

A high glucose condition sensitizes human hepatocytes to hydrogen peroxide-induced cell death

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Abstract. Oxidative stress is known to play a key role in the progression of liver disease, including non-alcoholic steatohepatitis (NASH), which is often accompanied by hyperglycemia. This study examined the influence of high glucose on oxidative stress-induced hepatic cell death. Hc cells, a normal human hepatocyte-derived cell line, were cultured in normal-to-high glucose (5.5-22 mM)-containing medium with varying concentrations (0.01-1 mM) of hydrogen peroxide. In certain experiments, cyclosporine A (CyA), which inhibits the mitochondrial permeability transition (MPT) pore, or Z-VAD-FMK (z-VAD), a pan-caspase inhibitor, were added to the medium. Cell viability was evaluated using a colorimetric assay. The mode of cell death was determined by nuclear staining methods using Hoechst 33258 and Sytox green. Neither high glucose (22 mM) nor 0.05-0.5 mM of hydrogen peroxide alone killed Hc cells. However, a combination of the two induced cell death, causing the nuclei of Hc cells to become expanded rather than condensed, and the nuclear membrane to become weak. CyA, but not z-VAD, blocked cell death. These results suggest that a high glucose condition may cause human hepatocytes to undergo hydrogen peroxide-induced necrotic cell death.

Introduction

Recently, oxidative stress has been recognized as the major cause of liver disease, including non-alcoholic steatohepatitis (NASH), alcoholic liver disease (ALD) and hepatitis C virus (HCV)-related liver disease (1). The pathogenesis of oxidative stress is currently under investigation. Reactive oxygen species such as (ROS), superoxide and hydrogen peroxide, which are induced in the normal liver, are among the causes of oxidative stress. During inflammation due to infection, activated macrophages and neutrophils undergo respiratory bursts and release ROS to destroy invading organisms (2,3). This function is an important normal immune reaction to infection in the liver. Alcohol also causes increased ROS production in the liver through several metabolic pathways, and the induced ROS accelerate hepatocyte cell death (4,5). In addition, metabolic syndrome, closely associated with NASH, generates ROS in the liver and other tissues (6). ROS generation resulting in abnormal mitochondrial function has been observed in NASH, and oxidative stress plays a role not only in hepatic steatosis but also in hepatic fibrosis (7). HCV also causes mitochondrial damage in hepatocytes and increases ROS (8,9), followed by ROS-induced hepatic steatosis (10,11). Hepatic cancer in HCV transgenic mice is moreover associated with the generation of ROS (12). In short, ROS are generated with various liver diseases and influence hepatocyte and hepatic stromal cells (13). Oxidative stress has potentially important implications for the progression of liver disease.

Oxidative stress induces insulin resistance in the liver (14) and muscle (15). It is known that insulin resistance in the liver is brought on by deficiencies in insulin signaling in hepatocytes (16) and causes hyperinsulinemia, hyperglycemia and other metabolic syndrome factors. Both hyperinsulinemia and hyperglycemia were recently noted as being a cause of liver damage (17). A previous study revealed that maintaining normoglycemia by the use of insulin reduces morbidity and mortality in critically ill patients. This phenomenon is assumed to be related to the protection of hepatocyte mitochondria ultra-structure and function by normoglycemia (18). On the other hand, hyperglycemia-related cell death is observed in various cell types. For instance, high glucose has been reported to induce endothelial cell death (19), cardiac myoblast cell death

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Abbreviations: NASH, non-alcoholic steatohepatitis; ALD, alcoholic liver disease; HCV, hepatitis C virus; ROS, reactive oxygen species; CTGF, connective tissue growth factor; CyA, cyclosporine A; MPT, mitochondrial permeability transition; CypD, cyclophilin D; Tac, tacrolimus

Key words: high glucose, reactive oxygen species, hepatocyte cell death, necrosis, cyclosporine A

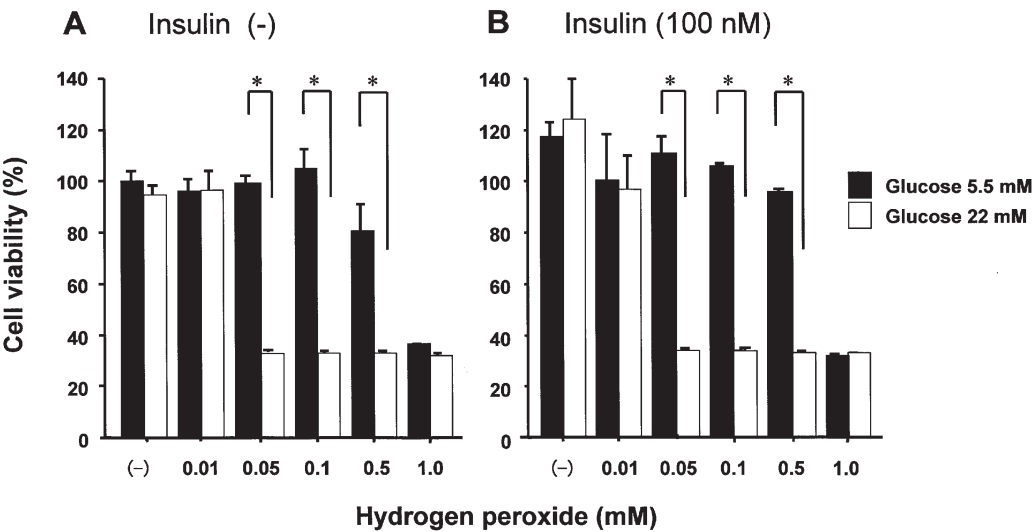


Figure 1. Effect of varying concentrations of hydrogen peroxide on Hc cell viability under normal or high glucose culture conditions in the absence (A) or presence (B) of insulin. Black bars indicate a normal glucose (5.5 mM) culture condition, and white bars a high glucose (22 mM) culture condition. After a 2-h exposure to hydrogen peroxide (0.01-1.0 mM), cell viability was determined using a colorimetric method. Cell viability in each culture condition is expressed as a percentage compared to the viability of the control (normal glucose medium without hydrogen peroxide and insulin). Data represent the mean \pm SD values of four independent experiments. * $p < 0.01$.

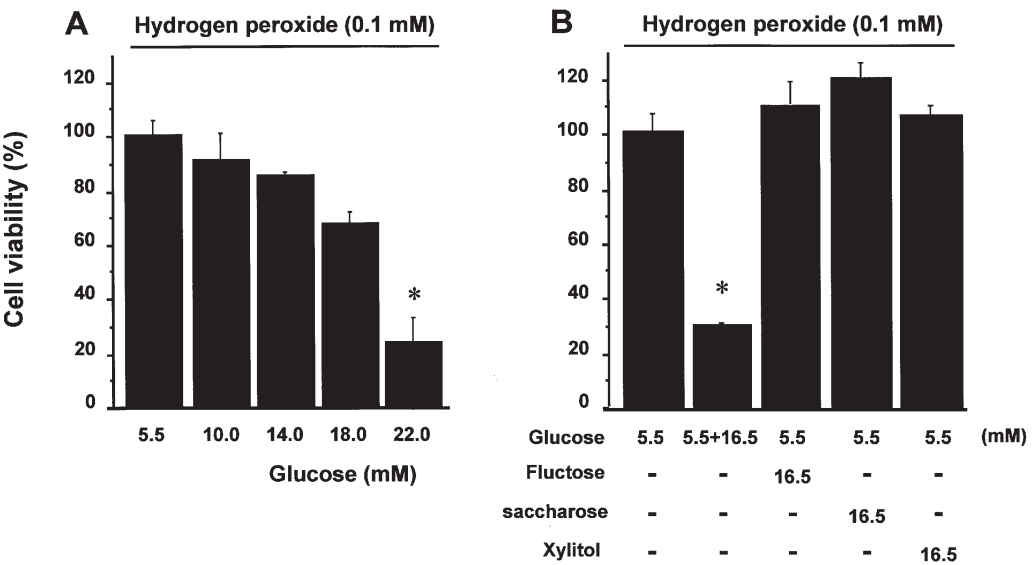


Figure 2. Effect of varying concentrations of glucose (A) and various types of saccharides (B) on hydrogen peroxide-induced Hc cell death. Hc cells were cultured in medium containing indicated concentrations of glucose with 0.1 mM hydrogen peroxide (A). Glucose, fructose, saccharose or xylitol (17.5 mM) were added to the culture medium containing 5.5 mM glucose with 0.1 mM hydrogen peroxide (B). Cell viability in each culture condition is expressed as a percentage compared to the viability of the control (5.5 mM glucose medium with 0.1 mM hydrogen peroxide). Data represent the mean \pm SD values of four independent experiments. * $p < 0.01$ vs. others.

(20), neuron death (21), muscle cell death (22) and podocyte cell death *in vitro* (23). In addition, high glucose stimulated connective tissue growth factor expression, a key factor of hepatic fibrosis, in hepatic stellate cells (24). This indicates that it might play an important role in hepatic fibrosis (25). However, the influence of high glucose on hepatocytes has not been thoroughly investigated.

Advanced liver disease by alcohol, NASH or HCV infection does not result from a single factor. The two-hit theory for the progression of NASH is a well-known hypothesis (26). Specifically, hepatic steatosis, which is correlated with oxidative stress caused by various etiologies (28), is known

to be a co-factor in other liver diseases (27). A previous report demonstrated that ROS determines susceptibility to TGF- β -induced apoptosis in cirrhotic hepatocytes (29) and to TNF- α -induced apoptosis in primary hepatocytes (30). Components of metabolic syndrome, such as hyperglycemia and hyperinsulinemia, may also be negative factors for liver disease (31,32). However, the influence of the combination of metabolic syndrome and ROS on hepatocyte cell death has yet to be examined.

This study attempted to examine the influence of high glucose on ROS-induced hepatocyte cell death using hydrogen peroxide as a representative ROS.

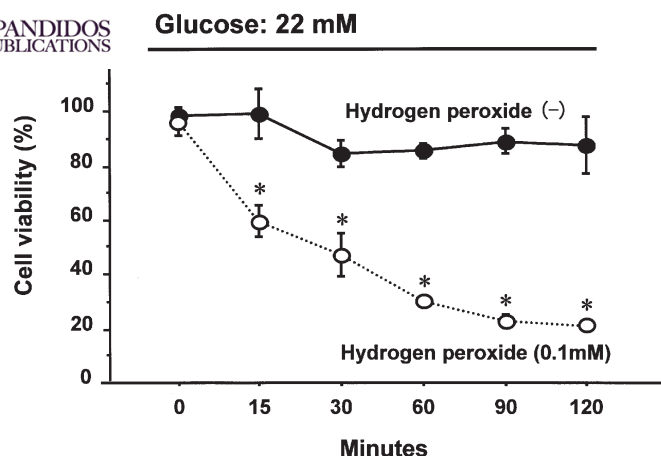
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Figure 3. Time course of cell viability after exposure to high glucose with or without hydrogen peroxide. White circles indicate exposure with 0.1 mM hydrogen peroxide, and black circles exposure without hydrogen peroxide. Cell viability is expressed as a percentage compared to the viability of the control (22 mM glucose without 0.1 mM hydrogen peroxide at 0 min). Data represent the mean \pm SD values of four independent experiments. * $p < 0.01$ vs. without hydrogen peroxide at each indicated time point.

Materials and methods

Reagents and cell culture. Insulin, cyclosporine A (CyA) and tacrolimus (Tac) were generous gifts from Novartis Pharma Co. (Basel, Switzerland) and Astellas Co. (Tokyo, Japan), respectively. Z-VAD-FMK (z-VAD), a pan-caspase inhibitor, was purchased from Sigma (St. Louis, MO, USA), and hydrogen peroxide, fructose, saccharose and xylitol from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Hc cells, a normal human hepatocyte-derived cell line, (Applied Cell Biology Research Institute, Kirkland, WA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 5.5 mM glucose supplemented with 10% fetal bovine serum. Cells (3×10^3) were placed into 96-well multiplates. After 8 h, the medium was replaced with fresh serum-free DMEM containing 5.5 mM of glucose, and the cells were incubated for 24 h. This was followed by treatment with varying concentrations of hydrogen peroxide in serum-free DMEM containing 5.5 mM (normal) or 22 mM (high) glucose for 15-120 min. Next, cell viability was determined. In certain experiments, 100 nM of insulin was added to the culture medium together with hydrogen peroxide. Varying concentrations of CyA, Tac or z-VAD were added to the culture medium 30 min before hydrogen peroxide treatment.

Cell viability assay. Cell viability was determined by the colorimetric method using a Cell Counting Kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). Data were expressed as the mean \pm SD. Statistical significance was assessed using the Student's t-test. Statistically significant difference was defined as $p < 0.05$. All numerical results are the mean of four independent experiments.

Fluorescent nuclear staining. The Hc cells were seeded onto 11-mm glass cover-slips in 24-well plates at 2×10^4 cells/well.

The next day, the medium was replaced with serum-free high or normal glucose DMEM, and the cells were treated with hydrogen peroxide. To determine the mode of cell death, Hc cells were incubated for 15 min with Sytox green nucleic acid stain (Molecular Probes, Eugene, OR, USA), which penetrates cells with compromised plasma membranes but does not cross the membranes of viable cells or apoptotic bodies. Hc cells were also stained with 1 mM of Hoechst 33258 (Invitrogen Japan K.K., Tokyo, Japan) for 30 min, and morphological changes in the nuclei were examined. Fluorescent nuclei were visualized using an Olympus BX50 microscope (Olympus, Tokyo, Japan) at 450-490 nm, and images captured using a Nikon DXM 1200 digital camera (Nikon, Tokyo, Japan).

Results

High glucose enhances sensitivity in hydrogen peroxide-induced Hc cell death. We examined the effects of a high glucose (22 mM) culture condition on the susceptibility of Hc cells to hydrogen peroxide (Fig. 1A). In normal glucose (5.5 mM) culture conditions, 0.05-0.5 mM hydrogen peroxide did not affect the viability of Hc cells. However, in high glucose (22 mM) culture conditions, Hc cells became susceptible to 0.05-0.5 mM hydrogen peroxide. Hydrogen peroxide (1 mM) killed Hc cells regardless of glucose concentrations. Since insulin stimulates the uptake and metabolism of glucose in cells, we added 100 nM insulin to the culture medium together with hydrogen peroxide (Fig. 1B). Although insulin seemed to increase cell viability in the absence of hydrogen peroxide, it could not restore the cell death induced by 0.05-0.5 mM hydrogen peroxide in a high glucose culture condition.

Effect of glucose on Hc cell death is dose dependent; other saccharides do not have a glucose-like effect at comparable concentrations. The relationship between hydrogen peroxide-induced Hc cell death and glucose concentrations in the culture medium was examined (Fig. 2A). With 0.1 mM of hydrogen peroxide, Hc cell viability decreased in higher glucose conditions compared to a normal glucose (5.5 mM) condition, and considerable cell death was detected in the highest glucose conditions (22 mM). There was a statistically significant difference between 22 mM glucose and the other glucose concentrations ($p < 0.01$). To ascertain whether this phenomenon was due to a specific effect of glucose or to non-specific effects of high concentrations of saccharides, cell viability with exposure to 0.1 mM hydrogen peroxide was compared among similar concentrations of four different saccharides: fructose, saccharose, xylitol and glucose, where 16.5 mM of each saccharide was added to DMEM containing 5.5 mM of glucose to adjust the concentration (Fig. 2B). Hydrogen peroxide (0.1 mM) induced Hc cell death at a high concentration of glucose only, and not at high concentrations of the other saccharides.

Hc cell viability is rapidly decreased by hydrogen peroxide in a high glucose condition. The decline of Hc cell viability was detected after 15 min exposure to hydrogen peroxide (0.1 mM) in a high glucose culture condition (Fig. 3). Thereafter, cell viability rapidly decreased. Viable cells were rarely observed after 120 min exposure to hydrogen peroxide.

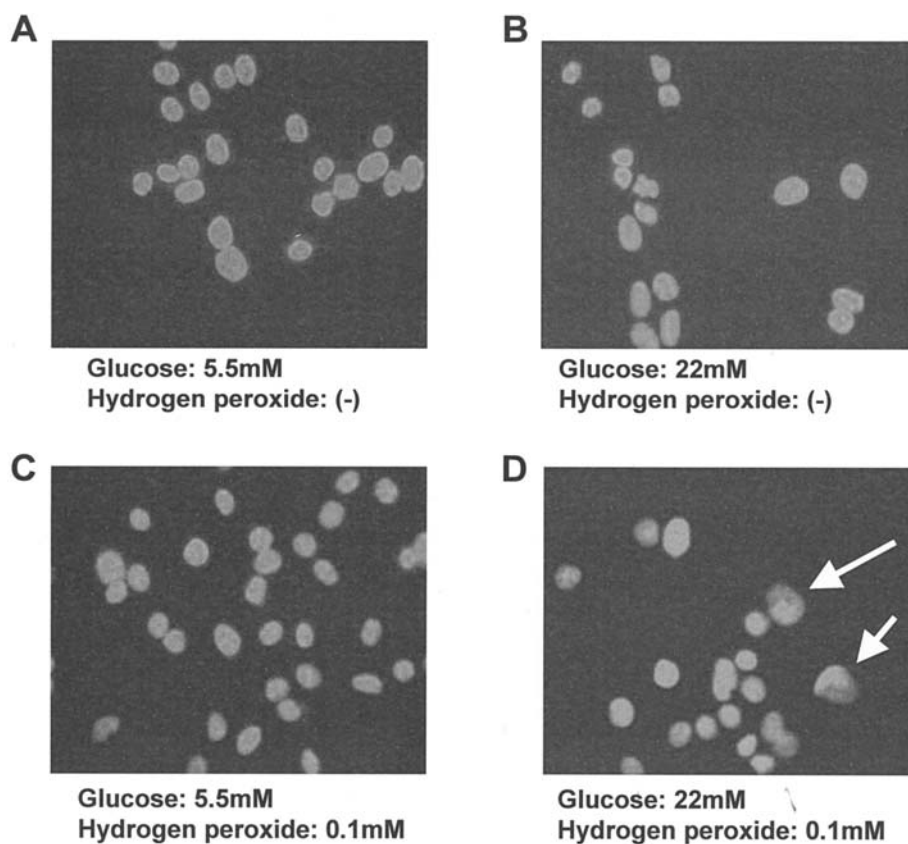


Figure 4. Hoechst staining of dying Hc cells. Hc cells were incubated for 120 min in 5.5 mM (A) and 22 mM (B) glucose without hydrogen peroxide, or 5.5 mM (C) or 22 mM (D) glucose with 0.1 mM hydrogen peroxide, then stained by Hoechst 33258 as described in Materials and methods. Representative data from four experiments are shown.

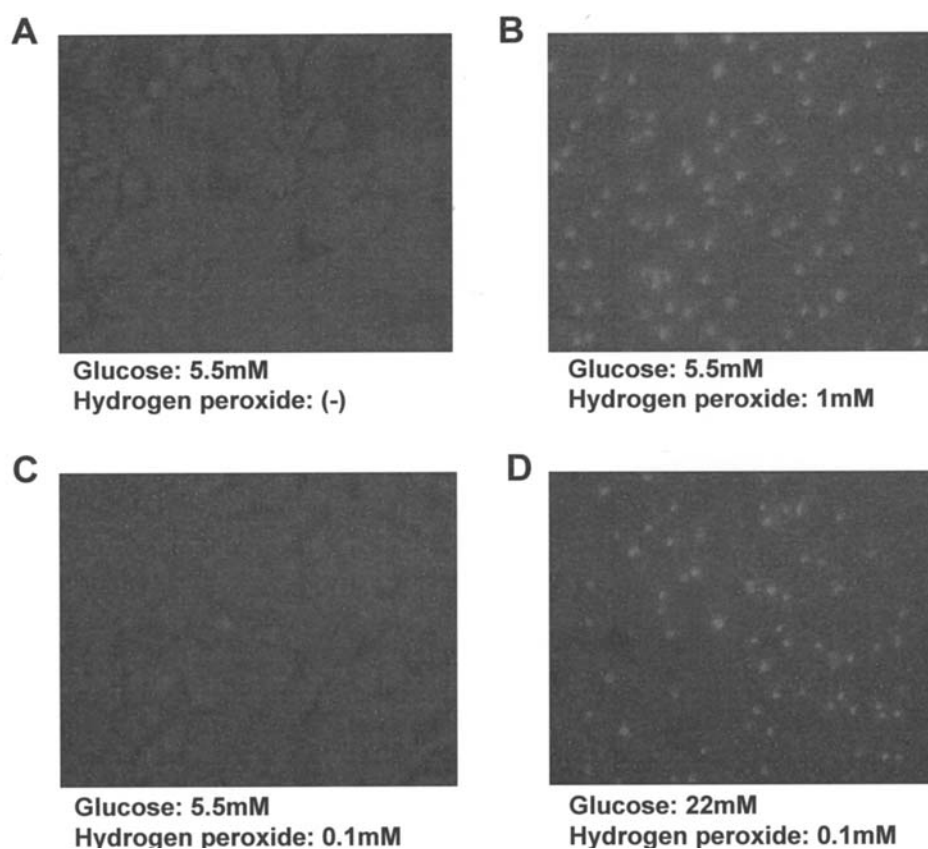


Figure 5. Sytox green staining of dying Hc cells. Hc cells were incubated for 120 min in 5.5 mM glucose without hydrogen peroxide (A), 5.5 mM glucose with 1 mM hydrogen peroxide (B), 5.5 mM glucose with 0.1 mM hydrogen peroxide (C) or 22 mM glucose with 0.1 mM hydrogen peroxide (D), then stained with Sytox green as described in Materials and methods. Representative data from four experiments are shown.

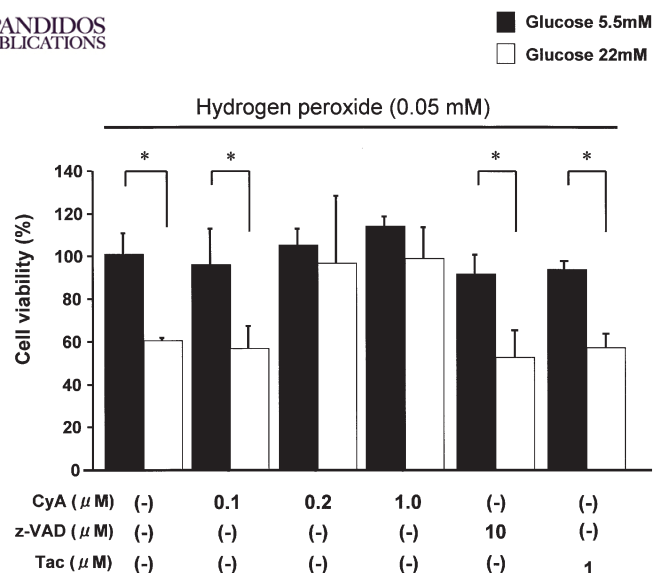


Figure 6. Effect of cyclosporine A (CyA), tacrolimus (Tac) or z-VAD on cell death induced by hydrogen peroxide under high glucose conditions. Hc cells were treated for 120 min with 0.05 mM hydrogen peroxide in medium containing 5.5 or 22 mM glucose. The indicated concentrations of CyA, Tac or z-VAD were added to the medium 30 min before treatment. Cell viability in each culture condition is expressed as a percentage compared to the viability of the control (5.5 mM glucose medium with 0.05 mM hydrogen peroxide). Data represent the mean \pm SD values of four independent experiments. * $p < 0.01$.

Hydrogen peroxide-treated Hc cells have enlarged and Sytox green-stained nuclei. To determine the mode of cell death, the nuclei of Hc cells were stained with Hoechst 33258 (Fig. 4) and Sytox green (Fig. 5) 2 h after hydrogen peroxide treatment. Hoechst 33258 staining indicated no structural changes in the nuclei of Hc cells in normal glucose, high glucose or normal glucose with hydrogen peroxide (0.1 mM) culture conditions (Fig. 4A, B and C). However, in high glucose culture conditions, Hc cells exposed to hydrogen peroxide (0.1 mM) showed expanded rather than condensed nuclei, indicating apoptosis (Fig. 4D). Hc cells were also stained with Sytox green, which penetrates cells with compromised plasma membranes but does not cross the membranes of apoptotic bodies. Sytox green-stained nuclei were detected in cells treated with 1 mM hydrogen peroxide in a normal glucose condition (Fig. 5B) or with 0.1 mM hydrogen peroxide in a high glucose condition (Fig. 5D), but not in the cells incubated in a normal glucose condition with or without 0.1 mM of hydrogen peroxide (Fig. 5A and C).

Cyclosporine A, but not z-VAD or tacrolimus, rescues Hc cells from hydrogen peroxide-induced cell death. We finally examined whether CyA or z-VAD restores Hc cell death induced by 0.05 mM of hydrogen peroxide in a high glucose condition. CyA, an inhibitor of calcineurin, has a property which inhibits mitochondrial permeability transition (MPT) pores, resulting in the inhibition of necrotic cell death (33). z-VAD, a pan-caspase inhibitor, blocks caspase-dependent cell death such as apoptosis. Pre-incubation with 0.2 and 1.0 μ M of CyA 30 min before exposure to 0.05 mM hydrogen peroxide in a high glucose condition recovered cell viability to the level observed in 0.05 mM hydrogen peroxide in a

normal glucose concentration, but z-VAD did not (Fig. 6). Tac, another calcineurin inhibitor that does not affect MPT activity, showed no effect on cell viability.

Discussion

Several mechanisms of cell death in response to hydrogen oxide have been proposed (34-38). Of these, Peiro *et al* reported that a high concentration of hydrogen oxide induces necrosis and a low one apoptosis in human aortic smooth muscle cells (38). Han *et al* showed that sub-lethal levels of hydrogen oxide sensitized cultured hepatocytes to TNF-induced apoptosis. High levels of hydrogen oxide triggered necrosis in hepatocytes regardless of whether TNF was present (34). In the present study, 0.05-0.1 mM of hydrogen peroxide did not have the ability to kill Hc cells under normal glucose culture conditions. However, in a high glucose culture condition, they effectively induced Hc cell death. This cell death was characterized by rapid induction, the expansion of nuclei (39), weakness of the nuclear membrane (40), no condensation of nuclei and no restoration of cell death by a pan-caspase inhibitor. These results suggest that the mode of cell death observed in this study could be necrosis rather than apoptosis (41). In addition to the above findings, the synergic effect of high glucose and hydrogen peroxide on cell death was blocked by CyA. Since CyA inhibits MPT pores, it is possible that the mechanism of cell death was MPT dependent. CyA also inhibited Hc cell death induced by a high concentration of hydrogen peroxide (1 mM) (data not shown). A similar observation was reported regarding high glucose-induced endothelial cell death through a MPT-dependent process, which was prevented in the presence of CyA (42). These findings suggest a synergistic mechanism where high glucose induces the inhibition of MPT activity, while the addition of low-dose hydrogen peroxide leads to MPT-related cell death. A high glucose condition might elevate the sensitivity of hepatocytes to ROS, including hydrogen peroxide. This is consistent with the observation reported by Bouvard *et al* that HeLa-tat cells, which are stably transfected with the tat gene from human immunodeficiency virus type 1 and have a decreased antioxidant potential, exhibit necrosis or apoptosis under high glucose (20 mM) culture conditions, while parental HeLa cells do not (42).

The relationship between CyA and cell death was recently clarified. Necrosis involves the opening of pores in the inner mitochondrial membrane, known as MPT pores (44). This process is triggered by the accumulation of calcium inside the mitochondria and an increase in oxygen-free radicals that accompanies reperfusion (45). MPT pores have three components, adenine nucleotide translocase, voltage-dependent anion transporter and cyclophilin D (CypD). CyA binds to CypD, preventing it from binding to the adenine nucleotide translocase and strongly inhibiting MPT activity (46), which leads to ATP depletion and necrotic cell death (47). MPT is a key in necrotic cell death caused by oxidative stress, but is rarely involved in apoptosis. The mechanism by which CyA inhibits MPT has been attributed to its inhibitory effect on the peptidyl-prolyl isomerase activity of CypD, believed to be required for the formation of the MPT complex and MPT activation (46).

The coexistence of high glucose and hydrogen peroxide is a situation of clinical importance. A previous report (24) revealed that the association of elevated liver enzymes and postprandial hyperglycemia is adequate for the early diagnosis of NASH (48). Additionally, it was reported that acarbose (49), an α -glucosidase inhibitor, improves postprandial hyperglycemia by delaying the absorption of glucose. In addition, a β -cell stimulator improves postprandial hyperglycemia by early stimulation of insulin and attenuates NASH. It is possible that hepatocytes escape cell death due to the improvement of postprandial hyperglycemia provided by these drugs. However, the influence of glucose concentrations on liver disease has not been thoroughly investigated. CyA, a useful immunosuppressant, might be suitable for immunosuppression following liver transplantation. This is because transplanted patients are susceptible to diabetes (50), and liver grafts are exposed to oxidative stress such as ischemic-reperfusion stress (51), suggesting that the damaged grafted liver, along with hyperglycemia, increases the risk of hepatocyte death.

This study demonstrated that the combination of high glucose and hydrogen peroxide causes necrotic hepatocyte death. This necrotic cell death is thought to be a MPT-related cell death because it is blocked by CyA. Clinically, hyperglycemia, a factor of metabolic syndrome, is often associated with ROS in the liver. Therefore, it is necessary to examine the influence of glucose concentrations on patients with HCV infection, NASH and ALD and who are undergoing liver transplantation.

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