Adenosine A$_{2a}$ receptor stimulation prevents hepatocyte lipotoxicity and non-alcoholic steatohepatitis (NASH) in rats

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A B S T R A C T

NEFA (non-esterified 'free' fatty acid)-mediated lipotoxicity plays a critical role in the pathogenesis of NASH (non-alcoholic steatohepatitis). In the light of the growing need for new therapeutic options for NASH, we investigated the action of A$_{2a}$R (adenosine A$_{2a}$ receptor) stimulation against lipotoxicity. The effects of the A$_{2a}$R agonist CGS21680 [2-p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxyamidoadenosine] were evaluated 'in vitro' in liver cells exposed to SA (stearic acid) and 'in vivo' in rats with NASH induced by 8 weeks of feeding with an MCD diet (methionine/choline-deficient diet). In cultured hepatocytes, SA promoted apoptosis by inducing MKK4 (mitogen-activated protein kinase kinase 4)/SEK1 (stress-activated protein kinase/extracellular-signal-regulated kinase kinase-1) and JNK-1/2 (c-Jun N-terminal kinase-1/2) activation. CGS21680 addition prevented JNK-1/2 activation and reduced apoptosis without interfering with lipid accumulation. CGS21680 action required PI3K (phosphoinositide 3-kinase)/Akt-mediated block of MKK4/SEK1. Consistently, PI3K inhibition with wortmannin abolished the cytoprotective action of CGS21680 and reverted MKK4 inhibition. SA lipotoxicity was also prevented by transfecting HTC cells with a specific MKK4/SEK1 siRNA (small interfering RNA). In rats receiving the MCD diet, the development of NASH was associated with MKK4/SEK1 and JNK-1/2 activation. CGS21680 (0.5 mg/kg of body weight, intraperitoneal) administration to MCD-fed rats prevented JNK-1/2 activation by acting on MKK4/SEK1. CGS21680 also effectively reduced NASH-associated ALT (alanine aminotransferase) release, hepatocyte apoptosis, liver inflammation and fibrosis without affecting hepatic steatosis. Taken together, these results demonstrate that, by inhibiting JNK-1/2, A$_{2a}$R stimulation reduces lipotoxicity and ameliorates NASH, giving a rationale to investigate A$_{2a}$R agonists as possible new therapeutic agents in preventing fatty liver progression to NASH.

INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is characterized by lipid accumulation within the hepatocytes and is considered the hepatic manifestation of the so-called 'metabolic syndrome' being associated with visceral obesity, insulin resistance and dyslipidaemia [1,2]. In approximately 15–20% of the patients, NAFLD can evolve from NASH (non-alcoholic steatohepatitis) with the possible progression to cirrhosis or hepatocellular

carcinoma [3]. Several mechanisms, including oxidative stress, pro-inflammatory cytokine production, unbalanced adipokine generation and mitochondrial dysfunctions, have been associated with the evolution of NAFLD to more severe liver injury [4,5]. Furthermore, increasing evidence implicates the direct toxicity of circulating NEFAs (non-esterified 'free' fatty acids) and their metabolites, a phenomenon known as lipotoxicity [6–8]. Indeed, because of insulin resistance, NAFLD subjects show increased NEFA release from both visceral and subcutaneous fat and high circulating NEFA levels [1,2]. Hepatocyte incapability to esterify such an excess of NEFAs triggers apoptosis through the induction of ER (endoplasmic reticulum) stress and JNK (c-Jun N-terminal kinase) activation [7,8]. Accordingly, JNK activation is evident in liver biopsies from NASH patients, and pharmacological or genetic JNK inhibition prevents lipotoxicity 'in vitro' and ameliorates steatohepatitis in rodent models of NASH [7–9]. Thus treatments targeting JNKs may be beneficial for preventing lipotoxicity.

Adenosine is a physiological modulator of a variety of tissue responses to injury. Stressed cells release adenosine in the extracellular spaces that, by interacting with one or more of the four adenosine receptors (A_1, A_{2a}, A_{2b} and A_3), modulates in both autocrine and paracrine fashion cell survival, immuno-inflammatory reactions and tissue repair [10,11]. In the liver, adenosine-mediated protection relays upon the stimulation of A_{2a}Rs (adenosine A_{2a} receptors) that activate different intracellular signals involving, among others, PI3K (phosphoinositide 3-kinase)/Akt (also known as protein kinase B) and PKC (protein kinase C)/p38 MAPK (mitogen-activated kinase), as well as HIF-1 (hypoxia-inducible factor-1) [12–15]. Since these survival signals can interfere with JNK-dependent death signals [16,17], we postulated that A_{2a}R stimulation could prevent NAFLD evolution by interfering with lipotoxicity. In this study, we investigated the effects of the A_{2a}R agonist CGS21680 against SA (stearic acid)-mediated hepatocyte lipotoxicity as well as against the development of NASH induced by feeding rats on an MCD diet (methionine/choline-deficient diet).

MATERIALS AND METHODS

Animals

Male Wistar rats were used for the present study (180–250 g in weight; Harlan-Nossan). All animal experiments were approved by the Italian Ministry of Health and by the University 'A. Avogadro' Commission for Animal Care and followed the criteria of the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health.

Hepatocyte preparation and treatment

Hepatocytes were isolated by collagenase (Sigma) perfusion from the rat livers and cultured on collagen-coated culture dishes in DMEM (Dulbecco's modified Eagle medium)/Ham's F12 medium [15]. For lipotoxicity assays, hepatocytes were incubated with fresh medium supplemented with SA (25 μmol/l; Sigma). CGS21680 (5 μmol/l), SP600125 (10 μmol/l) and wortmannin (250 nmol/l), all from Sigma, were added 1 h after SA treatment. The HTC rat hepatoma cell line was obtained from the European Collection of Cell Cultures and cultured in DMEM/Ham's F12 medium containing 10 % FBS (fetal bovine serum), 1 % penicillin/streptomycin and 1 % glutamine. Cell apoptosis was evaluated using a Becton Dickinson FACScan flow cytometer by measuring the binding FITC-conjugated annexin V (Annexin-V-Fluos kit; Roche) or DNA fragmentation following propidium iodide (20 μg/ml; Sigma) addition [18]. Lipid accumulation in the cells was evaluated by using the Steatosis Colorimetric Assay Kit (Cayman Chemical), according to the manufacturer's instructions.

In vivo studies

Male Wistar rats were fed on an MCD or an isocaloric control diet (Laboratorio Dottori Piccioni) for 8 weeks. After 2 weeks, rats were injected intraperitoneally twice a week either with sterile saline or CGS21680 (0.5 mg/kg of body weight in sterile saline). Rats were killed under sevoflurane anaesthesia and blood was collected. Livers fragments were immediately frozen in liquid nitrogen and kept at -80°C until analysed. Two portions of each liver were fixed in 10 % formalin for histology. Serum ALT (alanine aminotransferase) and total liver triacylglycerol content were determined spectrometrically using kits supplied by Radim and Sigma Diagnostics respectively. Oxidative stress was evaluated in liver homogenates by measuring thiobarbituric acid-reactive substances expressed as malondialdehyde equivalents. Liver TNFα (tumour necrosis factor α) and cleaved caspase 3 fragments (17 kDa) were evaluated by Western blotting with specific polyclonal antibodies (Millipore).

RNA interference experiments

MISSION® siRNA (small interfering RNA) against rat A_{2a}Rs and MKK4 (MAPK kinase 4)/SEK1 (stress-activated protein kinase/extracellular-signal-regulated kinase kinase-1) were purchased from Sigma. A_{2a}R sequences were as follows: sense 5′-GCUACAUCG-CCAUCCGAAU-3′ and antisense 5′-AUUCGGAU-GGCGAUGUAGC-3′. MKK4/SEK1 sequences were as follows: sense 5′-CAUCAAACCUUCCAAUAUU-dTdT-3′ and antisense 5′-AAUAUUGGAAGGUUUG-AUGdTdT-3′. An siRNA with no matching sequences in the rat genome (Ambion) was used as negative control. HTC cells were transfected using Lipofectamine™ 2000

(Invitrogen), according to manufacturer's recommendations. Transfection efficiency was analysed after 48 h using BLOCK-iT Fluorescent Oligo (Invitrogen) and was above 85 %.

mRNA extraction and real-time PCR

Total RNA was extracted from frozen rat livers with TRI reagent (Applied Biosystems), according to the manufacturer's instructions, and quantified at 270 nm. RNA (1 μg) was retro-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instruction. Real-time PCR was performed in a Techne TC-312 termalcycler (Tecne), using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for rat TGFβ1 (transforming growth factor β1), IL (interleukin)-1β, type I procollagen α- and β-actin (Applied Biosystems). The data were processed using 7000 System Software and normalized to the β-actin gene expression. All samples were run blind in duplicate and the results are expressed as arbitrary units.

Liver histopathology

Paraffin-embedded liver sections were stained with haematoxylin and eosin and Gomori's silver staining as described previously [19]. Steatosis, inflammation and liver cell injury were scored as described by Kleiner et al. [20]. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining was performed on paraffin liver sections using Apoptags Kit (Intergen), according to manufacturer's instructions. α-SMA (α-smooth muscle actin) was detected using a specific polyclonal antibody (Labvision; Bio-Optica) and peroxidase–polymer conjugates (Labvision; Bio-Optica). A total of ten microscopic fields on five different sections were analysed for each sample to detect TUNEL-positive apoptotic bodies and α-SMA positive cells.

Analysis of the phosphorylation state of Akt, JNK and MKK4

Protein extracts were electrophoresed by SDS/PAGE (10 % gel) and, after blotting on to nitrocellulose membranes, the membranes were probed with antibodies against phospho-Akt (Ser^{473}), Akt, phospho-JNK (Thr^{183}/Tyr^{185}), JNK, phospho-SEK1/MKK4 (Ser^{257}/Thr^{261}) and SEK1/MKK4 (Ser^{80}) and SEK1/MKK4 (Cell Signaling Technology). The antigens were detected by Western Lightning Chemiluminescence Reagent Plus (ECL) (PerkinElmer) and VersaDoc 3000 quantitative imaging system (BioRad Laboratories). The results were expressed as ratios.

Statistical analysis

Statistical analysis was performed by Instat-3 statistical software (GraphPad Software) using one-way ANOVA test (two-tailed P value) with Bonferroni's correction for multiple comparisons when more than two groups were analysed. Distribution normality was preliminarily verified using the Kolmogorov–Smirnov test. Significance was taken at 5 % level.

RESULTS

A$_{2a}$R stimulation protects hepatocytes against lipotoxicity

The effects of the A$_{2a}$R agonist CGS21680 [13–15] on hepatic lipotoxicity were evaluated using primary rat hepatocytes incubated 8 h with SA (25 μmol/l). SA exposure induced hepatocyte lipid accumulation, as evidenced by ORO (Oil Red O) staining (Figures 1A and 1B) and apoptosis, as detected by annexin V membrane binding (Figure 1C) and DNA fragmentation (Figure 1D). According to previous observations [7,8], SA-induced toxicity was associated with the activation of JNK, as evidenced by an increased phosphorylation at Thr^{183}/Tyr^{185} of both JNK-1 and JNK-2 (Figure 1E). Hepatocyte supplementation with CGS21680 (5 μmol/l) 1 h after SA treatment did not affect lipid accumulation (Figures 1A and 1B), but significantly protected against apoptosis (Figures 1C and 1D) and entirely abolished JNK-1/2 phosphorylation (Figure 1E). Consistently, blocking JNKs with SP600125 (10 μmol/l) significantly reduced SA-induced apoptosis (Figures 1C and 1D). The protective action of CGS21680 was also evident against lipotoxicity induced by oleic acid and palmitic acid supplementation [7,8] (see Supplementary Figure S1 at http://www.clinsci.org/cs/123/cs1230323add.htm). By using Akt phosphorylation as marker of PI3K activity [21], we observed that CGS21680-mediated protection against lipotoxicity involved PI3K/Akt activation (Figure 1F), and the PI3K inhibitor wortmannin (250 nmol/l) reverted CGS21680-mediated JNK-1/2 inhibition (Figure 1E) as well as the protection against SA-induced apoptosis (Figures 1C and 1D). However, wortmannin did not interfere with the action of JNK inhibitor SP600125 (Figures 1C and 1D), indicating that A$_{2a}$R/PI3K-dependent signals prevented hepatocyte lipotoxicity acting upstream to JNK-1/2. To confirm the specific role of A$_{2a}$Rs in CGS21680-mediated protection, A$_{2a}$R expression was knocked down in rat hepatocyte-derived HTC cells by transient transfection with a specific siRNA (A$_{2a}$R-siRNA). HTC cells receiving A$_{2a}$R-siRNA showed a significant decrease in A$_{2a}$R protein (Figure 2A) and lost CGS21680-mediated protection against SA lipotoxicity (Figures 2B and 2C). JNK-1/2 activation in response to cytokines or cell stress, involves a protein kinase cascade including three groups of MAPK kinases (MKK2, MKK4/SEK1 and MKK7) that, in turn, promote JNK-1/2 Thr^{183}/Tyr^{185} phosphorylation [22].
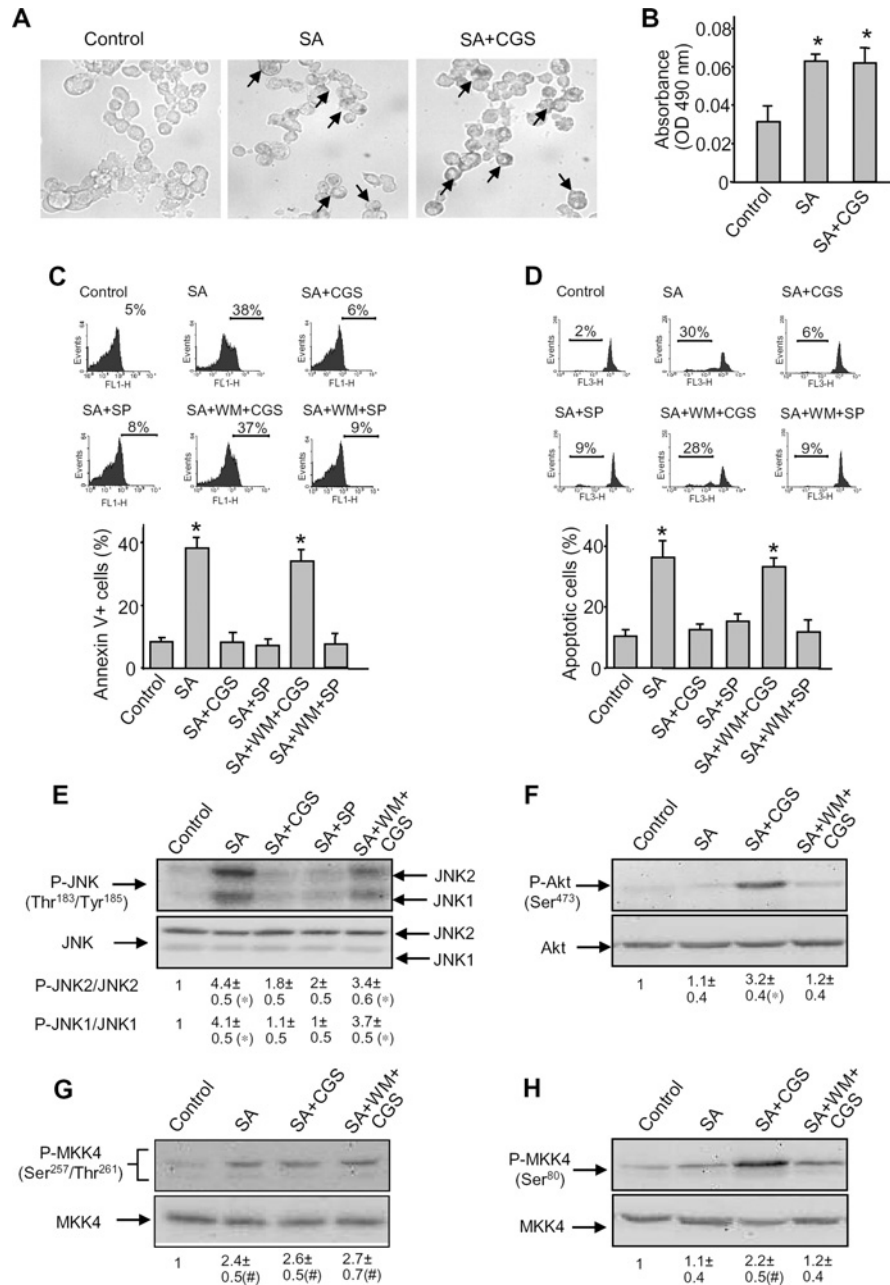
Figure 1   CGS21680 prevents rat hepatocyte lipotoxicity by interfering with JNK-1/2 activation

Rat hepatocytes were incubated with SA (25 μmol/l) and CGS21680 (CGS; 5 μmol/l) was added 1 h after SA. The JNK inhibitor SP600125 (SP; 10 μmol/l) and PI3K blocker worthmannin (WM; 250 nmol/l) were added together with CGS21680. Lipid accumulation was visualized as intra-cytoplasmatic droplets stained by ORO (A) and confirmed by colorimetric ORO determination at 490 nm (B). Hepatocyte apoptosis was determined by annexin V membrane binding (C) and flow-cytometric detection of cells showing fragmented DNA as sub-diploid peak (D). The activation of JNK-1/2 (E), Akt (F) and MKK4 (G) were evaluated as phosphorylation of Thr^{183}/Tyr^{185} (JNK), Ser^{437} (Akt) and Ser^{257}/Thr^{261} (MKK4), whereas the inhibition of MKK4 (H) was estimated as phosphorylation of Ser^{80}. The results are means $\pm$ S.D. for six experiments. [#]$P < 0.05$ and [*]$P < 0.001$ compared with the control, SA + CGS, SA + SP or SA + WM + SP group.

Interestingly, MKK4/SEK1 is a target of Akt and Akt-mediated MKK4/SEK1 Ser^{80} phosphorylation blocks its downstream signalling [23]. The analysis of MKK4/SEK1 phosphorylation state revealed that SA promoted the activating [22] Ser^{257}/Thr^{261} MKK4/SEK1 phosphorylation (Figure 1G). CGS21680 did not affect SA-induced phosphorylation at Ser^{257}/Thr^{261} (Figure 1G), but caused the inhibitory Ser^{80} phosphorylation of MKK4/SEK1 (Figure 1H). This latter effect was abolished by wortmannin (Figure 1H), suggesting that $A_{2a}R$/PI3K/Akt-dependent signals down-modulated JNK-1/2 by acting on MKK4/SEK1. To validate this hypothesis, HTC cells
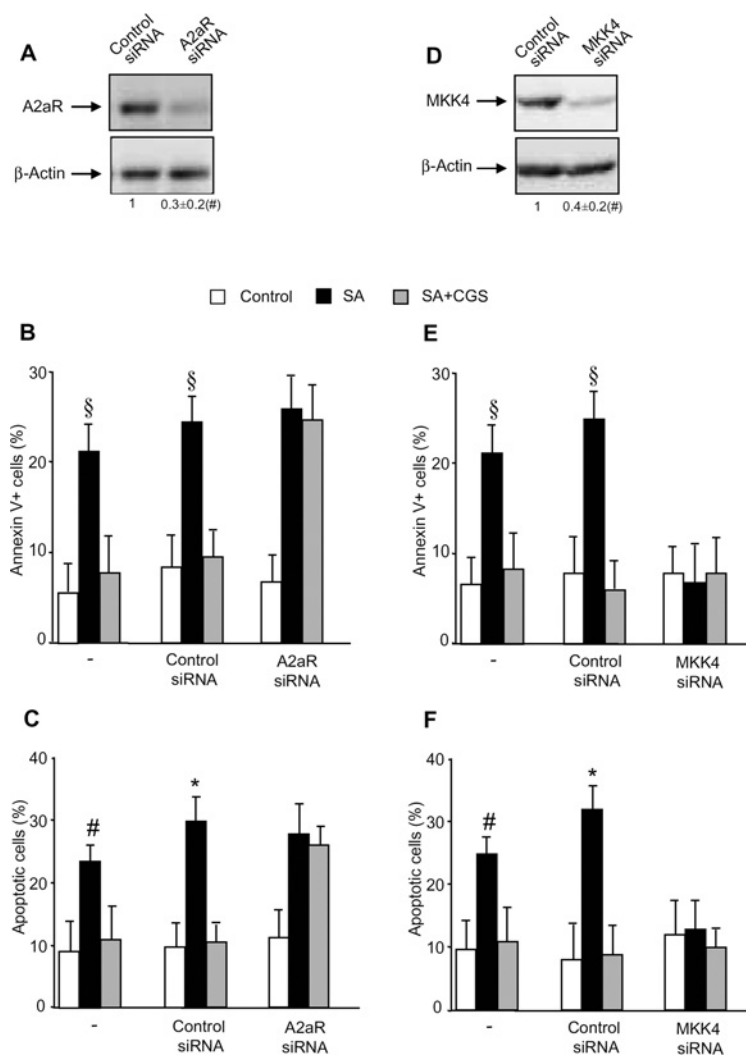
Figure 2   Role of A$_{2a}$R and MKK4 in mediating CGS21680 effects against HTC cell lipotoxicity

HTC cells were transfected with siRNA against A$_{2a}$Rs (A$_{2a}$R-siRNA) (A–C) and MKK4 (MKK4-siRNA) (D–F) or an unrelated control siRNA (A–F) and the effect on MKK4 and A$_{2a}$R expression was assessed by Western blotting 48 h after transfection (A and D). Untreated and transfected cells were incubated 8 h with SA (200 μmol/l) with or without CGS21680 (CGS; 5 μmol/l) added 1 h after SA. Apoptosis was measured by annexin V staining (B and E) and flow-cytometry detection of cells with fragmented DNA (C and F). Results are means $\pm$ S.D. for three experiments. $^{\#}P < 0.05$, $^{\S}P < 0.01$ and $^{*}P < 0.001$ compared with the control or SA + CGS group.

were transfected with MKK4/SEK1-siRNA or control siRNA and exposed to 200 μmol/l SA. Untransfected cells and cells receiving control siRNA showed a significant increase in apoptosis, while cells transfected with MKK4/SEK1-siRNA were protected against SA-induced lipotoxicity in a manner comparable with those receiving CGS21680 (Figures 2E and 2F).

A$_{2a}$R stimulation decreases hepatocyte apoptosis and ameliorates NASH in rats fed on an MCD diet

The capacity of A$_{2a}$R stimulation to prevent lipotoxicity in hepatocyte cultures led us to investigate whether CGS21680 could interfere with the development of NASH induced by feeding Wistar rats with an MCD

diet. In these experiments, CGS21680 (0.5 mg/kg of body weight) was administered twice a week by intraperitoneal injection starting at the beginning of the third week of the dietary treatment. Preliminary experiments showed that at this time point the rats receiving the MCD diet had extensive liver steatosis, without histological evidence of hepatocyte apoptosis or lobular inflammation and unmodified plasma ALT levels (results not shown). After 8 weeks on the MCD diet, rats showed increased ALT release (Figure 3A), liver triacylglycerol accumulation (Figure 3B) and signs of intrahepatic inflammation, such as elevated TNFα and IL-1β expression (Figures 4A and 4B). From the histology, we observed marked macrovesicular steatosis, most evident in centrolobular areas, accompanied by focal liver-cell necrosis, inflammatory infiltrates and
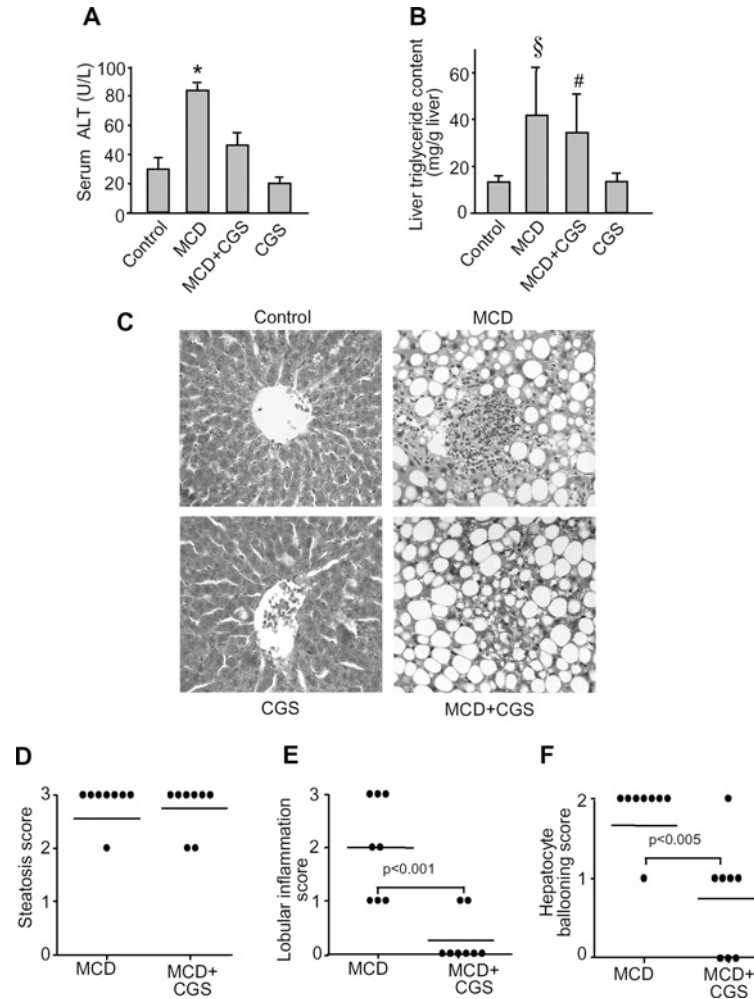
Figure 3   CGS21680 treatment prevents NASH induced by feeding rats with an MCD diet

Rats were fed on an MCD or an isocaloric control diet (control) for 8 weeks. After 2 weeks, rats were injected intraperitoneally twice a week either with sterile saline (control or MCD) or CGS21680 (0.5 mg/kg of body weight in sterile saline) (CGS or MCD + CGS). ALT release (A) and liver triacylglycerols (B) were measured as reported in the Materials and methods section. Liver damage was evaluated as by haematoxilin/eosin staining (C) and semi-quantitative scoring of steatosis (D), lobular inflammation (E) and hepatocyte ballooning (F) as described by Kleiner et al. [20]. The values are means $\pm$ S.D. from eight animals in each group. *$P < 0.001$ and §$P < 0.01$ compared with the control, CGS or MCD + CGS group; #$P < 0.05$ compared with the control or CGS group.

hepatocyte ballooning (Figures 3C–3F). These animals also presented initial signs of liver fibrosis, as shown by an elevation in TGFβ1 (Figure 4C) and type I procollagen α (Figure 4D) mRNA expression, as well as by an increase in α-SMA-positive activated HSCs (hepatic stellate cells) (Figures 3E and 3F) and of reticulin fibres (Figure 4G). The treatment with CGS21680 reduced ALT release ($P < 0.001$), hepatocyte ballooning ($P < 0.005$) and lobular inflammation ($P < 0.001$) (Figures 3A, 3F and 3E) without interfering with liver triacylglycerol accumulation (Figures 3B and 3D). NASH livers showed a significant increase in the number of TUNEL-positive cells and enhanced caspase 3 cleavage (Figures 5A and 5B) that were effectively prevented by CGS21680 treatment (Figures 5A and 5B). Furthermore, CGS21680 ameliorated intrahepatic inflammation and

fibrosis (Figures 4A–4G). As in cultured hepatocytes, the development of NASH was associated with JNK-1/2 (Thr[183]/Tyr[185]) and MKK4/SEK1 (Ser[257]/Thr[261]) phosphorylation (Figures 5C and 5E). CGS21680 treatment stimulated Akt activation (Figure 5D) with a concomitant lowering in JNK-1/2 activation (Figure 5C). Furthermore, in the livers of CGS21680-treated rats, we also observed the inhibitory MKK4/SEK1 Ser[80] phosphorylation (Figure 5F).

DISCUSSION

Thr results of the present study show that the pharmacological stimulation of $A_{2a}Rs$ prevents hepatocyte lipotoxicity and inhibits NAFLD evolution to
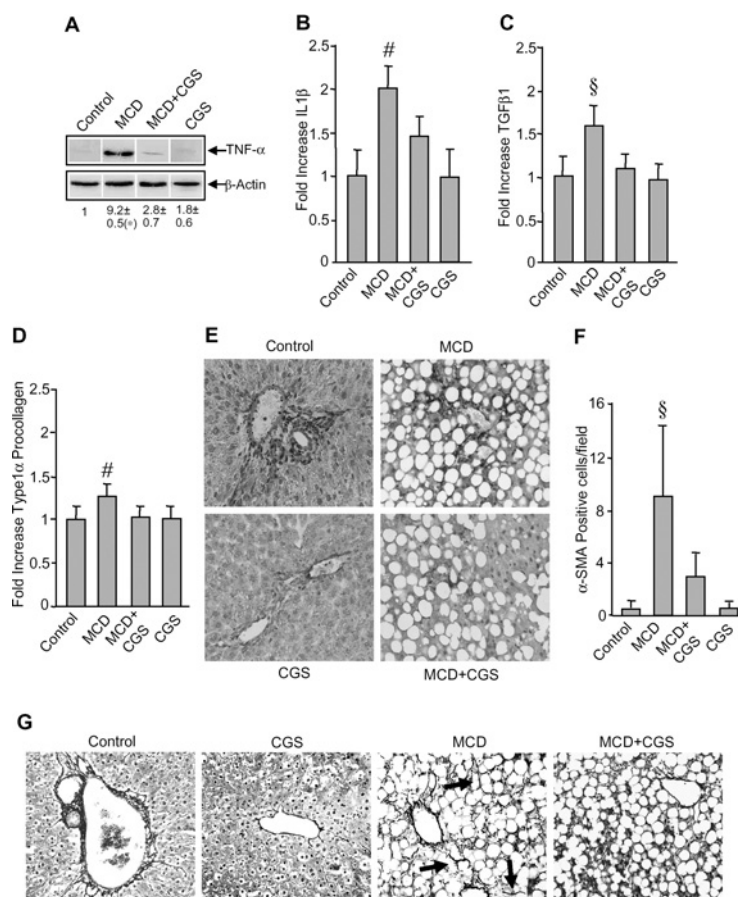
Figure 4   CGS21680 treatment prevents hepatic TNF, IL-1β, TGFβ1 and procollagen type 1α expression and liver fibrosis in rats fed on an MCD diet

Rats were fed on an MCD or an isocaloric control diet for 8 weeks. When reported, rats were injected either with sterile saline (control or MCD) or CGS21680 (0.5 mg/kg of body weight in sterile saline) (CGS or MCD + CGS). The hepatic levels of TNFα (A) were assessed by Western blot analysis, whereas the liver mRNA expression of IL-1β (B), TGFβ1 (C) and procollagen type 1α (D) were measured by real-time PCR. Liver fibrosis was evidenced by the presence of α-SMA-positive HSCs (E and F) and by reticulin fibre silver staining (G). The values are means ± S.D. from eight animals in each group. $^{\#}P < 0.05$ and $^{\S}P < 0.01$ compared with the control, CGS or MCD + CGS group.

NASH by interfering with JNK-1/2 activation. Recent evidence implicates JNK-1/2 in the pathogenesis of NAFLD/NASH [9]. In particular, the JNK-1 isoform interferes with insulin response and lipid metabolism, thus contributing to hepatic steatosis and development of steatohepatitis [24,25]. Moreover, JNK-1-specific activation in Kupffer cells promotes the production of pro-inflammatory and pro-fibrogenetic cytokines [26], whereas JNK-2 isoform influences hepatocyte apoptosis by modulating the activity of pro- and anti-apoptotic factors, such as Bim and Mcl-1 [27]. One aspect of the involvement of JNK in NASH that has received particular attention concerns its role in causing lipotoxicity. Studies by Gores's group have demonstrated that lipotoxicity can be reproduced 'in vitro' by exposing liver cells to saturated NEFAs such as SA [7,8]. In this experimental model, JNK promotes the induction of the PUMA (p53 up-regulated modulator of apoptosis) and the subsequent activation of Bax [7,8]. Moreover, JNK-

mediated phosphorylation inactivates the anti-apoptotic proteins Bcl-2 and Bcl-xL and sensitizes cells to the pro-apoptotic action of TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand) [7,8]. However, a few findings are available on the events responsible for JNK activation in NEFA-exposed cells. Herein, we show that in hepatocytes treated with SA, MKK4/SEK1 activation plays a key role in promoting JNK-1/2-dependent responses associated with lipotoxicity. This is consistent with a recent report showing that saturated fatty acid-induced JNK activation in liver cells requires the small GTP-binding proteins Cdc42 (cell division cycle 42) and Rac1 as well as the MLK3 (mixed-lineage protein kinase 3) [28]. In turn, MLK3 can phosphorylate dual specificity MAPK kinases (MKK4/SEK1 and MKK7) in the catalytic domains (Ser^{257}/Thr^{261}) promoting their subsequent action on JNK-1/2 [22].

The role of JNKs in the pathogenesis of NASH points them as possible targets for NASH therapy. In
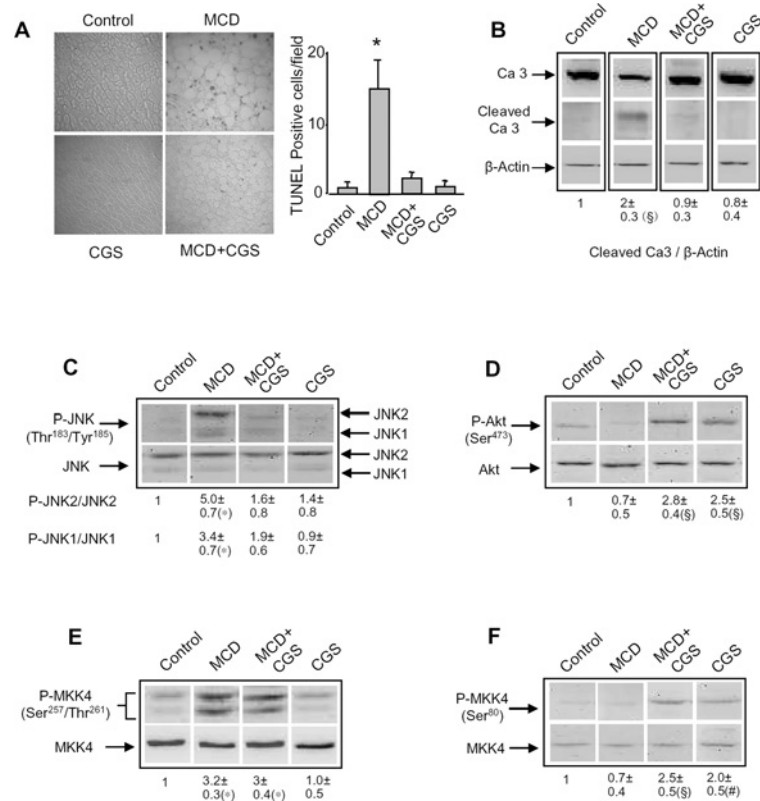
Figure 5   CGS21680 treatment prevents hepatocyte apoptosis and inhibits JNK-1/2 activation in rats receiving the MCD diet

Rats were fed on an MCD or an isocaloric control diet (control) for 8 weeks. After 2 weeks rats were injected intraperitoneally twice a week either with sterile saline (control or MCD) or CGS21680 (0.5 mg/kg of body weight in sterile saline) (CGS or MCD + CGS). Hepatocyte apoptosis was evaluated by TUNEL staining (A) and by Western blot analysis of cleaved caspase 3 (Ca3) fragments (B). The phosphorylation of JNK-1/2 (Thr^{183}/Tyr^{185}) (C), Akt (Ser^{437}) (D) and MKK4 (Ser^{257}/Thr^{261}) (E) and MKK4 (Ser^{80}) (F) were evaluated by Western blotting. The values are means $\pm$ S.D. from eight animals in each group. $^*P < 0.001$ compared with the control, MCD + CGS or CGS groups.

agreement with this hypothesis, we demonstrate that: (i) the $A_{2a}R$ agonist CGS21680 prevents JNK-dependent lipotoxicity in cultured hepatocytes exposed to SA; and (ii) CGS21680 cytoprotection involves a PI3K/Akt-mediated block of the JNK activator MKK4/SEK1. In our hands, CGS21680 prevents the activation of both JNK-1 and JNK-2 isoforms. Nonetheless, it is quite possible that only the action on JNK-1 accounts for the protective action of $A_{2a}R$ stimulation, as genetic deficiency of JNK-1 ameliorates NASH, whereas hepatic injury is worsened in JNK-2$^{-/-}$ mice [24,25]. Interestingly, JNK-1$^{-/-}$ mice also show a decreased JNK-2 phosphorylation [25], a condition resembling our findings. The coupling of $A_{2a}Rs$ with the PI3K/Akt signal pathway has been previously characterized in hepatocytes and involves direct PI3K activation through the interaction with inhibitory G_α-proteins and Src [14] as well as the down-modulation of PI3K regulatory mechanisms involving PTEN (phosphatase and tensin homologue deleted on chromosome 10) [29]. We observed that PI3K/Akt-mediated Ser^{80} phosphorylation of MKK4/SEK1 is

involved in CGS21680 protection against SA-induced lipotoxicity. This is in agreement with the data by Park et al. [23] showing that MKK4/SEK1 Ser^{80} phosphorylation blocks the formation of a MKK4/SEK1–JNK complex, thus preventing JNK activation. Nonetheless, we cannot exclude the possibility that the stimulation of the $A_{2a}R$/PI3K/Akt axis may also prevent lipotoxicity by acting downstream to JNK-1/2, since Akt is known to inhibit several death-inducing proteins, including GSK3 (glycogen synthase kinase 3), BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death), caspase 9 and the forkhead transcription factors [21].

NASH can be reproduced in rodents by the administration of an MCD diet. Although this experimental model lacks obesity and insulin resistance, it is widely used to reproduce parenchymal injury and inflammation that characterize the human disease [30]. Furthermore, this model reproduces 'in vivo' JNK activation observed in liver cells undergoing lipotoxicity [24]. By using MCD-fed rats, we observed that CGS21680 administration effectively prevents NAFLD

progression to NASH by reducing liver cell death, lobular inflammation and transaminase release. Such effects are associated with PI3K/Akt activation and JNK-1/2 inhibition. To our knowledge, this is the first demonstration that pharmacological stimulation of A$_{2a}$Rs has beneficial effects on NASH. In contrast with what is observed following genetic blocking of JNK-1 [24,25], the down-modulation of JNK-1/2 by CGS21680 does not affect hepatic steatosis. Such discrepancy could be explained considering that, in the present experiment, JNK inhibition was accomplished when steatosis was already evident after 2 weeks of feeding the MCD diet. This suggests the possibility that blocking JNKs avoids triacylglycerol accumulation by preventing insulin resistance or re-modulating NEFA metabolism [24,25], but it is unable to revert steatosis once it is established. Furthermore, A$_{2a}$R stimulation has been reported not to influence alcohol-induced steatosis in mice [31]. Hepatocyte apoptosis is a prominent feature of NASH and it correlates with the disease severity [7,8]. As observed in cultured hepatocytes exposed to SA, CGS21680 greatly lowers apoptosis markers in the liver of rats with MCD-induced NASH, thus it is possible that the anti-apoptotic action of CGS21680 'in vivo' might reflect an effect against lipotoxicity. However, additional factors likely contribute to the hepatoprotective action of CGS21680, as in a mouse NASH model the caspase inhibitor VX-166 fails to reduce transaminase release [32]. It is known that, during NAFLD/NASH evolution, JNK activation in Kupffer cells promotes the production of pro-inflammatory cytokines [26], whereas in hepatocytes JNK favours the production of IL-8 [33]. Thus it is probable that the prevention of these JNK-mediated pro-inflammatory responses may also be relevant for the protective action of CGS21680 in MCD-fed rats.

Some reports have shown that A$_{2a}$R deletion reduces liver fibrosis induced by CCl$_4$ and thiocetamide, whereas A$_{2a}$R stimulation increases collagen production by activated HSCs [34,35], indicating a role for these receptors in controlling tissue repair. Although in the rodent models of NAFLD/NASH the development of fibrosis is modest [30,36], in our hands, the administration of CGS21680 to rats fed on an MCD diet does not worsen fibrosis markers. On the contrary, CGS21680 significantly reduces the number of α-SMA-positive activated HSCs and the expression of TGFβ and type I procollagen α mRNAs in NASH livers. This is consistent with the observations showing that blocking JNK-1 in myeloid cells prevents fibrosis in mice receiving a choline-deficient L-type amino-acid-defined diet [26]. Furthermore, these results indicate that the anti-apoptotic and anti-inflammatory action of CGS21680 overcomes HSC stimulation by A$_{2a}$R agonists avoiding pro-fibrogenic responses.

In conclusion, our findings demonstrate that the stimulation of A$_{2a}$Rs preserves cultured hepatocytes from saturated fatty acid-induced lipotoxicity and ameliorates NASH 'in vivo'. These observations give a rationale to investigate the possible application of adenosine receptor agonists as new therapeutic agents to prevent NAFLD progression to NASH.

AUTHOR CONTRIBUTION

Chiara Imarisio and Elisa Alchera designed the research and performed the experiments. Salvatore Sutti and Francesca Boccafoschi contributed to the experiments and data analysis. Guido Valente performed the histological studies and contributed to the data analysis. Rita Carini supervised the research and drafted the paper. Emanuele Albano critically revised the paper.

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Adenosine A$_{2a}$ receptor stimulation prevents hepatocyte lipotoxicity and non-alcoholic steatohepatitis (NASH) in rats

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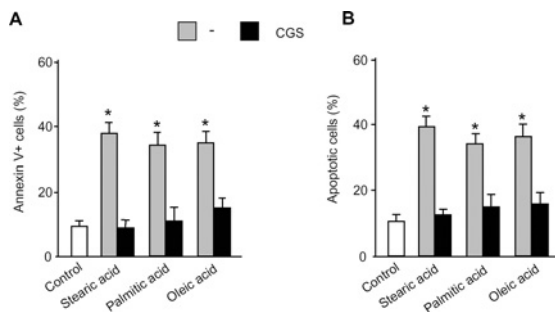
Figure S1   CGS21680 prevents rat hepatocyte lipotoxicity induced by SA, oleic acid and palmitic acid

Rat hepatocytes were incubated with SA (25 μmol/l), palmitic acid (PA; 25 μmol/l) or oleic acid (OL; 25 μmol/l) and CGS21680 (CGS; 5 μmol/l) was added 1 h after the fatty acids. Hepatocyte apoptosis was determined using annexin V membrane binding (A) and flow-cytometric detection of cells showing fragmented DNA as a sub-diploid peak. Results are means $\pm$ S.D. for three experiments. *$P < 0.001$ compared with control, SA, palmitic acid or oleic acid alone.

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