly second messengers (e.g., Ca^{2+} and cyclic adenosine 3′,5′monophosphate [cAMP]), between cells (Saez *et al.*, 2003; Spray *et al.*, 1994). The establishment of this flux is a prerequisite for normal liver functioning, including xenobiotic biotransformation (Hamilton *et al.*, 2001; Neveu *et al.*, 1994; Shoda *et al.*, 1999), albumin secretion, ammonia detoxification (Yang *et al.*, 2003), glycogenolysis (Saez *et al.*, 2003; Stumpel *et al.*, 1998), and bile secretion (Nathanson *et al.*, 1999; Temme *et al.*, 2001).

Gap junctions are composed of two connexons, in turn being built up by six connexin (Cx) proteins. More than 20 mammalian Cxs have been characterized, which are all expressed in a cell-specific way (Saez *et al.*, 2003; Spray *et al.*, 1994). In human and rat livers *in vivo*, hepatocytes express both Cx32 and Cx26. The former accounts for 90% of the hepatic Cx content, whereas the latter represents only a small fraction, being preferentially located in the periportal region. In contrast, Cx43 is the only Cx species produced by nonparenchymal liver cells, such as Kupffer cells, stellate cells, endothelial cells, cells of Glisson's capsule, as well as oval cells (Iwai *et al.*, 2000; Nakashima *et al.*, 2004; Saez *et al.*, 2003; Spray *et al.*, 1994). In primary cultures of hepatocytes, however, Cx32 protein levels decline rapidly, whereas Cx43 becomes detectable (Kojima *et al.*, 1995; Stutenkemper *et al.*, 1992; Willecke and Haubrich, 1996). This expression pattern switch is associated with the ultimate loss of GJIC between cultured hepatocytes (Stoehr and Isom, 2003; Willecke and Haubrich, 1996).

Several groups have focused on reestablishing GJIC in primary hepatocyte cultures. In this respect, three strategies have been proposed, namely (1) the introduction of an extracellular matrix, by cultivating hepatocytes on one layer or between two layers of extracellular matrix proteins (Fujita *et al.*, 1987; Hamilton *et al.*, 2001; Spray *et al.*, 1987), (2) the establishment of cell-cell contacts, by cocultivating hepatocytes with another cell type (Mesnil *et al.*, 1993), and (3) the addition of GJIC-promoting molecules to the culture medium, including dimethylsulfoxide (Kojima *et al.*, 1995), cAMP and derivatives

[1] To whom correspondence should be addressed at Doctoral research fellow of the Fund for Scientific Research Flanders (FWO-Vlaanderen), Belgium. Fax: +32-2-4774582. E-mail: mvinken@vub.ac.be.
[2] Present address: Fund for Scientific Research, Flanders (FWO-Vlaanderen), Belgium.

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(Saez *et al.*, 1989), glucocorticosteroids (Ren *et al.*, 1994), and antioxidants (Kojima *et al.*, 1996).

Our group is currently exploring the use of histone deacetylase (HDAC) inhibitors as culture medium components for primary hepatocytes. HDAC inhibitors interfere with the acetylation of core histones, which affects the chromatin structure and thus the gene expression (Papeleu *et al.*, 2005a; Vanhaecke *et al.*, 2004b). Previous research from our laboratory showed that Trichostatin A (TSA), a prototype hydroxamate HDAC inhibitor, alters gene expression in favor of the differentiated phenotype in primary hepatocyte cultures (Papeleu *et al.*, 2003; Vanhaecke *et al.*, 2004a). Here, we investigate whether the homeostatic modifications induced by TSA in these *in vitro* models are associated with effects on GJIC and Cx expression.

MATERIALS AND METHODS

Chemicals. TSA (7-[4-(dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide) (purity > 98%) was from Sigma (Belgium). All other chemicals were commercially available products of analytical grade. Specifications of primary antibodies used for immunoblotting and immunocytochemistry are summarized in Table 1.

Cell isolation and culture. Procedures for the isolation and cultivation of rat hepatocytes were approved by the local ethical committee of the Vrije Universiteit Brussel. Hepatocytes (viability > 80%) were isolated from adult male outbred (200–250 g) Sprague-Dawley rats (Charles River Laboratories, Belgium) by use of a two-step collagenase method (Papeleu *et al.*, 2005b) and cultivated either in the absence (condition C0) or presence (condition C1) of 1μM TSA dissolved in absolute ethanol (final concentration 0.0033% vol/vol). In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (condition C2). The medium used was Dulbecco's modified Eagle medium containing 0.5 IU/ml insulin, 7 ng/ml glucagon, 292 mg/ml L-glutamine, antibiotics (7.3 IU/ml benzyl penicillin, 50 μg/ml kanamycin monosulfate, 10 μg/ml sodium ampicillin, and 50 μg/ml streptomycin sulfate), and 10% vol/vol fetal bovine serum. After 4 h, the medium was removed and renewed with fresh medium supplemented with 25 μg/ml hydrocortisone hemisuccinate and 0.25 μg/ml amphotericin B. After 24 h, the medium was renewed daily with serum-free medium. Samples were taken from day 1, 4, and 7 (D1, D4, and D7, respectively) cultured cells.

Lactate dehydrogenase leakage assay of cell viability. Lactate dehydrogenase (LDH) leakage was measured according to the Bergmeyer procedure (Bergmeyer, 1974) using a commercial kit (Merck, Germany). Percentage of LDH leakage was calculated by the following equation: (100 × LDH activity in supernatant)/(LDH activity in [supernatant + cells]).

RNA extraction and quantitative reverse transcriptase–polymerase chain reaction. Cells were harvested from culture plates by scraping and washed twice with cold phosphate-buffered saline (PBS). Total cellular RNA was extracted using the SV Total RNA isolation system (Promega, USA) according to the manufacturer's instructions. Samples were subsequently subjected to DNase treatment (Ambion, USA). Reverse transcription of approximately 2 μg of total RNA was carried out using the iScript cDNA synthesis kit (Bio-Rad, Germany).

For conventional reverse transcriptase–polymerase chain reaction (RT-PCR), 2 μg cDNA was amplified by means of the iTaq DNA polymerase kit (Bio-Rad). The reaction mix (final volume 50 μl) was composed of standard 1× Taq polymerase buffer containing 2mM $MgCl_2$, deoxynucleotide triphosphates (0.2mM each), 1.25 U Taq polymerase, and 1mM of each primer. PCR primers are listed in Table 2 and were synthesized at Eurogentec (Belgium). PCR conditions used were as follows—for Cx26: 40 cycles (1 min at 95°C, 1 min at 53.2°C, and 1 min at 72°C); for Cx32: 35 cycles (1 min at 95°C, 1 min at 59.1°C, and 1 min at 72°C); for Cx43: 40 cycles (1 min at 95°C, 1 min at 59.6°C, and 1 min at 72°C), each followed by final elongation at 72°C for 7 min. Samples were separated by electrophoresis on a 1.8% wt/vol agarose gel. Visualization of the products was performed with ethidium bromide (0.005% vol/vol) under UV transillumination.

For real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), a reaction mix was prepared in RNase-free water (final volume 25 μl) containing 2 μl cDNA preparation, 2× Taqman universal PCR master mix (Applied Biosystems, USA), and 20× gene expression assay mix. Gene expression mixes for Cx26 (accession number NM_001004099.1), Cx32 (accession number NM_017251.1), Cx43 (accession number NM_012567.2), and 18S rRNA (accession number X03205) were from Applied Biosystems, with assay IDs Rn02376786_s1, Rn01641031_s1, Rn01433957_m1, and Hs99999901_s1, respectively. We tested some housekeeping genes in hepatocytes and found that 18S rRNA is a reliable internal standard to be used in the ΔΔCt method. Real-time quantifications were performed using the Applied Biosystems 7500 Real Time PCR system. Ct values were calculated using the system software. At indicated time points, Ct values of the target genes were normalized to those of 18S rRNA, for both the test samples (C1 and C2 cultures) and the calibrator samples (C0 cultures). The resulting ΔCt values of the test samples were then normalized to those of the calibrator samples, yielding ΔΔCt. Relative alterations (fold change) in mRNA levels were calculated according to the formula $2^{-(\Delta\Delta Ct)}$.

Preparation of cell lysates and immunoblotting. For immunoblotting, cells were harvested from culture plates by scraping and washed twice with cold PBS. Cells were homogenized in lysis buffer (50mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid pH 7.5, 150mM NaCl, 1mM ethylenediaminetetraacetic acid, 2.5mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 0.1% vol/vol Tween 20, and 10% vol/vol glycerol) supplemented with 0.1mM phenylmethylsulfonyl fluoride, 5mM dithiothreitol, and 1× protease inhibitor cocktail (Roche, Germany). Following sonication for 10 s, samples were left on

TABLE 1
Primary Antibodies Used for Immunoblotting and Immunocytochemistry

Antigen	Supplier	Cat. no.	Species	Type	Dilution
Cx26	Zymed (USA)	71-0500	Rabbit	Polyclonal	1/250 (WB and IC)
Cx32	Sigma (USA)	C3470	Rabbit	Polyclonal	1/500 (WB and IC)
Cx43	Sigma	C6219	Rabbit	Polyclonal	1/4000 (WB) and 1/50 (IC)
AcH4	Upstate	06-598	Rabbit	Polyclonal	1/50 (IC)

Note. AcH4, acetylated histone H4; IC, immunocytochemistry; WB, Western blotting.

TABLE 2
Primers Used for Conventional RT-PCR Analysis

Target	Primer sequence	Product size (bp)
Cx26	F: 5′-CGCGGCCGTCCGCTCTCCCAA-3′ R: 5′-GAAGTAGTGGTCGTAGCACAC-3′	481
Cx32	F: 5′-AGGTGTCGCAGTGCCAGGGAG-3′ R: 5′-CCCGTGCCCCTCAAGCCGTAG-3′	373
Cx43	F: 5′-GCGTGAGGAAAGTACCAAAC-3′ R: 5′-GTGAAGCCGCCGCCAAAGTTG-3′	128

Note. F, forward primer; R, reverse primer.

ice for 1 h. Cell lysates were centrifugated at 13,791 × g for 5 min. Protein concentrations in supernatants were determined according to the Bradford (1976) procedure, using a commercial kit (Bio-Rad) with bovine serum albumin as a standard. Proteins (25 or 50 μg) were fractionated on sodium dodecyl sulfate polyacrylamide gels (7.5%, 10%, or 12%) and blotted afterward onto nitrocellulose membranes (Amersham Pharmacia Biotech, United Kingdom). The efficiency of transfer as well as equal protein loading was controlled by Ponceau S staining. Subsequent blocking of the membranes was performed with 5% wt/vol nonfatty milk in Tris-buffered saline solution (20mM Tris pH 7.6, 135mM NaCl) containing 0.1% vol/vol Tween 20 (TBST). Membranes were incubated with primary antibodies (Table 1) for 2 h and with peroxidase-conjugated secondary antibodies (Dakocytomation, Denmark) for 1 h, both performed at room temperature. Excessive antibodies were removed by washing the membranes several times with TBST. Detection of proteins was carried out by means of an enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech).

Immunocytochemistry. Hepatocytes, grown on glass coverslips in 35-mm dishes, were fixed for 10 min with ice-cold ethanol (70% vol/vol). Following rehydration with PBS, cells were permeabilized with 0.1% vol/vol Triton X-100 and blocked with 0.1% wt/vol nonfatty milk, each for 30 min. Cells were then incubated with primary antibodies (Table 1) for 2 h, washed with PBS, and exposed to a tetramethyl rhodamine isothiocyanate–labeled secondary antibody (Jackson Immunoresearch Laboratories, USA) for 45 min. After extensive rinsing with PBS, samples were mounted with Vectashield (Vector Laboratories, USA). For Cx analysis, diamidinophenylindole was included. Samples were kept at 4°C prior to analysis. Detection was performed by fluorescence microscopy (Leica DMR/XA). For negative controls, an identical procedure was followed, but primary antibodies were omitted (data not shown).

GJIC assay. GJIC was studied by means of the scrape-loading/dye transfer assay (el-Fouly *et al.*, 1987) with some modification. Briefly, hepatocytes, plated in 60-mm dishes, were rinsed twice with PBS. Then, 4 ml of 0.25% wt/vol Lucifer Yellow CH (Sigma) was added, and three scrape lines were made by gently passing a scalpel across the cultures. Cultures were placed for 5 min at 37°C in a humidified 5% CO_2 incubator. Thereafter, the tracer solution was removed, and cells were rinsed three times with PBS. Hepatocytes were fixed with 4% wt/vol paraformaldehyde, and cultures were stored at 4°C. Dye transfer was evaluated by fluorescence microscopy (Leica DMR/XA). For negative controls, cells were incubated with 50μM 18α-glycyrrhetinic acid (Sigma) for 1 h prior to analysis (data not shown).

Statistical analysis. Data are expressed as mean values ± SDs of three independent experiments. Results were evaluated by ANOVA with *post hoc* Dunnett's tests (significance level 0.05) for statistical difference between treatments.

RESULTS

TSA Induces Accumulation of Acetylated Histone H4 in Primary Cultures of Adult Rat Hepatocytes

TSA is a well-known HDAC inhibitor, causing hyperacetylation of core histones in several cell line models including hepatoma cells (Papeleu *et al.*, 2005a; Vanhaecke *et al.*, 2004b). In these settings, accumulation of acetylated histones already becomes evident when using nanomolar concentrations of TSA. Our group previously showed that higher TSA concentrations (i.e., micromolar range) are required in order to be effective in primary cell systems *in casu* primary rat hepatocyte cultures (Papeleu *et al.*, 2003; Vanhaecke *et al.*, 2004a). Similarly, Ogawa *et al.* (2005) applied up to 2μM suberoylanilide hydroxamic acid (SAHA) to trigger hyperacetylation of histones H3 and H4 in primary cultures of human peritoneal mesothelial cells. We therefore handled TSA in 1μM concentration throughout the current study. As shown by immunocytochemistry analysis, 1μM TSA indeed caused hyperacetylation of histone H4 in primary cultured rat hepatocytes (Fig. 1). Measurements of LDH leakage, performed in parallel, showed that 1μM TSA dissolved in absolute ethanol (final concentration 0.0033% vol/vol) did not cause cytotoxicity during 7 days of culture (Fig. 2).

TSA Enhances GJIC in Primary Cultures of Adult Rat Hepatocytes

We evaluated the effect of 1μM TSA on GJIC in primary cultures of adult rat hepatocytes by using the scrape-loading/dye transfer method (el-Fouly *et al.*, 1987). Limited dye transfer was observed on the first day of hepatocyte cultivation (Fig. 3), a finding that was in line with the work of Stoehr and Isom (2003). This observation, present in all cultivation conditions, was likely to result from the hepatocyte isolation procedure during which gap junctions are disrupted (Stoehr and

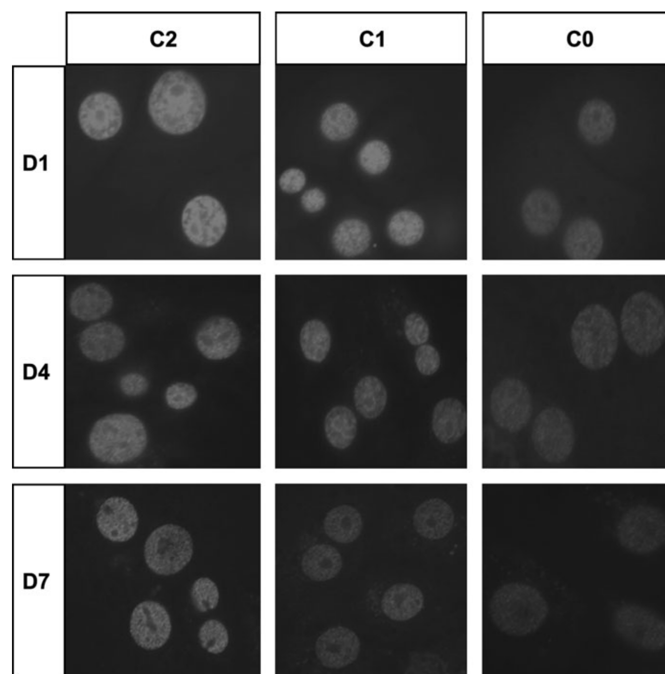

FIG. 1. Effects of TSA on acetylation of histone H4 in primary cultured adult rat hepatocytes. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells and subjected to indirect immunocytochemistry (see "Materials and Methods") using a primary antibody directed against acetylated histone H4 (Table 1) and a tetramethyl rhodamine isothiocyanate–labeled secondary antibody (red). Results shown are representative of at least three independent experiments (original magnification ×630).

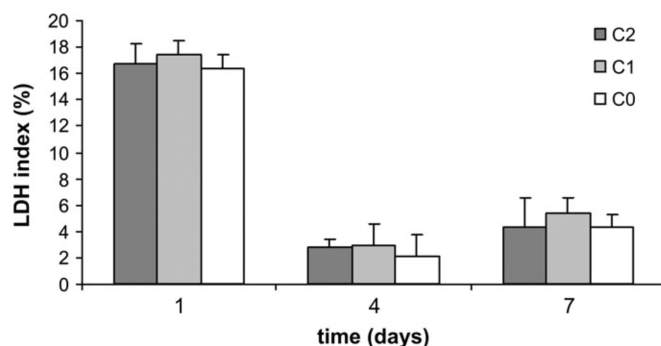

FIG. 2. Effects of TSA on the viability of primary cultured adult rat hepatocytes. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells and subjected to LDH assay as described in "Materials and Methods." Percentage of LDH leakage was calculated by the following equation: (100 × LDH activity in supernatant)/(LDH activity in [supernatant + cells]). Results are mean values ± SDs of three independent experiments. Statistical significance ($p < 0.05$) between C0 and C1 and between C0 and C2 conditions was tested by ANOVA with *post hoc* Dunnett's tests.

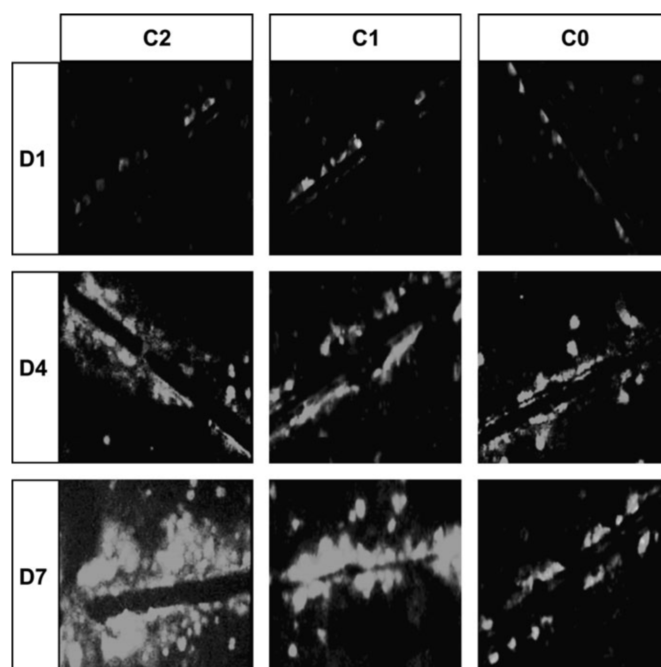

FIG. 3. Effects of TSA on GJIC in primary cultured adult rat hepatocytes. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells and subjected to scrape-loading/dye transfer analysis as described in "Materials and Methods." The distance traveled by Lucifer Yellow CH (green) from the scrape reflects the level of GJIC present in the culture system. Results shown are representative of at least three independent experiments (original magnification ×100).

Isom, 2003). In control cultures, dye transfer slightly increased towards day 4 but remained low at day 7. In TSA-treated cultures, however, dye transfer was higher at day 7, especially in C2 cultures (Fig. 3).

TSA Differentially Affects Cx Protein Levels and Localization in Primary Cultures of Adult Rat Hepatocytes

Following the observation that TSA improved GJIC between primary cultured rat hepatocytes, its effect on Cx protein levels and localization were investigated using immunoblotting and immunocytochemistry. When carrying out Western blot analysis, samples of rat liver homogenate were included in order to allow comparison between hepatic Cx protein steady-state levels *in vivo* and those of the cultivated hepatocytes.

In control cultures, Cx32 protein levels declined rapidly. Cx26 protein levels remained relatively unchanged during the whole cultivation period. Cx43, a Cx species not detectable in adult rat hepatocytes, progressively appeared and was predominantly expressed in its unphosphorylated form (Fig. 4). Immunocytochemistry analyses further showed alterations at the level of Cx protein localization during cultivation (Figs. 5A, 5B, and 5C). On day 1, both Cx32 and Cx26 were mainly located within the cytosol of hepatocytes in all cultivation conditions (Figs. 5A and 5B). Presumably, this was an inevitable consequence of the isolation procedure, which might also explain the lack of GJIC observed at that cultivation time point (Fig. 3). The membrane localizations of both Cxs were restored on day 4 in untreated cultures. Weak signals were observed on day 7, especially in case of Cx32 (Figs. 5A and 5B). In contrast, Cx43 staining became evident on day 4 and was clearly present on day 7. At each time point, Cx43 was almost uniquely present in the nucleus of primary cultured adult rat hepatocytes (Fig. 5C).

Upon exposure of primary rat hepatocytes to 1μM TSA, the decrease of Cx32 protein levels seemed delayed (Fig. 4), which was associated with the abundance of this protein on the cell membrane surface at later time points in culture (Fig. 5B). In contrast, TSA affected negatively the Cx26 protein levels, especially at earlier time points of culture (Fig. 4). Moreover, on days 4 and 7, Cx26 was preferentially located in the cytosol (near the nuclear compartment) of TSA-treated hepatocytes (Fig. 5A). TSA also promoted the appearance of Cx43 (Fig. 4) but did not affect its localization (Fig. 5C). Furthermore, the outcome of the production of the three Cx proteins under the influence of TSA was greatly enhanced by early drug treatment, namely, in C2-cultured hepatocytes (Figs. 4, 5A, 5B, and 5C).

TSA-Induced Alterations of Cx Protein Levels in Primary Cultures of Adult Rat Hepatocytes Are not Reflected at the Transcriptional Level

Since altered Cx protein levels were observed in primary rat hepatocyte cultures treated with TSA, the next logical step was

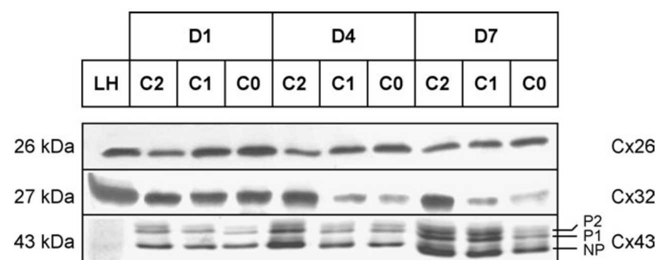

FIG. 4. Effects of TSA on Cx protein levels in primary cultured adult rat hepatocytes. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells and subjected to immunoblot analysis (see "Materials and Methods") using primary antibodies (Table 1) and peroxidase-conjugated secondary antibodies. Detection of proteins was performed by means of enhanced chemiluminescence. Blots shown are representative of at least three independent experiments (LH, liver homogenate; NP, nonphosphorylated Cx43; P1 and P2, phosphorylated Cx43 variants).

to investigate whether these effects were also reflected at the transcriptional level. Initially, conventional RT-PCR analysis was used to assess Cx gene expression. At no single time point, major differences in band intensities between the different cultivation conditions could be noticed (Fig. 6), suggesting that Cx gene expression in primary rat hepatocytes was not affected by TSA. In order to allow more appropriate quantification of Cx mRNA levels, we also performed real-time qRT-PCR analysis. As depicted in Table 3, TSA did not significantly alter Cx mRNA levels, which is in line with the conventional RT-PCR analysis. Most importantly, a poor correlation existed between Cx mRNA and protein levels in TSA-treated cultures. At day 4 of culture, for instance, TSA tended to decrease Cx32 mRNA levels, whereas increased Cx32 protein levels, particularly in C2 cultures, were observed at that time point.

TSA-Mediated Modulation of Cx Protein Levels Is Partly Initiated during the Hepatocyte Isolation Procedure

Since the TSA-mediated effects on Cx protein levels were greatly enhanced upon early drug treatment, we examined whether TSA altered Cx protein levels as early as during hepatocyte isolation. As shown by immunoblot analysis (Fig. 7), TSA downregulated Cx26 protein levels during hepatocyte isolation and continued to do so upon subsequent cultivation of the hepatocytes (Fig. 4). Compared to conventional hepatocyte isolation, liver perfusion performed in the presence of TSA was associated with a more abundant presence of Cx32 protein (Fig. 7). On day 1 in culture, however, Cx32 was present at the same protein levels in all culture conditions (Fig. 4). A temporary enhancement of the (unphosphorylated) Cx43 protein levels was also observed when rat hepatocytes were exposed early to TSA (Fig. 7).

DISCUSSION

The aim of this study was to investigate whether the prototype HDAC inhibitor TSA could be of use to maintain GJIC and physiological Cx protein levels in primary cultures of adult rat hepatocytes. In this respect, the HDAC inhibitor 4-phenylbutyrate was recently found to increase GJIC and Cx43 protein levels in cultured human glioblastoma cells (Asklund *et al.*, 2004). In a similar way, both TSA and SAHA were shown to enhance GJIC in *ras*-transformed WB-F344 rat liver epithelial cells as well as in primary cultures of human peritoneal mesothelial cells. The SAHA-mediated enhancement of GJIC in these *in vitro* systems was also accompanied by elevated Cx43 expression (Ogawa *et al.*, 2005).

We found that Cx32 protein levels were positively affected by TSA in primary cultured rat hepatocytes. In contrast, TSA downregulated Cx26 protein levels and induced, at the same time, its perinuclear localization. At present, the relevance of these opposite actions on Cx26 and Cx32 is not clear. It has been suggested that Cx32-containing gap junctions are mainly involved in hepatocyte differentiation, whereas their Cx26-based counterparts could play a role in hepatocyte proliferation (Kojima *et al.*, 2001; Yano *et al.*, 2001). On the other hand, previous work from our group showed that TSA favors the differentiated state of primary hepatocytes at the expense of their proliferative capacity (Papeleu *et al.*, 2003; Vanhaecke *et al.*, 2004a). Thus, our results might point to distinct regulation and/or biological roles of Cx32 and Cx26 in the control of the hepatocellular homeostatic balance.

As also described by others (Kojima *et al.*, 1995; Stutenkemper *et al.*, 1992; Willecke and Haubrich, 1996), we found that Cx43, a Cx species not physiologically present in adult rat hepatocytes, progressively appeared in primary culture. Cx43 was preferentially located in the nuclear compartment of primary cultured adult rat hepatocytes and was mainly expressed in its nonphosphorylated form. The biological significance of this phenomenon remains elusive. It has been proposed that newly appearing Cx43 in primary cultured hepatocytes could be due to proliferation and differentiation of nonparenchymal liver cells (e.g., oval cells), which are present in isolated hepatocyte preparations (Stutenkemper *et al.*, 1992). Alternatively, the progressive appearance of Cx43 might be considered as part of a dedifferentiation process, whereby primary cultured adult rat hepatocytes gradually adopt a more fetal-like Cx expression pattern (Willecke and Haubrich, 1996). Anyhow, TSA was found to increase Cx43 protein levels in this culture system. This finding is in agreement with the aforementioned works of Asklund *et al.* (2004) and Ogawa *et al.* (2005).

Overall, the TSA-induced alterations of Cx protein levels and their localization were associated with the enhancement of GJIC, as evidenced by increased dye coupling. It seems reasonable to assume that Cx32-based gap junctions accounted for elevated GJIC, since Cx26 and Cx43 were mainly found in the

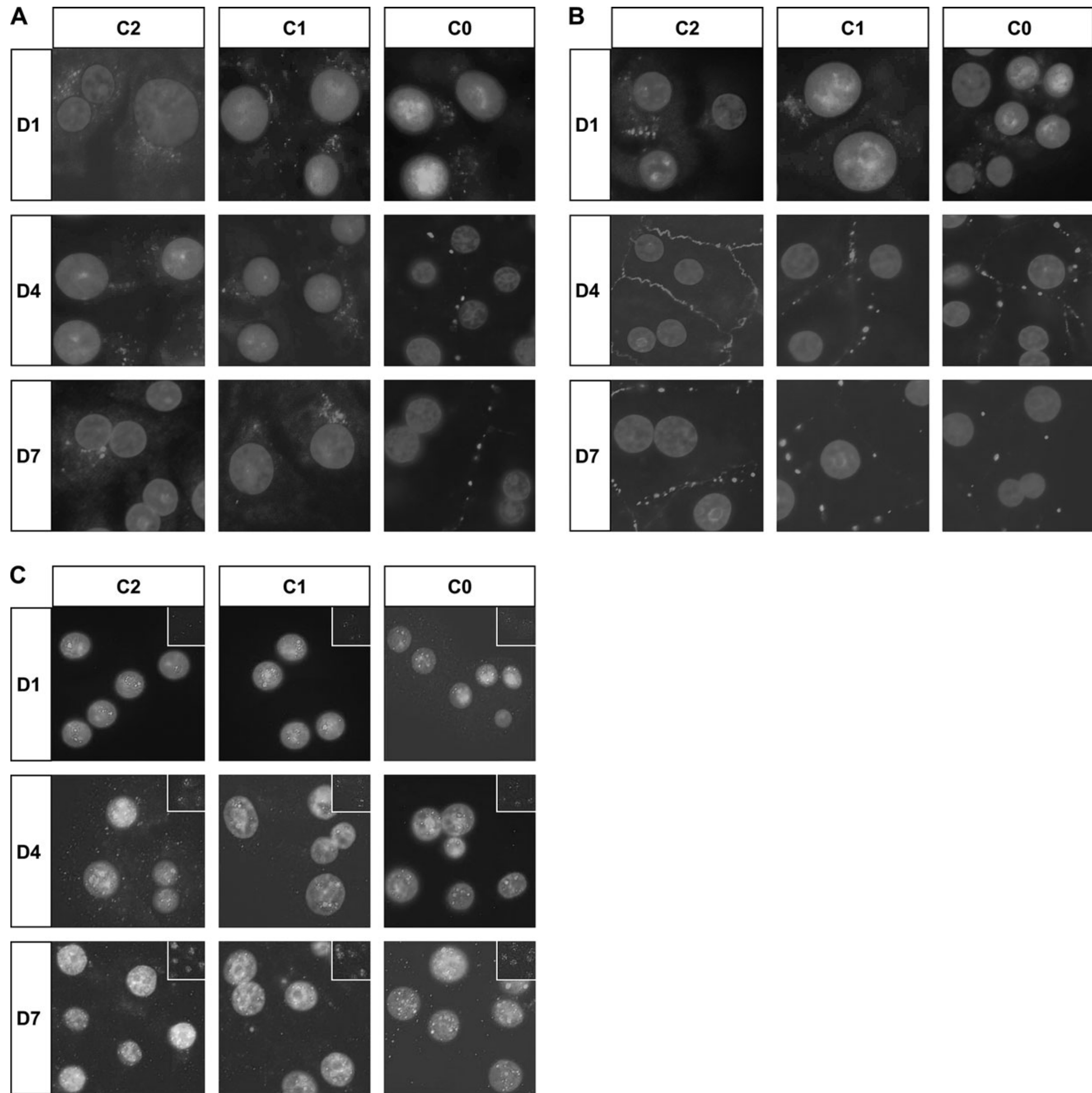

FIG. 5. Effects of TSA on the cellular localizations of Cx26 (A), Cx32 (B), and Cx43 (C) in primary cultured adult rat hepatocytes. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells and subjected to indirect immunocytochemistry (see ''Materials and Methods'') using primary antibodies (Table 1) and a tetramethyl rhodamine isothiocyanate–labeled secondary antibody (red). Nuclear counterstaining was performed with diamidinophenylindole (blue). For Cx43, unmerged images are provided in the right upper corners. Results shown are representative of at least three independent experiments (original magnification ×630).

cytosol and nucleus, respectively, of TSA-treated hepatocytes. In support of this hypothesis are some recent experiments performed in our laboratory, showing that ilimaquinone does not affect GJIC in TSA-treated primary rat hepatocyte cultures (data not shown). Ilimaquinone specifically inhibits GJIC in Cx43-HeLa cells, when compared to HeLa cells transfected with other Cx genes (e.g., Cx32; Cruciani and Mikalsen, 2005).

Strikingly, the influence of TSA on Cx protein levels and GJIC was greatly enhanced when drug treatment was started during the hepatocyte isolation procedure. It has been well documented that hepatocytes reenter the cell cycle upon their isolation, a process that is unavoidably accompanied by strongly modulated gene expression patterns (Baker *et al.*, 2001). We previously showed that cell cycle reentry can be prevented by performing hepatocyte isolation in the continued presence of TSA (Papeleu *et al.*, 2003). The results of this study demonstrate that this early inhibition of hepatocyte cell cycling by TSA is associated with its improved efficacy in relation to Cx

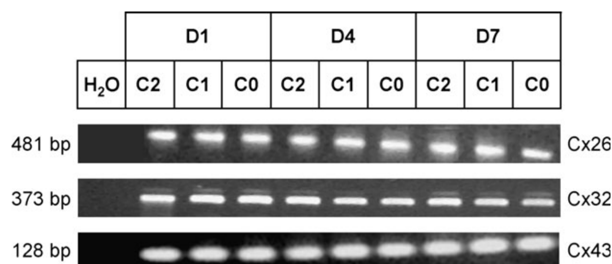

FIG. 6. Effects of TSA on Cx gene expression in primary cultured adult rat hepatocytes. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells. Total RNA was extracted, followed by DNase treatment, and samples were subjected to conventional RT-PCR analysis as described in ''Materials and Methods.'' Samples were separated by electrophoresis on agarose gel, and visualization of the products was performed with ethidium bromide under UV transillumination. Results shown are representative of at least three independent experiments (H_2O, template replaced by water).

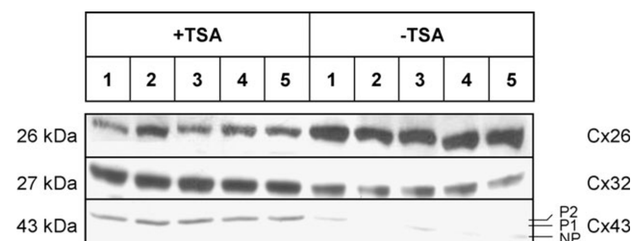

FIG. 7. Effects of TSA on Cx protein levels during hepatocyte isolation from adult rat liver. Isolation of hepatocytes from freshly removed adult rat liver was performed in the constant presence (+TSA) or absence (−TSA) of 1μM TSA. Samples were taken after dissociation of the cells from the liver (1), decantation of the supernatants from the cells (2), the first wash in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (3), the second wash in antibiotics-supplemented culture medium (4), and at the time of plating (5). Samples were subjected to immunoblot analysis (see ''Materials and Methods'') using primary antibodies (Table 1) and peroxidase-conjugated secondary antibodies. Detection of proteins was performed by means of enhanced chemiluminescence. Blots shown are representative of at least three independent experiments. (NP, nonphosphorylated Cx43; P1 and P2, phosphorylated Cx43 variants).

protein production and GJIC. Although the outcome already became evident during the hepatocyte isolation procedure, clear effects of TSA on Cx protein levels, especially on those of Cx32 and Cx43, were postponed to later cultivation time points. The occurrence of this ''gap phase'' was possibly attributed to the drastic environmental change that hepatocytes undergo upon their isolation from the liver. During the initial phases of cultivation, the hepatocyte machinery is mainly aimed at recovering from isolation-related stress (Baker *et al.*, 2001), which could delay the outcome of TSA efficacy.

TABLE 3
Effects of TSA on Cx Gene Expression in Primary Cultured Adult Rat Hepatocytes

	D1		D4		D7	
	C1	C2	C1	C2	C1	C2
Cx26	2.7 ± 0.6	2.4 ± 0.8	0.6 ± 0.2	0.4 ± 0.2	1.6 ± 0.7	1.3 ± 0.2
Cx32	2.8 ± 1.0	2.6 ± 0.8	0.5 ± 0.2	0.5 ± 0.3	1.6 ± 0.4	1.5 ± 0.3
Cx43	1.4 ± 0.2	1.6 ± 0.5	0.7 ± 0.1	1.0 ± 0.5	1.2 ± 0.3	1.2 ± 0.2

Note. Freshly isolated rat hepatocytes were cultivated either in the absence (C0) or presence (C1) of 1μM TSA. In another set of experiments, the isolated rat livers were perfused in the presence of 1μM TSA, and drug treatment was continued during subsequent cultivation of the hepatocytes (C2). Samples were taken from day 1, 4, and 7 (D1, D4, and D7) cultured cells. Total RNA was extracted, followed by DNase treatment, and samples were subjected to real-time qRT-PCR analysis as described in ''Materials and Methods.'' Ct values of the target genes were normalized to those of 18S rRNA, for both the test samples (C1 and C2 cultures) and the calibrator samples (C0 cultures). The resulting ΔCt values of the test samples were then normalized to those of the calibrator samples, yielding ΔΔCt. Relative alterations in mRNA levels were calculated according to the formula $2^{-(\Delta\Delta Ct)}$. Results represent fold change of controls (C0-cultured rat hepatocytes) at indicated time points and are mean values ± SDs of three independent experiments. Statistical significance ($p < 0.05$) between C0 and C1 and between C0 and C2 conditions was tested by ANOVA with *post hoc* Dunnett's tests.

The molecular mechanisms underlying the TSA-mediated effects on gap junction expression and activity in primary cultures of rat hepatocytes remain to be established. Ogawa and colleagues demonstrated that the SAHA-mediated induction of GJIC in *ras*-transformed WB-F344 rat liver epithelial cells and primary cultures of human peritoneal mesothelial cells is preceded by increased Cx43 gene transcription and, subsequently, enhanced Cx43 protein production (Ogawa *et al.*, 2005). There are at least three indications that this scenario does not fully apply to our experimental setting. First, the TSA-induced alterations were largely restricted to the protein level. Second, the influence of TSA on Cx protein levels was greatly enhanced by bringing the exposure forward, while Cx mRNA levels remained unaffected. Third, alterations of Cx protein levels already appeared early upon performing hepatocyte isolation in the presence of TSA. Thus, it seems likely that the mechanisms involved in the actions of TSA on Cx expression in primary cultures of adult rat hepatocytes are located at a level other than the transcriptional one (e.g., Cx protein processing).

In summary, two major conclusions can be drawn from the current study. (1) TSA differentially affects Cx protein levels and localization in primary rat hepatocyte cultures, thereby suggesting distinct regulation and/or potentially distinct roles of different Cx species in the control of hepatic homeostasis. (2) TSA improves GJIC between primary cultured adult rat hepatocytes, consequently showing the usefulness of HDAC inhibitors in the further optimization of liver-based *in vitro* models for pharmacotoxicological testing. For that purpose, HDAC inhibitor treatment is preferentially initiated during hepatocyte isolation.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES

Asklund, T., Appelskog, I. B., Ammerpohl, O., Ekstrom, T. J., and Almqvist, P. M. (2004). Histone deacetylase inhibitor 4-phenylbutyrate modulates glial fibrillary acidic protein and connexin 43 expression, and enhances gap-junction communication, in human glioblastoma cells. *Eur. J. Cancer* **40,** 1073–1081.

Baker, T. K., Carfagna, M. A., Gao, H., Dow, E. R., Li, Q., Searfoss, G. H., and Ryan, T. P. (2001). Temporal gene expression analysis of monolayer cultured rat hepatocytes. *Chem. Res. Toxicol.* **14,** 1218–1231.

Bergmeyer, H. U. (1974). Lactate dehydrogenase. In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, Ed.), Vol. 2, pp. 574–579. Academic Press, New York.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.

Cruciani, V., and Mikalsen, S.-O. (2005). Ilimaquinone inhibits gap junctional communication in a connexin isotype-specific manner. *Exp. Cell Res.* **304,** 136–148.

el-Fouly, M. H., Trosko, J. E., and Chang, C. C. (1987). Scrape-loading and dye transfer: A rapid and simple technique to study gap junctional intercellular communication. *Exp. Cell Res.* **168,** 422–430.

Fujita, M., Spray, D. C., Choi, H., Saez, J. C., Watanabe, T., Rosenberg, L. C., Hertzberg, E. L., and Reid, L. M. (1987). Glycosaminoglycans and proteoglycans induce gap junction expression and restore transcription of tissue-specific mRNAs in primary liver cultures. *Hepatology* **7,** 1S–9S.

Hamilton, G. A., Jolley, S. L., Gilbert, D., Coon, D. J., Barros, S., and LeCluyse, E. L. (2001). Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. *Cell Tissue Res.* **306,** 85–99.

Iwai, M., Harada, Y., Muramatsu, A., Tanaka, S., Mori, T., Okanoue, T., Katoh, F., Ohkusa, T., and Kashima, K. (2000). Development of gap junctional channels and intercellular communication in rat liver during ontogenesis. *J. Hepatol.* **32,** 11–18.

Kojima, T., Fort, A., Tao, M., Yamamoto, M., and Spray, D. C. (2001). Gap junction expression and cell proliferation in differentiating cultures of Cx43 KO mouse hepatocytes. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281,** G1004–G1013.

Kojima, T., Mitaka, T., Mizuguchi, T., and Mochizuki, Y. (1996). Effects of oxygen radical scavengers on connexins 32 and 26 expression in primary cultures of adult rat hepatocytes. *Carcinogenesis* **17,** 537–544.

Kojima, T., Mitaka, T., Paul, D. L., Mori, M., and Mochizuki, Y. (1995). Reappearance and long-term maintenance of connexin32 in proliferated adult rat hepatocytes: Use of serum-free L-15 medium supplemented with EGF and DMSO. *J. Cell Sci.* **108,** 1347–1357.

Mesnil, M., Piccoli, C., and Yamasaki, H. (1993). An improved long-term culture of rat hepatocytes to detect liver tumour-promoting agents: Results with phenobarbital. *Eur. J. Pharmacol.* **248,** 59–66.

Nakashima, Y., Ono, T., Yamanoi, A., El-Assal, O. N., Kohno, H., and Nagasue, N. (2004). Expression of gap junction protein connexin32 in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. *J. Gastroenterol.* **39,** 763–768.

Nathanson, M. H., Rios-Velez, L., Burgstahler, A. D., and Mennone, A. (1999). Communication *via* gap junctions modulates bile secretion in the isolated perfused rat liver. *Gastroenterology* **116,** 1176–1183.

Neveu, M. J., Babcock, K. L., Hertzberg, E. L., Paul, D. L., Nicholson, B. J., and Pitot, H. C. (1994). Colocalized alterations in connexin32 and cytochrome P450IIB1/2 by phenobarbital and related liver tumor promoters. *Cancer Res.* **54,** 3145–3152.

Ogawa, T., Hayashi, T., Tokunou, M., Nakachi, K., Trosko, J. E., Chang, C.-C., and Yorioka, N. (2005). Suberoylanilide hydroxamic acid enhances gap junctional intercellular communication *via* acetylation of histone containing connexin 43 gene locus. *Cancer Res.* **65,** 9771–9778.

Papeleu, P., Elaut, G., Rogiers, V., and Vanhaecke, T. (2002). Cell cultures as *in vitro* tools for biotransformation studies. In *Recent Research Developments in Drug Metabolism and Disposition* (S. G. Pandalai, Ed.), Vol. 1, pp. 199–234. Transworld Research Networks, Kerala, India.

Papeleu, P., Loyer, P., Vanhaecke, T., Elaut, G., Geerts, A., Guguen-Guillouzo, C., and Rogiers, V. (2003). Trichostatin A induces differential cell cycle arrests but does not induce apoptosis in primary cultures of mitogen-stimulated rat hepatocytes. *J. Hepatol.* **39,** 374–382.

Papeleu, P., Vanhaecke, T., Elaut, G., Vinken, M., Henkens, T., Snykers, S., and Rogiers, V. (2005a). Differential effects of histone deacetylase inhibitors in tumor and normal cells: What is the toxicological relevance? *Crit. Rev. Toxicol.* **35,** 363–378.

Papeleu, P., Vanhaecke, T., Henkens, T., Elaut, G., Vinken, M., Snykers, S., and Rogiers, V. (2005b). Isolation of rat hepatocytes. In *Methods in Molecular Biology: Cytochrome P450 Protocols* (I. R. Phillips and E. A. Shephard, Eds.), Vol. 320, pp. 229–238. Humana Press Inc., Totowa, NJ.

Ren, P., de Feijeter, A. W., Paul, D. L., and Ruch, R. J. (1994). Enhancement of liver cell gap junction protein expression by glucocorticoids. *Carcinogenesis* **15,** 1807–1813.

Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D., and Beyer, E. C. (2003). Plasma membrane channels formed by connexins: Their regulation and functions. *Physiol. Rev.* **83,** 1359–1400.

Saez, J. C., Gregory, W. A., Watanabe, G. T., Dermietzel, R., Hertzberg, E. L., Reid, L., Bennett, M. V. L., and Spray, D. C. (1989). cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. *Am. J. Physiol.* **257,** C1–C11.

Shoda, T., Mitsumori, K., Onodera, H., Toyoda, K., Uneyama, C., Imazawa, T., and Hirose, M. (1999). The relationship between decrease in Cx32 and induction of P450 isozymes in the early phase of clofibrate hepatocarcinogenesis in the rat. *Arch. Toxicol.* **73,** 373–380.

Spray, D. C., Fujita, M., Saez, J. C., Choi, H., Watanabe, T., Hertzberg, E., Rosenberg, L. C., and Reid, L. M. (1987). Proteoglycans and glycosaminoglycans induce gap junction synthesis and function in primary liver cultures. *J. Cell Biol.* **105,** 541–551.

Spray, D. C., Saez, J. C., Hertzberg, E. L., and Dermietzel, R. (1994). Gap junctions in liver: Composition, function, and regulation. In *The Liver: Biology and Pathobiology*, 2nd ed. (I. M. Arias, I. L. Boyer, N. Fausto, W. B. Jakoby, D. A. Schachter, and D. A. Shafritz, Eds.), pp. 951–967. Raven Press Ltd., New York.

Stoehr, S. A., and Isom, H. C. (2003). Gap junction-mediated intercellular communication in a long-term primary mouse hepatocyte culture system. *Hepatology* **38,** 1125–1135.

Stumpel, F., Ott, T., Willecke, K., and Jungermann, K. (1998). Connexin 32 gap junctions enhance stimulation of glucose output by glucagon and noradrenaline in mouse liver. *Hepatology* **28,** 1616–1620.

Stutenkemper, R., Geisse, S., Schwarz, H. J., Look, J., Traub, O., Nicholson, B. J., and Willecke, K. (1992). The hepatocyte-specific phenotype of murine liver cells correlates with high expression of connexin32 and connexin26 but very low expression of connexin43. *Exp. Cell Res.* **201,** 43–54.

Temme, A., Stumpel, F., Sohl, G., Rieber, E. P., Jungermann, K., Willecke, K., and Ott, T. (2001). Dilated bile canaliculi and attenuated decrease of nerve-dependent bile secretion in connexin32-deficient mouse liver. *Pflugers Arch.* **442,** 961–966.

Vanhaecke, T., Henkens, T., Kass, G. E. N., and Rogiers, V. (2004a). Effect of the histone deacetylase inhibitor Trichostatin A on spontaneous apoptosis in various types of adult rat hepatocyte cultures. *Biochem. Pharmacol.* **68,** 753–760.

Vanhaecke, T., Papeleu, P., Elaut, G., and Rogiers, V. (2004b). Trichostatin A-like hydroxamate histone deacetylase inhibitors as therapeutic agents: Toxicological point of view. *Curr. Med. Chem.* **11,** 1629–1643.

Willecke, K., and Haubrich, S. (1996). Connexin expression systems: To what extent do they reflect the situation in the animal? *J. Bioenerg. Biomembr.* **28,** 319–326.

Yang, J., Ichikawa, A., and Tsuchiya, T. (2003). A novel function of connexin 32: Marked enhancement of liver function in a hepatoma cell line. *Biochem. Biophys. Res. Commun.* **307,** 80–85.

Yano, T., Hernandez-Blazquez, F. J., Omori, Y., and Yamasaki, H. (2001). Reduction of malignant phenotype of HepG2 cell is associated with the expression of connexin 26 but not connexin 32. *Carcinogenesis* **22,** 1593–1600.