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ORIGINAL ARTICLE

Protective effect of adenosine A_{2A} receptor activation in small-for-size liver transplantation

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Summary

The aim of the present study was to investigate the potential role of adenosine A_{2A} receptor ($A_{2A}R$) activation in small-for-size liver transplantation. A rat orthotopic liver transplantation model was performed by using 40% (range: 36–46%) liver grafts. Recipients were given either saline (control group) or CGS 21680 (2-p-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride, a selective $A_{2A}R$ agonist), or CGS 21680+ ZM 241385 (a selective $A_{2A}R$ antagonist) immediately after reperfusion for 3 h. Compared with control group, CGS 21680 used at both low dose (0.05 μg/kg/min) and high dose (0.5 μg/kg/min) increased the survival rate from 16.7% (2/12) to 83.3% (10/12) and 66.7% (8/12), respectively. These effects correlated with improved liver function and preserved hepatic architecture. CGS 21680 effectively decreased neutrophil infiltration, suppressed pro-inflammatory (TNF-α, IL-1β and IL-6) expression, promoted expression of antiapoptotic molecules, and inhibited apoptosis. The effects of CGS 21680 were prevented when ZM 241385 was co-administrated. In conclusion, the present study showed that $A_{2A}R$ activation alleviated portal hypertension, suppressed inflammatory response, reduced apoptosis, and potentiated the survival of small-for-size liver grafts. Our findings provide the rationale for a novel therapeutic approach using $A_{2A}R$ activation to maximize the availability of small-for-size liver grafts.

Introduction

Partial liver transplantation is a valuable strategy to solve the problem of organ shortage. However, small-for-size liver grafts encounter several risks of graft failure after reperfusion, including microcirculatory damage [1], more severe inflammatory responses [2], and accelerated acute rejection process [3]. At the time of reperfusion, excessive blood inflow (in relation to the graft size) carries more oxygen to the cells in small-for-size grafts, subsequently generating more reactive oxygen species [4]. This increases the susceptibility of liver cells to the apoptotic stimuli and the mechanical injury associated with transient portal hypertension in small-for-size grafts. In addition, the microcirculatory and cellular injuries in small-for-size allografts might trigger profound immune responses [5]. However, effective therapeutic strategies to alleviate hepatic ischemia/reperfusion injury (IRI) have not yet been investigated thoroughly in liver transplantation using small-for-size grafts.

Adenosine is produced in response to ischemia or inflammation and protects tissues from injury. Exposure of tissues to adenosine before ischemia reduces subsequent injury in the central nervous system (CNS) [6] and myocardium [7] because of preconditioning mediated by A_1 or A_3 adenosine receptors. In contrast, activation of the A_{2A} adenosine receptor ($A_{2A}R$) before ischemia does not reduce subsequent ischemic injury, but $A_{2A}R$ activation during reperfusion reduces tissue damage to kidney [8], heart [9], skin [10], lung [11], and spinal cord [12]. The protection of $A_{2A}R$ involves dilation of vascular smooth muscle cells, suppression of superoxide generation, reduction of

neutrophil adherence to endothelial cells and inhibition of apoptosis. Recently, using bone marrow transplantation to create chimeric mice, $A_{2A}R$ activation on bone marrow-derived cells was found to play an important role in protecting the liver from reperfusion injury [13].

The present study was designed to examine whether $A_{2A}R$ activation could protect small-for-size liver transplantation and the possible mechanism underlying. Here, we demonstrated that $A_{2A}R$ activation prevented IRI in small-for-size liver grafts by attenuating transient portal hypertension, suppressing inflammatory response and decreasing apoptotic cell death. To best of our knowledge, this study is the first to provide evidence that $A_{2A}R$ activation protects small-for-size liver grafts from hepatic IRI.

Materials and methods

Animals and drugs

Male inbred rats (Sprague–Dawley weighing 180–240 g from the Laboratory Animal Center of Jiangsu Province, Nanjing, China) were used as donors and recipients. Animals were fed with a standard rodent diet and water and cared for according to guidelines approved by the China Association of Laboratory Animal Care.

The selective $A_{2A}R$ agonist CGS 21680 and $A_{2A}R$ antagonist ZM 241385 were obtained from Tocris Cookson Ltd, Bristol, UK, and they were dissolved in 0.9% saline solution for i.v. infusion.

Study design

A rat nonarterialized orthotopic liver transplantation without veno-venous bypass was used as described by Man *et al.* [14] previously. The lobe ligation technique was used to reduce the graft size on the backtable. The median lobe of the liver was selected to be the graft and the median ratio of the graft weight to recipient liver weight (graft weight ratio) was 40% (range: 36–46%). There were mainly four treatment groups. In all groups, liver grafts were storage at 4 °C for 80-min ischemic time in cold saline. Both donors and recipients were anesthetized with ether following an i.m. injection of atropine (0.05 mg/kg). Each recipient rat was fitted with a catheter in the right external jugular vein for i.v. infusion via a peristaltic pump (MS-Reglo, Ismatec, Glattbrugg, Switzerland). Immediately before reperfusion, recipients were infused with saline (control group), CGS 21680 (at two doses of 0.05 and 0.5 μg/kg/min), or CGS 21680 (0.5 μg/kg/min) + ZM 241385 (0.5 μg/kg/min) continuously for 3 h under the anesthesia. Additional group (six rats) was killed at 6 h after reperfusion. All experiments were approved by the Nanjing Medical University Animal Care and Use Committee.

Hemodynamic study

Three rats in each group were used for hemodynamic study. The left femoral artery and ileocolic vein were cannulated by a catheter for measurement of the mean arterial pressure and portal pressure, respectively. A cardiotachometer (model 13-4615-66, Gould, Cleveland, OH, USA) triggered by the pressure signal was used to monitor heart rate. All catheters were connected via the pressure transducers (model P23 XL, Viggo-Spectramed, Oxnard, CA, USA) for continuous pressure monitoring and recording.

Assay of serum aspartate aminotransferase (AST) level

Blood samples were collected into test tubes from the recipient's tail vein after reperfusion. The serum samples were stored at −80 °C until the next analysis. Serum AST was measured by using a transaminase kit (Pointe Scientific, Inc. Canton, MI, USA). A total of 100 μl 20×-diluted serum was added to a prewarmed (37 °C) mixture of 1.0 ml L-aspartate-α-ketoglutarate and then incubated in a 37 °C water bath. The absorbance at 340 nm was measured every minute for the next 3 min. The average absorbance difference per minute was calculated and multiplied by the factor 35360 will yield results in U/L of prediluted serum.

Histomorphometric analysis

Liver specimens were fixed in a 10% buffered formalin solution and embedded in paraffin. Sections were cut at 4 μm and stained with hematoxylin and eosin. The histological severity of IRI was graded according to Suzuki's criteria [15].

For electron microscopic analysis, liver tissues were fixed in 2.5% dialdehyde for 2 h, postfixed in osmium tetroxide, stained *en bloc* in aqueous uranyl acetate, dehydrated in ethanol, and embedded in Epon-812 epoxy resin; ultrathin sections were stained in aqueous uranyl acetate and Reynolds' lead citrate, and were examined by using a JEM-1200 electromicroscopy (Jeol Ltd, Tokyo, Japan).

Tissue myeloperoxidase (MPO) activity assay

We used an MPO activity assay to determine neutrophil infiltration into the grafts at 6 h after reperfusion. Frozen tissue was thawed and suspended in an iced solution of 0.5% hexadecyltrimethyl-ammonium and 50 mM potassium phosphate buffer solution, with pH adjusted to 5.0. Samples were homogenized for 30 s and centrifuged at 20 000 *g* for 15 min at 4 °C. The supernatant (0.1 ml)

was mixed in a solution of hydrogen peroxide-sodium acetate and tetramethyl benzidine. The change in absorbance at 460 nm was measured with a Beckman DU spectrophotometer (Beckman Institute, Fullerton, CA, USA). The quantity of enzyme degrading 1 mM peroxide per minute at 37 °C per gram of tissue was defined as 1 unit of MPO activity.

Tissue cytokine determination by ELISA

Livers were prepared freshly for TNF-α, IL-1β and IL-6 determination by enzyme-linked immunosorbent assay (ELISA) at 6 h and 24 h after reperfusion. liver tissue was lyzed in buffer (25 mM Hepes, pH 7.4, 0.1% CHAPS, 5 mM $MgCl_2$, 1.3 mM EDTA, 1.0 mM EGTA) containing protease and phosphatase inhibitors. Cleared lyzates were measured by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacture's instructions and adjusted to total protein concentration.

Western blots for caspase-3, Bcl-2, Bag-1 and α-tubulin

Protein extracted from liver tissue samples was separated on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (30 μg per sample) and transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL, USA). The membrane was blocked with 5% nonfat dry milk and 0.1% Tween 20 in TBS and incubated with primary antibody against Caspase-3, Bcl-2, Bag-1 (Cell Signaling Technology Inc., Boston, MA, USA) and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The filters were washed and then incubated with horseradish peroxidases antibody (Invitrogen Inc., South San Francisco, CA, USA). Relative quantities of protein were determined by using a densitometer (Kodak Digital Science 1D Analysis Software, Rochester, NY, USA).

Detection of apoptosis

Apoptosis was detected by using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay according to the instructions of the commercial kit (ApopTag, Intergen Co., Purchase, NY, USA). Ten random fields were counted for each TUNEL-stained tissue sample. Classic TUNEL positivity was characterized by focal nuclear staining, with nuclear and cell membrane integrity in apoptotic cells intact.

Statistical analysis

Results were expressed as mean ± SEM. Statistical analysis was performed with Student's *t*-test or ANOVA where appropriate. Chi-squared test was used to compare 7-day survival rates. $P < 0.05$ was considered statistically significant.

Results

CGS 21680 attenuated portal hypertension after reperfusion at a high dose

The dose of 0.5 μg/kg/min CGS 21680 has the profound effects on vasodilation with a less impact on heart rate. As shown in Fig. 1b, the portal pressure of the control group was at the level of 17.5 cmH_2O immediately after liver transplantation and maintained at 13–16 cmH_2O during the first hour after liver transplantation. After CGS 21680 treatment (at the high dose of 0.5 μg/kg/min), the portal pressure decreased to the basal level after reperfusion and was significantly lower than that of the control group, while ZM 241385 restored the portal hypertension. The recipients received the low dose of CGS 21680 at 0.05 μg/kg/min had similar portal pressure as control.

$A_{2A}R$ activation improved IRI in small-for-size grafts

As shown in Fig. 2a, 7-day survival rate was 16.7% (2/12) in control group; after CGS 21680 treatment, the 7-day

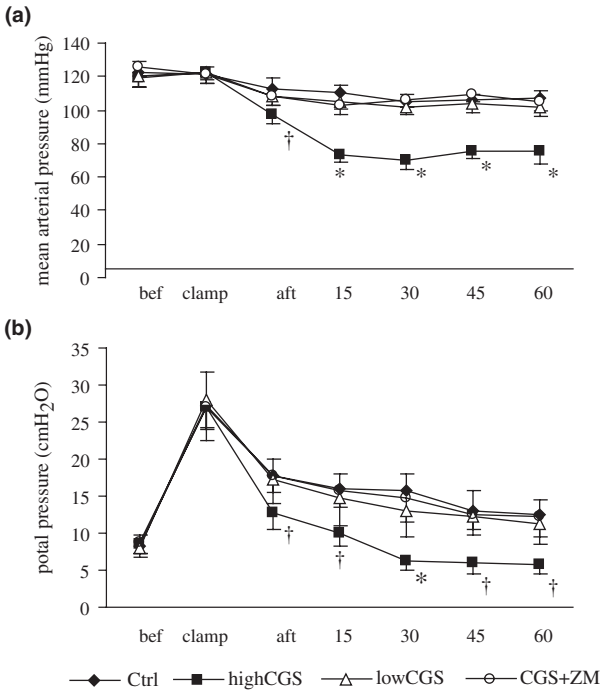

Figure 1 Time course of the mean arterial pressure (a) and portal pressure (b) of the rats ($n = 3$ per group; *$P < 0.01$, †$P < 0.05$ compared with control). bef, pressure before portal vein clamping; clamp, pressure during portal vein clamping; aft, pressure after reperfusion; 15′, 15 min after reperfusion.

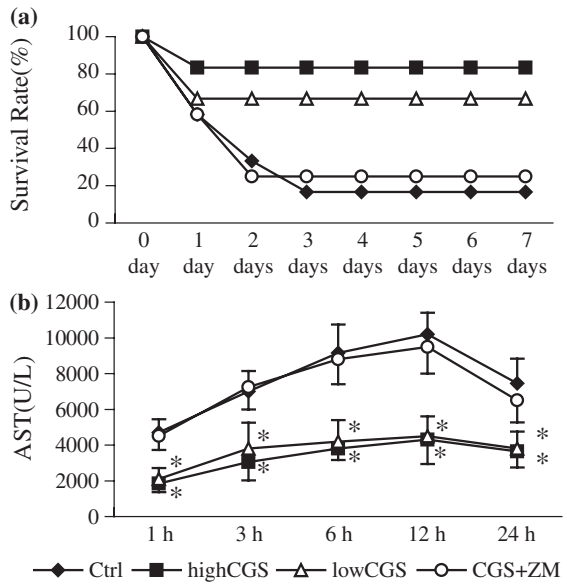

Figure 2 Survival rate (a) and serum assay of serum aspartate aminotransferase (AST) concentration (b) in rats. It should be noted that the 7-day survival rate (12 rats in each group) is improved significantly by CGS treatment (control: 16.7% vs. lowCGS: 66.7% or highCGS: 83.3%; $P < 0.05$) (a). The serum AST concentration (six rats in each time point) was lower (*, $P < 0.01$) in both lowCGS and highCGS than that in Ctrl and CGS + ZM (b). There are no significant differences between highCGS and lowCGS and between Ctrl and CGS + ZM in a and b.

survival rate was significantly improved to 66.7% (8/10, at a low dose of 0.05 μg/kg/min), and 83.3% (10/12, at the high dose of 0.5 μg/kg/min). To evaluate hepatocellular injury, we measured the serum AST levels at 1, 6, 12 and 24 h after reperfusion. The AST levels in both lowCGS and highCGS groups were significantly lower than that in control group (Fig. 2b), while ZM 241385 prevented the effect of CGS 21680.

A_{2A}R activation preserved hepatic architecture

The extent of hepatic IRI was also assessed by histology and electron microscopy (Fig. 3). Six hours after reperfusion, livers in control group showed severe central vein congestion and extensive areas of necrosis with polymorphonuclear neutrophil (PMN) infiltration adjacent to interactions of PMNs and monocytes with endothelium necrotic tissue (H-E staining, Fig. 3a), and severe mitochondria and endoplasmic reticulum swelling of hepatocytes accompanied by loss of microvilli (electron microscopy, Fig. 3b). In response to CGS 21680 treatment, hepatic architecture was preserved and hepatocyte had minimal reduction in mitochondria at 6 h after reperfusion (Suzuki's score at 6 h after reperfusion 1.45 ± 0.62 in highCGS group and 1.89 ± 0.58 in lowCGS group vs. 6.18 ± 0.89 in control; $P < 0.01$). Furthermore, in CGS + ZM group, grafts showed similar pattern as that in control group (Suzuki's score 5.72 ± 0.81). We also examined MPO as a biochemical marker of neutrophils and macrophages. As shown in Fig. 4, animals treated with CGS 21680 have much less MPO activity than that in control or CGS + ZM group.

A_{2A}R activation reduced pro-inflammatory cytokine expression

As shown in Fig. 5, CGS 21680-treated grafts were characterized by lower expression of TNF-α, IL-1β and IL-6 at 6 h after reperfusion. No significant difference was detected between CGS + ZM and control group for these cytokines.

A_{2A}R activation depressed hepatocellular apoptosis

To detect apoptotic cells, we performed TUNEL labeling in liver grafts. Results of TUNEL staining of liver grafts subjected to 6 h after reperfusion are shown in Fig. 6. The frequency of TUNEL+ cells was marked lower in sections from rats treated with CGS 21680, when compared with that of control and CGS + ZM groups. Consequently, an apoptotic index, calculated as the percentage of TUNEL-nuclei divided by the counter-stained nuclei, was significantly diminished in the groups treated with CGS 21680 when compared with control.

To evaluate whether CGS 21680 affected intragraft apoptotic networks, we assessed the expression of antiapoptotic Bcl-2/Bag-1and proapoptotic (caspase-3) gene products in liver grafts by western blots. As shown in Fig. 7, CGS 21680 induced a down-regulation of caspase-3 expression compared with control at 6 h after reperfusion. Expression of antiapoptotic Bcl-2 and Bag-1 was enhanced in CGS 21680-treated grafts at 6 h after reperfusion compared with control group.

Discussion

Development of new effective strategies to attenuate early-phase hepatic IRI in liver transplantation using small-for-size grafts is necessary to expand the liver donor pools. We report here the results of our studies on the effects of adenosine A_{2A} receptor (A_{2A}R) activation in small-for-size liver transplantation. A_{2A}R selectively activated by CGS 21680 (i) improved liver function and preserved hepatocyte integrity with resultant prolongation of animal survival; (ii) attenuated transient portal hypertension (at a high dose); (iii) decreased neutrophil infiltration and suppressed inflammatory response; (iv)

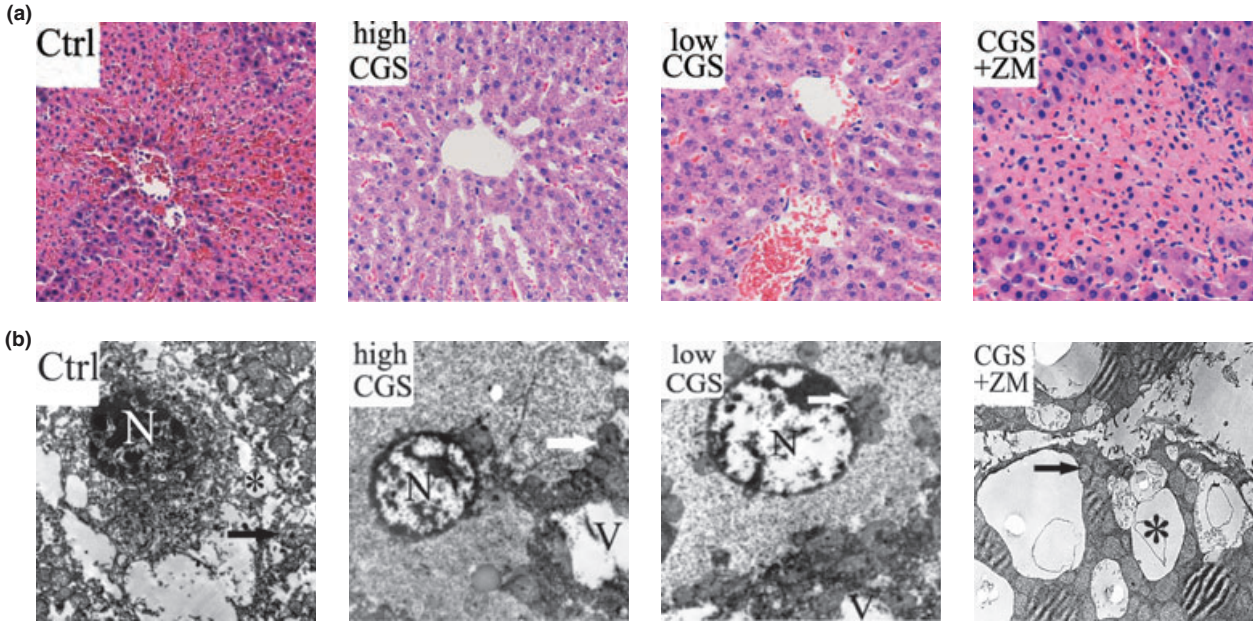

Figure 3 Photomicrographs of livers harvested at 6 h after reperfusion. In control and CGS + ZM groups, H-E staining shows severe disruption of lobular architecture, significant periportal edema, congestion and necrosis, while in highCGS and lowCGS groups there is minimal degeneration (a Magnification, × 200). Likewise, in control and CGS + ZM groups, electron microscopy shows severe mitochondria and endoplasmic reticulum swelling of hepatocytes accompanied by loss of microvilli; while in highCGS and lowCGS groups, both the cell nucleus and cellular organelles have no significant breakdown (b Magnification, ×5000). arrow, mitochondria; *, endoplasmic reticulum; N, nucleus; V, microvilli.

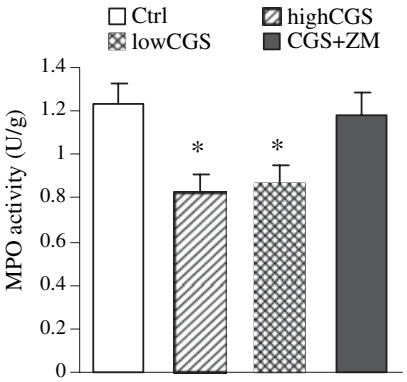

Figure 4 Reduction of myeloperoxidase (MPO) activity by CGS 21680. Liver MPO activity was measured at 6 h after reperfusion. Results are expressed as units of MPO activity per gram of tissue (n = 6/group, $^*P < 0.01$ compared with control).

inhibited apoptosis. Co-administration of ZM 241385, a specific $A_{2A}R$ antagonist, prevented these beneficial effects by CGS 21680, suggesting the direct involvement of $A_{2A}R$ activation in the protection.

Adenosine is a primordial signaling molecule that elicits numerous physiological responses in all mammalian tissues. The receptor-mediated effects of adenosine are mediated by four G protein-coupled receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) and all of them are expressed in rat live, although at an intermediate to low level [16,17]. For example, the A_1 receptor can have proinflammatory effects, including neutrophil chemotaxis and neutrophil adherence to endothelial cells [18]; activation of $A_{2B}R$ induces vasodilation in both smooth muscle and endothelium [19]; A_3R may suppress lipid peroxidation in endothelial and smooth muscle cells after ischemic injuries [20]. Thus, we chose a selective $A_{2A}R$ analog CGS 21680 in our experiment to avoid these effects. $A_{2A}Rs$ have a wide-ranging tissue distribution, with high expression in spleen, thymus, leukocytes (both lymphocytes and granulocytes), platelets and the CNS, intermediate level in Heart, lung and blood vessels, and low expression in skin, kidney and liver [16,17]. Hepatocyte preincubation with CGS21680 greatly improved cell viability during hypoxic incubation [21]. In the vasculature, $A_{2A}Rs$ have been described on both the smooth muscle and endothelium, where they are associated with vasodilation [16]. $A_{2A}R$ activation also has a mitogenic effect on endothelial cells [22]. Besides, it is noteworthy that stimulation of $A_{2A}R$ protects liver sinusoidal endothelial cells against reperfusion injury [23] and reduces TNF-α release by lipopolysaccharide (LPS)-stimulated Kupffer cells [24]. CGS21680 has also been shown to inhibit neutrophil elastase release

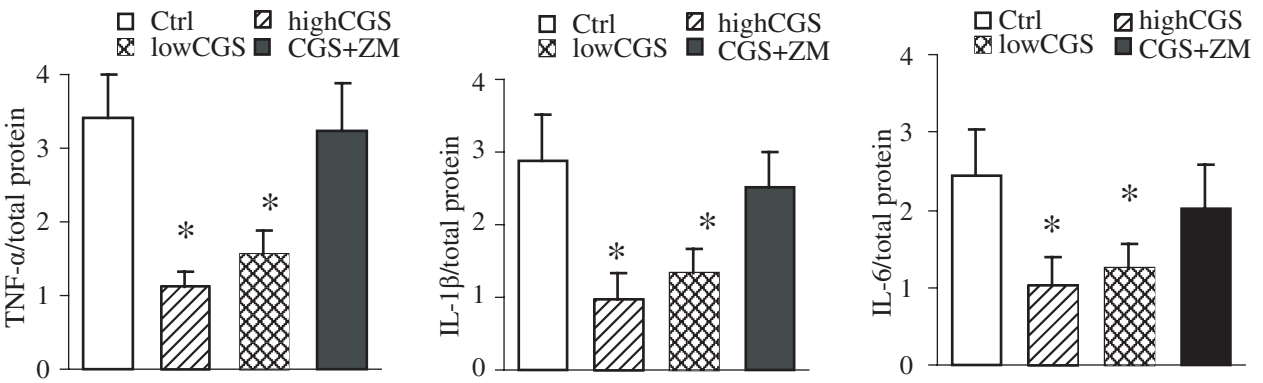

Figure 5 Expression of pro-inflammatory cytokines in liver grafts. Tissue protein was extracted and TNF-α, IL-1β and IL-6 were determined by ELISA. The levels of TNF-α, IL-1β and IL-6 were significantly decreased by CGS 21680 compared with that of control and CGS + ZM groups. (D; $*P < 0.01$ compared with controls).

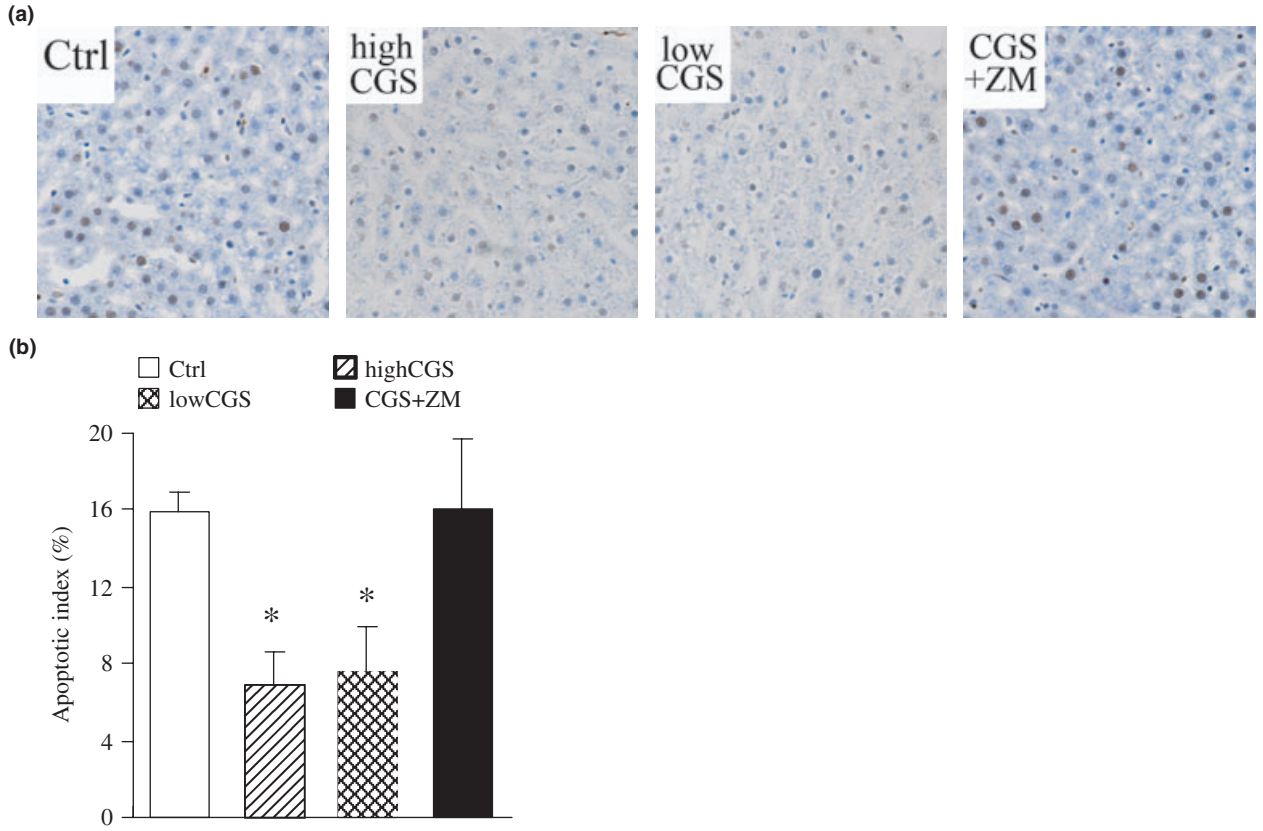

Figure 6 Detection of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay shows that the frequency of TUNEL+ cells (brown spot) was diminished in highCGS and lowCGS groups as compared with control and CGS + ZM groups (a Magnification, ×200). The part figure (b) shows the apoptotic index, calculated as the percentage of TUNEL-nuclei divided by the counter stained nuclei, was significantly diminished in highCGS and lowCGS groups ($*P < 0.01$ compared with control).

in isolated neutrophils and reduce TNF-α production by monocytes [25]. Our results demonstrated that CGS 21680 protects small-for-size liver grafts via vasodilation, inflammation suppression and apoptosis inhibition; however, the exact cell type(s) mediating the protection remains to be further investigated.

The mechanism of small-for-size liver graft failure has been studied in clinical transplantation and animal

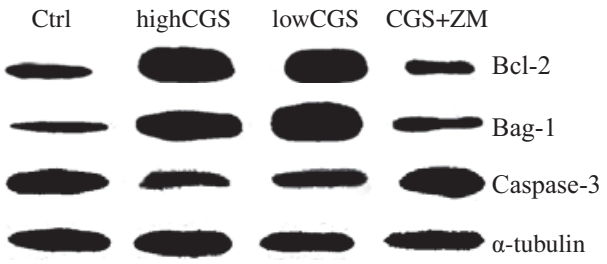

Figure 7 Western blotting for Bcl-2 Bag-1, caspase-3, and α-tubulin on liver samples obtained at 6 h after reperfusion. The antiapoptotic Bcl-2 and Bag-1 were found in higher levels in CGS 21680-treated livers when compared with that of control and CGS + ZM groups. Pro-apoptotic caspase-3 was found markedly decreased in both highCGS and lowCGS groups compared with control and CGS + ZM groups.

experiments [26,27]. Hepatic sinusoidal damage because of transient portal hypertension from hemodynamic force, and severe inflammatory response triggered by shear stress, accompanied with early activation of cell death signaling pathways result in small-for-size liver graft failure at the early phase after liver transplantation. Experimental and clinical studies have demonstrated that hepatic artery flow is mainly regulated by portal vein flow; increased portal vein flow decreasing hepatic artery flow. In addition, flow injury to sinusoidal endothelial cells and activation of Kupper's cells has been described after extended liver resections in rats [28]. Transient portal hypertension at the early phase after liver transplantation and subsequent up-regulation of vasoconstriction genes aggravate small-for-size graft injury [29]. Indeed, attenuation of shear stress by vasodilatory FK409 ameliorated portal hypertension and potentiate the survival of small-for-size grafts [30].

Although high dose of A_{2A} agonist produces vasodilation by acting on receptors on vascular smooth muscle, a much lower dose of CGS 21680 at 0.05 μg/kg/min without obvious change in portal pressure was also found to produce tissue protection in our present study. These findings indicate that other protective effects of A_{2A}R activation (e.g. inflammatory modulation and apoptotic inhibition) also contribute to the prevention of IRI in small-for-size grafts. Indeed, compelling evidence has indicated that leukocytes are the principal effector cells of reperfusion injury. Reperfusion induces a vigorous inflammatory response and a dramatic increase in neutrophil adherence to the reperfused endothelium, which leads to capillary plugging, edema, and a reduction in hepatic blood flow [31]. A_{2A}Rs are ubiquitous membrane receptors found on blood-borne cells (including T lymphocytes, monocytes, neutrophils, macrophages, and platelets) [32]. Activation of A_{2A}Rs on immune cells produces a series of responses that, in general, can be categorized as anti-inflammatory effects [33]. *In vivo* studies have reported that activation of A_{2A}Rs attenuates ischemia-reperfusion injury in heart, lung, liver, and kidney by reducing neutrophil accumulation, superoxide generation, expression of the adhesion molecules and endothelial adherence [11,34–36]. The mechanism by which A_{2A}R activation decreases inflammation in liver is still controversial. While A_{2A}R activation prevented IRI in isolated, buffer-perfused livers [35] where there are no circulating neutrophils and negligible numbers of resident tissue neutrophils, a recent study using chimeric mice showed that protection of liver from IRI by A_{2A}R agonists is mostly attributable to A_{2A}Rs on bone marrow-derived cells rather than vascular endothelial cells [13]. Nevertheless, the suppression is important, because the reduction of the influx of PMNs and monocytes from the circulation into the liver could contribute greatly to the reduced burst of oxygen radicals, decreased secretion of pro-inflammatory cytokines, and improvement of resistance in the graft caused by lobular ballooning, hepatocyte swelling, and sinusoidal congestion.

The exact mechanisms whereby free radicals, calcium entry, or inflammation might result in fulminant IRI are currently unknown, although cellular apoptosis has been suggested as a key early event [37]. A_{2A}R activation inhibited apoptosis in isolated liver [27], consistent with our present TUNEL-based findings of decreased frequency of apoptotic cells in CGS 21680-treated liver grafts. The cellular and physiological mechanisms by which A_{2A}R exerted cytoprotective functions against IRI at the graft site might involve antiapoptotic protein expression. Indeed, adjunctive treatment with ZM 241385, an A_{2A}R antagonist, prevented the expression of Bcl-2 and promoted the activation of the caspase 3 in this study. Caspase 3 activation is the key step in the apoptosis. Bcl-2 prevents the release of apoptogenic factors such as cytochrome *c* and apoptosis-inducing factor from mitochondria into the cytosol [38]. Moreover, Bcl-2 interacts with apoptosis-related family members, such as Bax, and several non-family member proteins including Raf-1 and Bag-1. Bag-1 cooperates with Bcl-2 to suppress apoptosis [39]. Thus, the inhibition of the apoptotic pathway by A_{2A}R activation also seems to contribute to the improvement of IRI in small-for-size grafts.

In summary, the mechanical injury of hepatic sinusoids related to transient portal hypertension as well as acute phase inflammatory response and apoptosis probably contributed to graft damage in small-for-size graft at the early phase after liver transplantation. Our data show that the A_{2A}R agonist CGS 21680 provided potent protection against small-for-size graft injury after liver transplantation. A_{2A}R activation significantly attenuated transient portal hypertension, downregulated inflammatory

response, and inhibited hepatic apoptosis. The present study may provide the rationale for a novel therapeutic approach using $A_{2A}R$ agonist to maximize the function and thus the availability of small-for-size liver grafts.

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