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Taurine preserves gap junctional intercellular communication in rat hepatocytes under oxidative stress

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Abstract: Gap junctional intercellular communication (GJIC) between hepatocytes is important for the maintenance of differentiated liver functions. Taurine is known to be cytoprotective, and is used clinically to improve liver functions. We evaluated the effect of taurine on GJIC in hepatocyte doublets under oxidative stress. Hepatocyte doublets were isolated from female Wistar rats, using a collagenase perfusion technique, and cultured in Leibovitz-15 medium containing fetal bovine serum (10%). H_2O_2 (2 mM) and/or taurine (0.1–1 mM) were added 2 h after inoculation, and the culture was incubated for 3 h. Fluorescent dye (Lucifer Yellow CH) coupling between adjacent cells was evaluated by microinjection. The distribution and quantity of connexin 32 (Cx32) in hepatocytes were detected using indirect immunofluorescence analysis and Western blotting. Steady state mRNA levels of Cx32 were detected by Northern blotting. The percentage of dye coupling 5 h after inoculation was 88 ± 6.3% in the control, however, this was decreased to almost half the control value by H_2O_2. Taurine prevented the decrease caused by H_2O_2 in a dose-dependent manner. Immunofluorescence analysis for Cx32 demonstrated numerous punctate fluorescent spots along the intercellular plasma membrane in controls, which were significantly decreased by H_2O_2. Taurine prevented the decrease of Cx32. Western blot analysis also showed the decrease of Cx32 protein levels by H_2O_2 treatment, which decrease was prevented by taurine. Interestingly, H_2O_2 and/or taurine treatments did not affect Cx32 mRNA levels. Our findings indicated that H_2O_2 treatment decreased GJIC between hepatocytes, most likely due to augmenting the degradation of Cx32 proteins, whereas taurine prevented this process. This effect of taurine is beneficial for the preservation of differentiated functions in the liver under oxidative stress.

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Introduction

Gap junctions are clusters of plasma membrane channels that connect the interiors of adjacent cells, and which permit the diffusion of small (<1-kDa) ions and second messengers, such as Ca^{2+}, ATP, and cyclic AMP, and inositol 1,4,5-triphosphate between neighboring cells. Gap junctions consist of hexametric structures of a transmembrane protein named connexin,[1–4] and these structures (connexon) form membrane pores between neighboring cells. In hepatocytes, two types of connexin proteins, Cx32 and Cx26, have been identified.[5–7] Gap junctional intercellular communication (GJIC) is one of the important functions of well differentiated cells. GJIC allows not only electronic coupling between excitable cells (ie, neuron and muscle cells) but also metabolic coupling between non-excitable cells. Further, GJIC is believed to regulate cell proliferation. For example, gap junctions transiently disappear in the regenerating liver, where hepatocytes are proliferating. Moreover, GJIC most likely plays an important role in carcinogenesis, since most cancer cells have lost or diminished GJIC.[8,9]

Taurine (2-aminoethanesulfonic acid), a sulfur-containing β-amino acid, is known to possess various physiological and pharmacological properties. In the liver, taurine is utilized for the conjugation of bile acids, one of the important processes in bile acid metabolism. Taurine is also known as a regulator of intracellular Ca^{2+}, and as an antioxidant and cytoprotectant.[10] Supplementation of taurine in chemically defined medium has been reported to maintain differentiated functions, such as albumin secretion, in primary cultured hepatocytes.[11] Taurine is, therefore, used clinically, in

particular in Japan, to improve liver function in pathophysiological conditions such as chronic viral hepatitis and liver cirrhosis.

Oxidative stress is involved in various pathological conditions in the liver. For example, reactive oxygen species (ROS) produced by Kupffer cells and infiltrating neutrophils play a pivotal role in the development of ischemia-reperfusion and alcohol-induced injury of the liver. It is also known that oxidative stress is one of the regulating factors of gap junctions.[12] Antioxidants have been shown to alleviate the down-regulation of GJIC caused by phenobarbital and DDT, and paraquat-generated oxygen radicals are reported to inhibit GJIC.[8,13–16] In this study, we focused on the function of gap junctions in hepatocytes under oxidative stress. The specific aim of this study was to evaluate the effect of taurine on GJIC in primary cultured hepatocyte doublets under oxidative stress. A preliminary account of this work has been presented in abstract form.[17]

Methods

Isolation and culture of rat hepatocytes

Hepatocyte doublets were isolated from female Wistar rats, weighing 200–250 g (Charles River Japan, Saitama, Japan), by collagenase perfusion and differential centrifugation, based on the method of Seglen,[18] with some modifications. Briefly, the liver was perfused in situ through the portal vein with Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution (HBSS) for 5 min. The liver was then perfused with Leibovitz-15 (L-15) medium (GIBCO/BRL; Life Technologies, Grand Island, NY, USA) containing 0.05% type-I collagenase (Sigma Chemical, St Louis, MO, USA) for approximately 10 min. The liver was then excised, and the dispersed cells were passed through serial nylon mesh filters and resuspended in the medium. Subsequently, non-parenchymal cells were removed by centrifugation at 50 g performed three times. Initial viability of the isolated hepatocytes was always above 90% by the trypan blue exclusion test. This isolation procedure provided approximately 20%–30% of isolated hepatocytes as doublet cells. Isolated hepatocytes were inoculated in L-15 medium with 10% fetal bovine serum (FBS), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES), 100 U/l penicillin, and 100 mg/ml streptomycin (GIBCO/BRL; Life Technologies) on 60-mm uncoated polystyrene dishes (Corning, Corning, NY, USA) at a density of 1×10^6 viable cells/dish. Hepatocytes were then cultured at 37°C in humidified air for 2 h prior to experiments.

Fluorescent dye transfer in hepatocyte doublets

For the measurement of fluorescent dye transfer, a phase contrast microscope equipped with a microinjector (IMP-2; Olympus, Tokyo, Japan) was used. Micropippettes were prepared using an electric pipette puller (Narishige Scientific Instrument Laboratory, Tokyo, Japan). Lucifer Yellow CH (10 mM in 150 mM LiCl solution; Sigma Chemical) was microinjected into one hepatocyte of the doublet cells and dye coupling was assessed under a fluorescence microscope. Twenty hepatocyte doublets were examined in each condition and the same experiments were repeated seven times to calculate the dye transfer rates.

Immunofluorescent analysis of Cx32

Indirect immunofluorescent analysis of Cx32 was performed using a monoclonal antibody for rat liver Cx32, according to the method of Takeda et al.,[19] with some modifications. Briefly, hepatocyte cultures were were rinsed with saline solution, frozen in liquid nitrogen, and then freeze-dried at −20°C overnight. Hepatocyte cultures were rinsed with 10-mM phosphate-buffered saline (PBS) three times and blocked with normal sera at 37°C for 30 min to avoid non-specific binding sites. The cells were then incubated with a mouse anti-rat Cx32 antibody (generous gift from Nippon Shinyaku, Kyoto, Japan) at 37°C for 60 min. Subsequently, hepatocyte cultures were rinsed with PBS three times and then incubated with the secondary fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:200 dilution in PBS at 37°C for 45 min. After incubation, the cells were rinsed with PBS three times and mounted in 3:2 glycerol/PBS solution containing 0.1% *p*-phenylenediamine. Photographs were taken using an epifluorescence microscope (Zeiss, Oberkochen, Germany).

Western blot analysis of Cx32

Cultured cells were homogenized in a buffer containing 66 mM Tris, pH 8.0; 5 mM ethylenediaminetetraacetic acid (EDTA); 1% Triton X-100; and protease/phosphatase inhibitors (Bestatin, Roche Diagnostics, Mannheim, Germany), 40 μg/ml; β-glycerophosphate (Sigma Chemical), 20 mM; P-nitrophenyl phosphate [PNPP] (Roche Diagnostics), 10 mM; Pefabloc (Roche Diagnostics), 500 μM; Pepstatin A (Roche Diagnostics), 700 ng/ml; Aprotinin (Roche Diagnostics), 2 μg/ml; Na_3VO_4 (Sigma Chemical), 500 μM; Leupeptin (Roche Diagnostics), 500 ng/ml, and the protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Thirty μg of protein was separated by 10% sodium dodecylsulfate-polyacrylamide gel elecrophoresis (SDS-PAGE) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Hybond-P; Amersham-

Pharmacia Bio Tech, Denver, CO, USA). The membranes were soaked with nonfat dry milk solution in Tris-buffered saline (TBS) overnight with gentle shaking and then incubated with a polyclonal rabbit anti-Cx32 (1:1000 dilution in TBS; Zymed Laboratories, South San Francisco, CA, USA) for 3 h at room temperature. After three washes with TBS containing 0.05% Triton X-100 (TBS-T), the membranes were incubated with a secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG; Santa Cruz Biotechnology) at 1:1500 dilution in TBS with 5% dry milk for 1 h at room temperature and washed three times. An enhanced chemiluminescence (ECL) detection kit (Amersham-Pharmacia Bio Tech) was empolyed, according to the manufacturer's instructions, for the detection of specific bands.

Northern blot analysis of Cx32

Total RNA from cultured hepatocytes was prepared by a single-step guanidine isothiocyanate-phenol-chloroform extraction method (Trizol; GIBCO/BRL Life Technologies). Twenty Micrograms of total RNA was separated in 1% agarose gel containing 2.2 mM formaldehyde and capillary-transferred to a nylon membrane (Magna-Graph; Osmonics Inc, Minnetonka, MN, USA) and fixed by ultraviolet light cross-linking. The membranes were prehybridized in a solution containing 50% formamide, 5 × standard saline citrate (SSC), 1 × Denhardt's solution, and 100 μg/ml salmon sperm DNA at 42°C for 90 min, and then hybridized with a ^{32}P-labeled cDNA probe for rat Cx32 in the same solution at 42°C overnight. The membranes were then washed in low stringency salt solutions and exposed to X-ray films (X-Omat AR; Kodak, Rochester, NY, USA). Subsequently, the membranes were reprobed for glycerol-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

Statistical analysis

Data values for the dye-coupling experiments were expressed as the means ± SD of seven separate experiments. Significant differences between mean values were verified by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. $P < 0.05$ was selected prior to the experiment to determine significance.

Results

Morphological changes in hepatocyte doublets

Morphological changes in primary cultured hepatocyte doublets were observed under the phase-contrast microscope. The hepatocyte doublets were pre-incubated for 2 h after inoculation, and subsequently incubated with medium containing H_2O_2 (2 mM) and/or taurine (1 mM) for 3 h. Figure 1A–D shows representative photographs of hepatocyte doublets under phase-contrast microscopy. In the control group, the shape of the cells was round and the surface of the plasma membrane was smooth (Fig. 1A). However, the cell surface became rough with marked bleb formation after 3-h incubation with H_2O_2 (Fig. 1B). In the group with taurine alone, the shape of cells was round and clear, as in the control (Fig. 1C). Interestingly, taurine dramatically prevented the bleb formation caused by H_2O_2 (Fig. 1D).

Dye-coupling between hepatocytes

To assess GJIC between hepatocytes, we performed a fluorescent dye transfer assay, using Lucifer Yellow CH. The lower panels of Fig. 1 show representative photographs of hepatocyte doublets after the microinjection of Lucifer Yellow CH under fluorescence microscopy. As shown in Fig. 1E, overt dye transfer was observed immediately after the injection of Lucifer yellow CH in control hepatocyte doublets 5 h after inoculation. However, the dye transfer was markedly diminished in the H_2O_2-treated group (Fig. 1F). In the group with taurine alone, immediate dye transfer was observed, similar to findings in controls (Fig. 1G), and taurine prevented the decrease of dye transfer due to H_2O_2 (Fig. 1H). Figure 2 shows the percentages of dye-coupled hepatocyte doublets in the presence of H_2O_2 and/or taurine. The dye-coupling rate of untreated hepatocyte doublets 5 h after inoculation was above 90%. However, the dye-coupling rate was decreased significantly by H_2O_2 treatment, to levels almost half of the control. Taurine prevented this decrease in a dose-dependent manner, with almost complete reversal at 1 mM.

Indirect immunofluorescence analysis of Cx32

Connexin 32 (Cx32), the major gap junction protein in hepatocytes, was detected by immunofluorescent staining, using an anti-rat Cx32 antibody (Fig. 3). In the control group, numerous Cx32-specific punctate spots were observed, predominantly along the plasma membrane between doublet cells (Fig. 3A), confirming the localization of Cx32 in intact cells. However, H_2O_2 treatment for 3 h markedly decreased the Cx32-specific spots as compared with findings in controls (Fig. 3B). In the group with taurine alone, the positive punctuate spots were observed to almost the same extent as in controls (Fig. 3C). Taurine (1 mM) completely prevented the decrease of Cx32-specific spots due to H_2O_2 (Fig. 3D). The lower concentration of taurine (0.1 mM) was less effective than 1 mM taurine (data not shown).

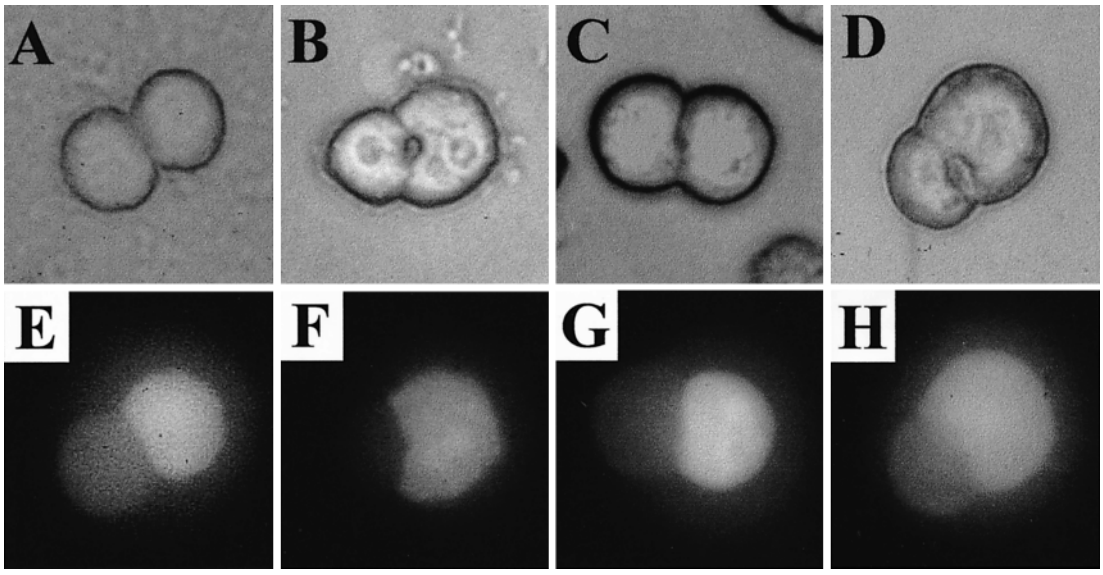

Fig. 1A–H. Morphological changes and dye-coupling of hepatocyte doublets. Hepatocyte doublets were preincubated for 2 h after inoculation and then incubated with media containing H_2O_2 (2 mM) and/or taurine (1 mM) for 3 h prior to experiments. Morphological changes in hepatocyte doublets were observed under a phase-contrast microscope (*upper panels*, **A–D**). Subsequently, dye-coupling of doublet cells was assessed by the microinjection of Lucifer yellow CH (*lower panels*, **E–H**). **A,E** Untreated hepatocyte doublets 5 h after inoculation. **B,F** Doublets incubated with 2 mM H_2O_2 for 3 h. **C,G** Doublets incubated with taurine (1 mM) for 3 h. **D,H** Doublets incubated with both H_2O_2 and taurine. ×600

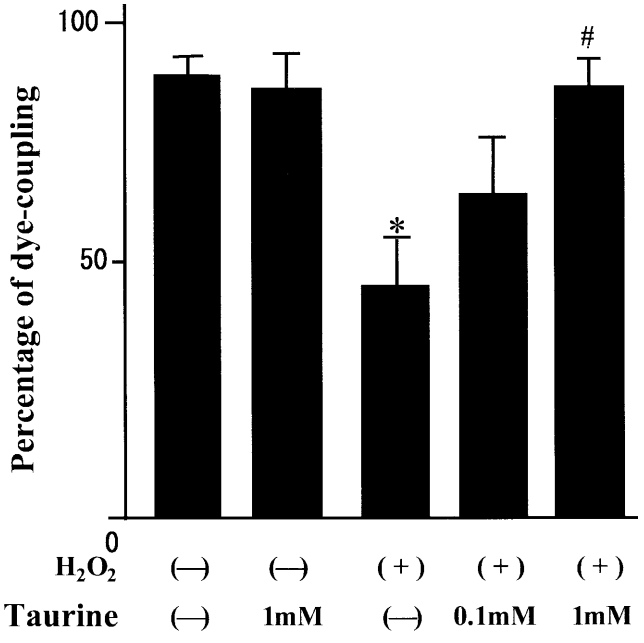

Fig. 2. Effect of taurine on intercellular communication in hepatocyte doublets. Experimental conditions were as noted in *Fig. 1*. The percentage of dye-coupled doublets was calculated by microinjection of Lucifer yellow CH to 20 hepatocyte doublets in each condition ($n = 7$; mean ± SD; $^*P < 0.05$ vs control; $\#P < 0.05$ vs H_2O_2 alone using one-way analysis of variance [ANOVA] and Tukey's post-hoc test)

Western blot analysis of Cx32

To evaluate the Cx32 protein levels quantitatively in each condition, we performed Western blot analysis using polyclonal antibodies for rat Cx32. Figure 4 shows representative photographs of Cx32-specific 27-kDa bands. In the control group 5 h after inoculation, a strong band was detected (first lane). However, the specific band was markedly diminished in H_2O_2-treated groups (second lane). Taurine treatment alone did not affect the protein level of Cx32 as compared with findings in controls (third lane); however, taurine almost completely prevented the decrease in Cx32 protein levels caused by H_2O_2 (fourth lane).

Northern blot analysis of Cx32

Cx32 mRNA steady state levels were determined by Northern blot analysis to clarify possible changes in Cx32 mRNA levels produced by H_2O_2 and/or taurine (Fig. 5). The intensities of the Cx32-specific bands, however, were similar in each treatment group, indicating that H_2O_2 and/or taurine (1 mM) did not alter Cx32 mRNA levels in primary cultured hepatocytes. Further, higher concentrations of taurine, of up to 50 mM, had no effect on Cx32 mRNA levels (data not shown).

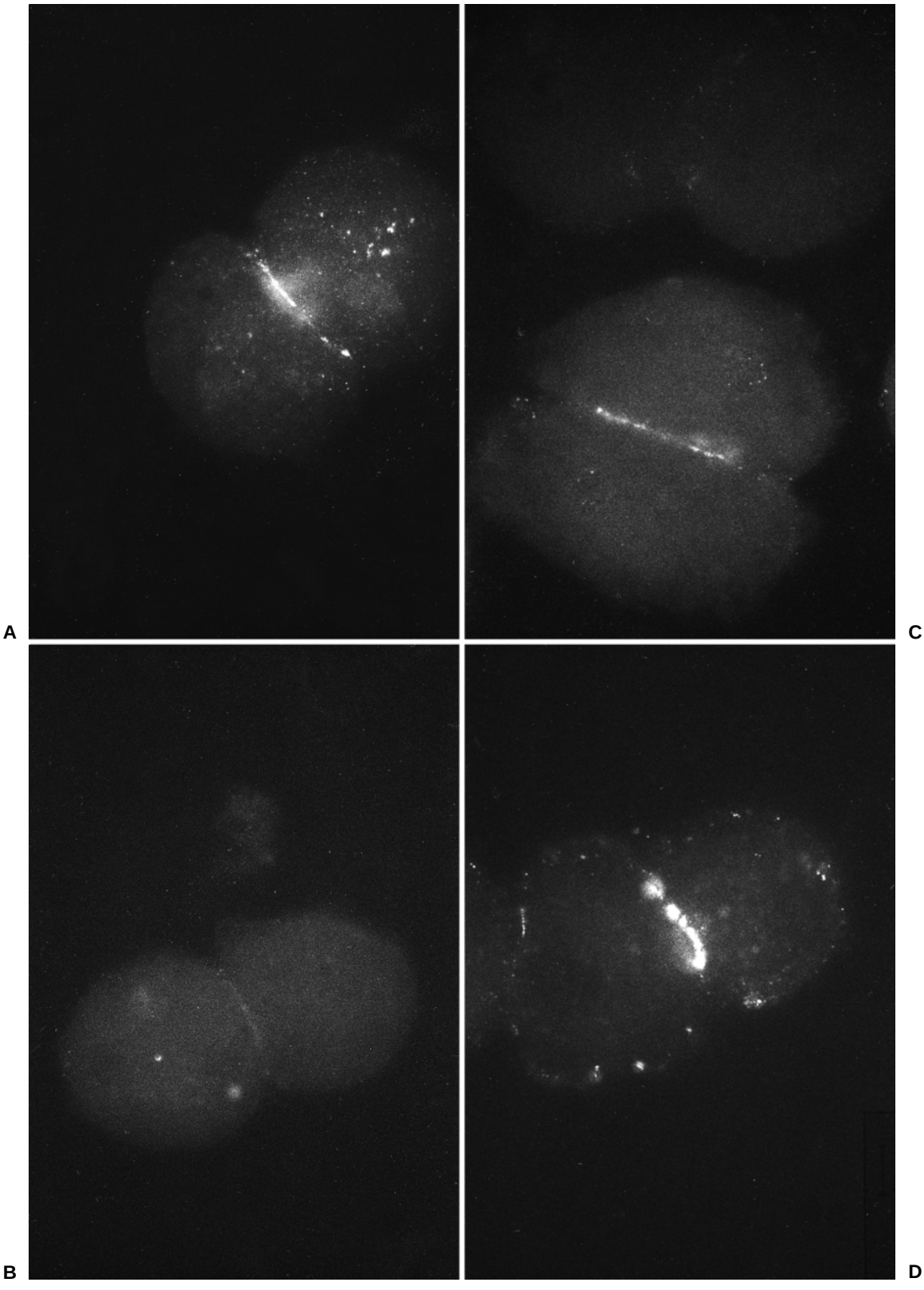

Fig. 3A–D. Immunofluorescence analysis of Cx32. Experimental conditions were as noted in *Fig. 1*. Immunofluorescence analysis was performed using a monoclonal antibody for rat liver Cx32. **A** Untreated hepatocyte doublets 5 h after inoculation. **B** Doublets incubated with 2 mM H_2O_2 for 3 h. **C** Doublets incubated with taurine (1 mM) for 3 h. **D** Doublets incubated with both H_2O_2 and taurine. Representative photographs from four individual preparations are shown. ×1000

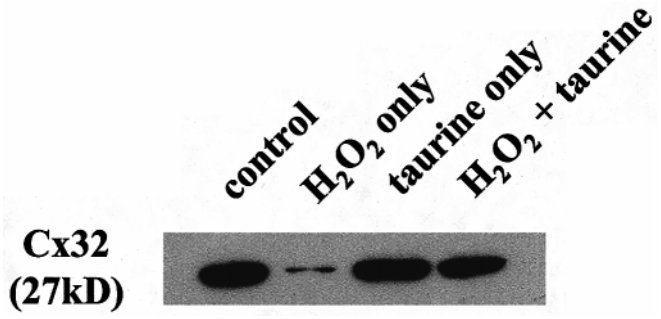

Fig. 4. Western blot analysis of Cx32. Whole cell protein extracts (30 μg) from cells in each condition were separated on 10% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blotting, using a polyclonal antibody for rat Cx32. Representative photographs of specific bands at 27 kDa from four individual experiments are shown. *First lane*, control; *second lane*, H_2O_2 alone (2 mM); *third lane*, taurine alone (1 mM); *fourth lane*, H_2O_2 plus taurine

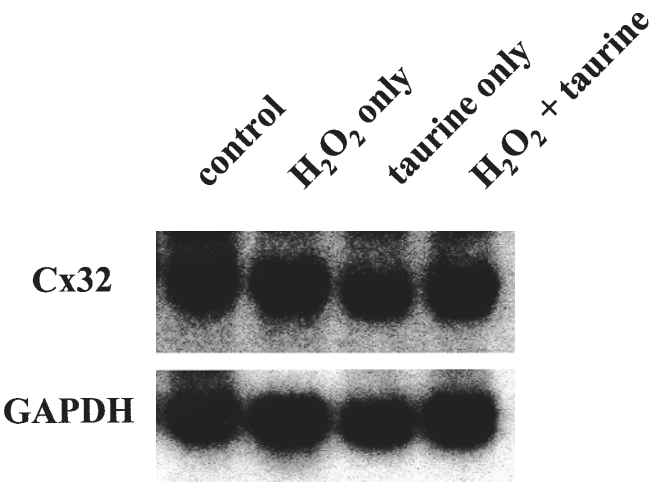

Fig. 5. Northern blot analysis of Cx32. Total RNA (20 μg) from cells in each condition was analyzed by Northern blotting for Cx32. Subsequently; membranes were reprobed for glycerol-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. *First lane*, control; *second lane*, H_2O_2 alone (2 mM); *third lane*, taurine alone (1 mM); *fourth lane*, H_2O_2 plus taurine

Discussion

In this study, primary cultured hepatocyte doublets were utilized to analyze gap junctions as a marker of differentiated functions in hepatocytes. Hepatocyte doublets are believed to maintain differentiated functions in hepatocytes in vivo, since they preserve gap junctions, tight junctions, and functional bile canaliculi between adjacent cells. Previous studies have demonstrated that hepatocyte doublets lose polarity and differentiated functions (ie, bile secretion, contraction of bile canaliculi, and albumin synthesis) in an early time course of culture. It was reported that hepatocyte doublets just after isolation preserved GJIC, measured by electrical conductance and dye coupling, which diminished gradually within 24 h after inoculation.[20,21] Further, hepatocyte growth factor (HGF), which is a strong growth promoter of hepatocytes, has been shown to accelerate the disappearance of GJIC in hepatocyte doublets.[20] These observations indicate that GJIC represents cellular differentiation status. In the present study, it was demonstrated that GJIC in hepatocyte doublets decreased rapidly with H_2O_2 treatment (Fig. 1E, F and Fig. 2), indicating that the cells had lost one of their differentiated functions because of the H_2O_2 treatment. This observation was consistent with the report of Brad et al.[12] that H_2O_2 inhibits GJIC in liver epithelial cells. Further, radical scavengers were reported to preserve GJIC in hepatocytes cultured for an extended time, by preventing the loss of Cx32.[22,23] These findings support the hypothesis that oxidative stress suppresses the differentiated functions of hepatocytes, including GJIC.

In the present study, taurine was demonstrated to be protective against the H_2O_2-induced inhibition of GJIC. Taurine is known to act as an antioxidant in vivo, but the precise mechanism of this action is still unclear. Taurine has the capacity to react directly with hypochlorous acid (HOCl), a well known oxidant, to generate taurine chloroamine.[24] It was demonstrated, however, that taurine did not react with H_2O_2 and hydroxyl radical (·OH) directly; suggesting that taurine in the present study did not elicit its effect by acting as a direct radical scavenger. Instead, taurine prevented the loss of Cx32 protein due to H_2O_2 treatment (Figs. 3 and 4), indicating that taurine maintained GJIC by regulating the amount of Cx32 protein rather than by regulating the gating of gap junction channels. The decrease in Cx32 protein levels produced by H_2O_2 was mainly due to enhanced degradation of the protein, since mRNA steady state levels were not decreased by H_2O_2 treatment (Fig. 5). To assess the precise turnover of Cx32 levels, however, more complicated techniques, such as pulse labeling and chasing, are required. It is concluded, therefore, that taurine most likely prevented the degradation or denaturation of Cx32 protein due to H_2O_2 treatment.

The effect of taurine in maintaining cell differentiation under oxidative stress was further demonstrated morphologically. In primary cultured hepatocytes, cell shape is closely correlated with differentiated functions. Cells just after inoculation, which preserve differentiated functions, as in vivo, have a round shape;

however, they become flattenned and spread during the time course of culture. In this study, hepatocyte doublets 5 h after inoculation preserved their round shape, with a smooth plasma membrane (Fig. 1A), suggesting that the differentiated functions were well preserved in these cells. In contrast, doublets treated with H_2O_2 showed a spread form with bleb formation (Fig. 1B), which most likely indicated that H_2O_2 impaired differentiated cell functions. Indeed, bleb formation represents derangement of the cytoskeleton and is often observed in cells that are becoming necrotic. Taurine, however, dramatically prevented this morphological change (bleb formation) in hepatocyte doublets treated with H_2O_2 (Fig. 1D), presumably due to its antioxidant properties. These findings also support the hypothesis that taurine is beneficial for the maintenance of the differentiated functions in hepatocytes.

Changes in GJIC under physiological and/or pathological conditions affect coordinated cellular activities in many organs. It is likely that GJIC allows synchronization of metabolic functions and coordination of organ-level responses in the liver, as in excitable tissues. Recent studies using Cx32 knockout mice demonstrated that sympathetic nerve stimuli did not propagate efficiently in hepatic lobules, leading to lower levels of glucose mobilization from glycogen stores in the livers of these animals.[25,26] Since it is well known that oxidative stress plays a crucial role under various pathological conditions, such as in the presence of alcoholic liver diseases, and in liver surgery and transplantation, it is possible that liver dysfunction under oxidative stress may be, at least in part, a consequence of impaired GJIC in hepatocytes. Indeed, it has been shown that gap junctions are decreased after ischemia-reperfusion of the liver in vivo.[27] Further, it was also demonstrated that the facilitation of GJIC by irsogladine, a chemical known to increase GJIC, protected gastric mucosa against ischemia-reperfusion.[28] The protective effect of taurine against the loss of GJIC in hepatocytes under oxidative stress shown in the present study may be beneficial for the maintenance of liver-specific differentiated functions under oxidative stress.

In conclusion, H_2O_2 inhibited the gap junctional intercellular communication (GJIC) in rat primary hepatocyte doublets, whereas taurine preserved GJIC in these cells under oxidative stress in a dose-dependent manner. Based on the lines of evidence reported previously and the findings in the present study, it is suggested that taurine preserves the differentiation of hepatocytes through the maintenance of active gap junction proteins. This effect of taurine on GJIC may be helpful for the preservation of differentiated functions in liver under oxidative stress.

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