Effect of chronic CB₁ cannabinoid receptor antagonism on livers of rats with biliary cirrhosis

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ABSTRACT

Recent studies have shown that the activated endocannabinoid system participates in the increase in IHR (intrahepatic resistance) in cirrhosis. The increased hepatic production of vasoconstrictive eicosanoids is involved in the effect of endocannabinoids on the hepatic microcirculation in cirrhosis; however, the mechanisms of these effects are still unknown. The aim of the present study was to investigate the effects of chronic CB₁ (cannabinoid 1) receptor blockade in the hepatic microcirculation of CBL (common bile-duct-ligated) cirrhotic rats. After 1 week of treatment with AM251, a specific CB₁ receptor antagonist, IHR, SMA (superior mesenteric artery) blood flow and hepatic production of eicosanoids [TXB₂ (thromboxane B₂), 6-keto PGF₁α (prostaglandin F₁α) and Cys-LTs (cysteinyl leukotrienes)] were measured. Additionally, the protein levels of hepatic COX (cyclo-oxygenase) isoforms, 5-LOX (5-lipoxygenase), CB₁ receptor, TGF-β₁ (transforming growth factor β₁), cPLA₂ [cytosolic PLA₂ (phospholipase A₂)] and sPLA₂ (secreted PLA₂) and collagen deposition were also measured. In AM251-treated cirrhotic rats, a decrease in portal venous pressure was associated with the decrease in IHR and SMA blood flow. Additionally, the protein levels of hepatic CB₁ receptor, TGF-β₁, cPLA₂ and hepatic collagen deposition, and the hepatic levels of 5-LOX and COX-2 and the corresponding production of TXB₂ and Cys-LTs in perfusates, were significantly decreased after 1 week of AM251 treatment in cirrhotic rats. Furthermore, acute infusion of AM251 resulted in a decrease in SMA blood flow and an increase in SMA resistance in CBL rats. In conclusion, the chronic effects of AM251 treatment on the intrahepatic microcirculation were, at least partly, mediated by the inhibition of hepatic TGF-β₁ activity, which was associated with decreased hepatic collagen deposition and the activated PLA₂/eicosanoid cascade in cirrhotic livers.

INTRODUCTION

Δ⁹-Tetrahydrocannabinol is the major active constituent of marijuana, a popular recreational drug of abuse [1]. In addition to its neurobehavioural effects, Δ⁹-tetrahydrocannabinol elicits certain cardiovascular effects in experimental animals and humans [2,3]. Cannabinoid receptors

Key words: cannabinoid receptor, cirrhosis, eicosanoid, increased intrahepatic resistance.

Abbreviations: AA, arachidonic acid; BF, blood flow; BW, body weight; CB₁, cannabinoid 1; CBL, common bile-duct ligation; COX, cyclo-oxygenase; HRP, horseradish peroxidase; IHR, intrahepatic resistance; LOX, lipoxynegenase; LT, leukotriene; Cys-LT, cysteinyll LT; LW, liver weight; MAP, mean arterial pressure; PG, prostaglandin; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; sPLA₂, secreted PLA₂; PVP, portal venous pressure; RI, resistance index; SMA superior mesenteric artery; TGF-β₁, transforming growth factor β₁; TX, thromboxane.

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are present throughout the body and are normally bound by endocannabinoids [4,5]. The two endocannabinoids isolated so far, anandamide and 2-arachidonylglycerol, are lipid in nature [6,7]. Sugiura et al. [8] reported that CB1 (cannabinoid 1) receptors were found on human vascular endothelial and smooth muscle cells and could possibly modulate vascular tone. Furthermore, Randall et al. [9] observed that anandamide induced CB1-receptor-mediated vasorelaxation in normal rat isolated perfused mesenteric arteries. Circulating anandamide and vascular and hepatic CB1 receptors are markedly up-regulated in animals and humans with cirrhosis [10–14]. Acute administration of a CB1 receptor antagonist has been shown to reduce portal pressure and improve mesenteric vasodilatation in rats with CCl4-induced cirrhosis [12,13].

In cirrhotic livers, an increase in the hepatic production of vasoconstrictive eicosanoids [TX (thromboxane) A2 and Cys-LTs (cysteinyl LTs (leukotrienes))] and the activation of the corresponding enzymes [COX (cyclo-oxygenase) and LOX (lipoxygenase)] play important roles in the mechanisms underlying increased IHR (intrahepatic resistance) [15,16]. Our recent study [17] of cirrhotic livers has suggested that acute administration of anandamide significantly increased IHR, which was associated with an increase in the hepatic production of vasoconstrictive eicosanoids. Indeed, the production of eicosanoids is dependent on the activity of PLA2 (phospholipase A2), a rate-limiting calcium-dependent enzyme in the AA (arachidonic acid) cascade, plus COX and LOX activities [18]. In almost every cell studied, multiple PLA2 isoforms exist, but sPLA2 (secreted PLA2) and cPLA2 (cytosolic PLA2) are the crucial enzymes involved in the selective release of AA and eicosanoids [19–21]. PLA2 activation depends on an increase in oxidative stress, such as lipid peroxidation, in tissue, which has been shown to be markedly increased in cirrhotic livers [22–24]. Previous studies have shown that cannabinoids can activate the activity of PLA2 and increase the production of AA-derived eicosanoids in several cell types [25–27]; however, the interaction in cirrhotic livers is still unclear.

The aim of the present study was to evaluate the chronic effects of AM251, a CB1 receptor antagonist, on splanchnic haemodynamics and the intrahepatic microcirculation in cirrhotic rats.

**MATERIALS AND METHODS**

**Animals**

Adult male Sprague–Dawley rats (250–350 g) were used in all experiments. Cirrhotis with portal hypertension was produced by CBL (common bile-duct ligation), as described previously [28]. Sham-operated rats had their bile duct exposed, but not ligated. All rats were caged at 24°C, with a 12-h light/dark cycle and were allowed free access to food and water. The animal studies were approved by the Animal Experiment Committee of the University and conducted humanely.

**Chemicals and reagents**

Polyclonal antibodies to CB1 receptor, COX isoforms, 5-LOX and TXB2, and enzyme immunoassay kits for 6-keto PGF1α [PG (prostaglandin) F1α] and Cys-LTs were purchased from Cayman Chemicals. The rabbit polyclonal antibody to low-molecular-mass PLA2 (sPLA2) was purchased from Upstate Biotechnology. Monoclonal/polyclonal antibodies to mouse TGF-β1 and cPLA2 were purchased from Santa Cruz Biotechnology. HRP (horseradish peroxidase)-conjugated affinity goat anti-rabbit IgG (H+L) sera were purchased from Jackson Immunoresearch Laboratories, and the anti-rabbit HRP antibody was from Bio-Rad Laboratories. Substances other than those described above were purchased from Sigma. AM251 was dissolved in 100 % ethanol to 15 mg/ml and was then diluted in 0.9 % saline to 3 mg/ml immediately prior to use. The vehicle (10 % ethanol in 0.9 % saline) was administered at 0.1 ml/100 g of BW (body weight), which was equivalent to the volume used for the administration of AM251. Control experiments indicated that all the solvents used in the study had no direct effect on PPP (portal perfusion pressure) and IHR at the concentrations used.

**Study protocol**

At 3 weeks after (23–25 days) surgery, both CBL and sham-operated rats were randomly assigned to receive either vehicle or AM251 (3 mg·kg⁻¹ of BW·day⁻¹), a selective CB1 receptor antagonist [29], for 7 consecutive days. All rats were starved for 18 h before experiments. In a preliminary study (results not shown), 7 days of sequential doses of AM251 (1, 3, 5, 7 mg·kg⁻¹ of BW·day⁻¹) were given intraperitoneally with the implantation of Azert Osmotic Pump (Cayman Chemical) to all animals. We found that 1 mg·kg⁻¹ of BW·day⁻¹ did not significantly affect PVP (portal venous pressure), whereas 3 mg·kg⁻¹ of BW·day⁻¹ resulted in a significant reduction in PVP. The administration of 5 or 7 mg·kg⁻¹ of BW·day⁻¹ did not decrease PVP further. Therefore the dose of 3 mg·kg⁻¹ of BW·day⁻¹ was chosen for further studies. In the present study, the overall mortality rate for AM251-treated CBL rats were similar to that in vehicle-treated CBL rats (3–6 %).

**Experiment 1: effects of CB1 receptor blockade on splanchnic haemodynamics**

Two groups of CBL rats and two groups of sham-operated rats (n = 7 in each group) were included in this study. All of the rats were anaesthetized with 100 mg of ketamine/kg of BW. The ileocolic vein was cannulated with PE-10 for PVP measurement, and PVP was monitored using a polygraph (Gould) via strain-gauge transducers (Viggo-Spectramed). Meanwhile, a flow probe connected with a flowmeter (Transonic) was
placed around the SMA (superior mesenteric artery) for continuous monitoring of SMA BF (blood flow) [30,31].

Experiment 2: effects of CB₁ receptor blockade on PPP and hepatic release of TXB₂, 6-keto PGF₁α and Cys-LTs

Cirrhotic and normal livers were obtained from the same set of CBL and sham-operated rats in Experiment 1. Then, the isolated liver perfusion study was done as described previously [17]. Briefly, the re-circulating liver perfusion study was conducted with 100 ml of Krebs solution [118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l MgSO₄, 1.2 mmol/l KH₂PO₄, 25 mmol/l NaHCO₃, 0.5 mmol/l EDTA, 11.1 mmol/l glucose and 2.5 mmol/l CaCl₂ (pH 7.4) and was gassed with 95% O₂/5% CO₂] with a constant rate of 35 ml/min. After the system was stable, PPP was monitored for 30 min. In addition, 3 ml of the perfusates were collected before and 30 min after the re-circulating system was started to measure the basal production of eicosanoids. Before analysis, the perfusates were acidified to pH 3.5 with 1 mol/l HCl, mixed, centrifuged at 2500 × g and probed with primary antibodies (rabbit polyclonal antibody) and secondary antibodies [goat anti-rabbit HRP and peroxidase-conjugated affinipure goat anti-rabbit IgG (H + L)]. Pre-stained protein markers were used to estimate the masses of the protein bands. Subsequent detection of the specific protein bands was visualized by ECL* (enhanced chemiluminescence; Amersham Biosciences). Finally, binding of a GADPH (glyceraldehyde-3-phosphate dehydrogenase) antibody to the same membrane was used to confirm that the amount of protein loading was equal. Densitometric quantification of the signal intensity of autoradiograms was performed using a densitometric analysis program (KDS1D20; Kodak Digital Science).

In the present study, an immunoprecipitation protocol was used to measure the hepatic expression of low-molecular-mass PLA₂(sPLA₂). Briefly, 500 μg–1 mg (1 μg of protein/litre) of cell lysate was added to a microcentrifuge tube and was mixed with 5–10 μg of anti-sPLA₂ sera. After gently rocking the reaction mixture overnight at 4°C, the immunocomplex was captured by 10 μl of washed Protein A–agarose beads. After draining the supernatant, the agarose beads were rocked gently in the residual reaction mixture for 2 h at 4°C. The beads were washed three times with ice-cold PBS (in the absence of SDS and deoxycholic acid) and were suspended in 50 μl of 2× Laemmlli sample buffer. After boiling for 5 min and centrifuging, the supernatant was used for immunoblot analysis, as described above.

Experiment 4: measurement of hepatic collagen deposition

In this experiment, a portion of liver tissue was obtained from each CBL rat receiving AM251 or vehicle for 1 week. In addition, another group of sham-operated rats receiving vehicle alone was included for comparison. Liver tissues were homogenized in acetic acid (0.5 mol/l) at 4°C. The fraction of insoluble collagen after acid extraction, composed of cross-linked collagen, was then heated at 80°C for 60 min to convert it into soluble gelatin. The gelatin content of the acid extracts was assayed using a Sirocol collagen assay kit (Biocolor), according to the manufacturer’s instructions and the method described in our previous study [34].

Experiment 5: haemodynamic effects of acute infusion of AM251

In this experiment, another group of CBL and sham-operated rats (n = 6 in each group) were included. Under
ketamine anaesthesia, the femoral artery and vein were cannulated with a PE-50 tube to monitor MAP (mean arterial pressure) and to infuse the drugs. PVP and SMA BF were measured as in Experiment 1. Meanwhile, SMA RI (SMA resistance index; in dyn·s·cm⁻²·10⁻⁵/100 g of BW) was calculated as: (MAP – PVP) × 80/SMA BF/BW. After an initial stable period of 30 min, basal values were obtained. Subsequently, sequential doses of AM251 (1, 3, 5, 7 mg/kg of BW) were administered intravenously in CBL and sham-operated rats. The maximal changes of each parameter after the injection of each dose of AM251 were recorded in each animal. The total duration of the experiment was up to 100 min. Rats with unstable haemodynamic status (such as hypotension or tachypnoea) were not included in the analysis.

**Statistical analysis**
Values are means ± S.E.M. One-way ANOVA (for BW, LW and haemodynamics) and unpaired Student’s t test (for PPP, TXB₂, 6-keto PGF₁α and Cys-LTs production) were used to determine any statistical significance.

**RESULTS**

**Effects of CB₁ receptor blockade on splanchnic haemodynamics**
All CBL rats had liver cirrhosis, ascites and splenomegaly by gross inspection. Higher LWs, PVP and SMA BF were observed in CBL rats compared with sham-operated rats (Table 1). Treatment for 1 week with AM251 significantly decreased PVP and SMA BF in CBL rats compared with those receiving vehicle. However, splanchnic haemodynamics were not changed by treatment for 1 week with AM251 in sham-operated rats.

**Effects of CB₁ receptor blockade on PPP, and hepatic release of TXB₂, 6-keto PGF₁α and Cys-LTs**
In vehicle-treated groups, IHR and hepatic production of eicosanoids (TXB₂ and Cys-LTs) were higher in cirrhotic livers than in normal livers (Table 1 and Figure 1). In CBL rats receiving AM251, a significant decrease in IHR was associated with a suppression in the hepatic production of TXB₂ and Cys-LTs compared with CBL rats receiving vehicle (Table 1 and Figure 1). However, hepatic production of 6-keto PGF₁α was not changed in cirrhotic livers by treatment for 1 week with AM251 (Table 1 and Figure 1). Similarly, IHR and the hepatic production of eicosanoids in normal livers were not changed by AM251 treatment in sham-operated rats.

**Effects of CB₁ receptor blockade on the protein expression of hepatic COX-1, COX-2, 5-LOX, CB₁ receptor, TGF-β₁, cPLA₂ and sPLA₂**
In vehicle-treated groups, protein levels of hepatic CB₁ receptor, 5-LOX, COX-1, COX-2, TGF-β₁, cPLA₂ and sPLA₂ in cirrhotic rats were higher than those in sham-operated rats (Figures 2–4). In AM251-treated cirrhotic rats, the protein levels of hepatic CB₁ receptor, 5-LOX, COX-2, TGF-β₁ and cPLA₂ were lower than those in vehicle-treated cirrhotic rats. However, the protein level of sPLA₂ was similar between the AM251- and vehicle-treated CBL rats. Additionally, the expression of various proteins was not changed by treatment with AM251 in sham-operated rats.

**Measurement of hepatic collagen deposition**
In vehicle-treated groups, hepatic collagen deposition in cirrhotic livers were significantly higher than those in normal livers (Table 1). In AM251-treated cirrhotic rats, hepatic collagen deposition was lower than those in vehicle-treated cirrhotic rats.

**Haemodynamic effects of acute infusion of AM251**
In the basal condition, higher PVP and SMA BF, and lower MAP and SMA RI, were observed in CBL rats than those in sham-operated rats. Following AM251 infusion,
Effects of cannabinoid receptor inhibition in cirrhotic rats

The pathophysiological hallmarks of the non-structural and reversible dynamic component of increased IHR in cirrhosis are the increased production of local vasoconstrictors, the increased response to vasoconstrictors and the reduced release of intrahepatic vasodilators, such as NO. These changes in cirrhotic livers result in an imbalance between these vasoconstrictor/vasodilator forces and the development of portal hypertension [15–17]. In the present study, a significant decrease in PVP was associated with a significant decrease in IHR both in acute and chronic AM251-treated cirrhotic rats. In contrast with sham-operated rats, the acute infusion of AM251 resulted in a significant decrease in SMA BF and an increase in SMA RI in CBL rats. This indicated that AM251 could induce mesenteric vasoconstriction in cirrhotic rats. In other words, the decrease in portal pressure in CBL rats by CB1 receptor blockade was the result of a decrease in the resistance of the hepatic microcirculation and an amelioration of splanchnic vasodilatation. A recent study [14] in three models of cirrhotic rats induced by CCl4, thioacetamide and bile-duct ligation showed that pharmacological inactivation of CB1 receptors can be achieved by acute and chronic administration of a CB1 receptor antagonist. Similarly, the present study has shown that the overexpression of hepatic CB1 receptors in cirrhotic rats was effectively inhibited by treatment for 1 week with AM251. This indicated that the dose and duration of AM251 used in the present study was adequate. A recent study [14] has shown that hepatic CB1 receptors could be up-regulated by endogenous ligands, such as endocannabinoids or inflammatory cytokines. As for other G-protein-coupled receptors, the expression of CB1 receptors may also be regulated by the efficiency of the binding between the ligand and receptor [35]. Thus the inhibition of hepatic CB1 receptors by chronic administration of AM251 may be attributed to blocking of ligand–receptor binding and a simultaneously suppression of TGF-β1-related cytokines release. Teixeira-Clerc et al. [14] reported a similar finding in CBL rats after chronic CB1 receptors antagonism. Indeed, it has been established that the contribution of cannabinoids to the splanchnic vasodilatation in cirrhosis is mostly due to an up-regulation of CB1 receptors on rat cirrhotic mesenteric arteries [36]. Additionally, CB1 receptors have also been shown to be up-regulated in cirrhotic livers [11,37,38]. Our recent study [17] suggested that anandamide plays a role in increased IHR in cirrhotic livers through the release of intrahepatic vasoconstrictive eicosanoids. In the present study, we confirm further the role of hepatic CB1 receptors in increased IHR in CBL-induced cirrhotic rats.

The families of TXs, PGs and LTs are called eicosanoids, because they are derived from C20 essential fatty acids, such as 5,8,11,14-eicosatetraenoic acid (AA). Hormones, autacoids and physical stimuli, such as oxidative stress, augment the biosynthesis of eicosanoids by interacting with plasma-membrane-bound G-protein-coupled receptors. This results in elevated cytosolic concentrations of calcium, thereby activating PLA2. Thus
PLA$_2$ hydrolyses the membrane phospholipids resulting in the release of AA. Once released, AA is metabolized rapidly by COX or LOX to eicosanoids. It is obvious that the release of AA and, subsequently, eicosanoids are dependent mainly on the activation of calcium-dependent PLA$_2$ [18,39]. Therefore any alteration in
PLA2 activation may directly influence the production of eicosanoids.

It has been confirmed that cannabinoids activate PLA2 activity and promote the release of AA from membrane phospholipids in a variety of mammalian somatic cells [25–27,40]. The detailed mechanism has been suggested to involve the binding of cannabinoids to CB1 receptors, which are also G-protein-coupled receptors, to decrease intracellular calcium concentrations and stimulate the release of AA [41–43]. The present study has shown that the increased hepatic production of TXB2 and Cys-LTs, which are important AA-derived vasoconstrictors in cirrhotic livers [15,16], was associated with the up-regulation of hepatic PLA2, 5-LOX and COX-2. In addition, a simultaneous up-regulation of hepatic CB1 receptors in cirrhotic livers was observed. Furthermore, the present study has also shown that the increased hepatic protein levels of PLA2, COX-2 and 5-LOX and the production of AA-derived eicosanoids (TXB2 and Cys-LTs) in cirrhotic livers were also simultaneously and significantly suppressed by chronic CB1 receptor blockade. This indicated that the activation of these substances in cirrhotic livers might be mediated by an up-regulation of hepatic CB1 receptors and the increase in circulating cannabinoids [10]. Recently, cannabinoids have been found to induce necrosis of activated hepatic

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**Table 2** Haemodynamic data before and after acute infusion of AM251 in CBL and sham-operated rats

Values are means ± S.E.M. *P < 0.05 compared with basal. 1 dyn = 10⁻⁵ N.

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stellate cells and, subsequently, to increase intracellular reactive oxygen species, which may activate PLA2 system [44]. Previous studies have detected the expression of PLA2 mRNA and protein in either homogenates or macrophages of rat livers [45,46]. Another study has also shown that the activation of the PLA2 system was detected in the kidney and liver of cirrhotic rats [47]. Thus increased circulating endocannabinoids may activate the hepatic PLA2 system, via an up-regulation of hepatic CB1 receptors and an increase in oxidative stress in cirrhotic livers.

A recent study [14] in knock-out mice has shown that genetic inactivation of the CB1 receptor resulted in a significant reduction in hepatic myofibroblasts in vivo. The same study [14] also showed that acute and chronic pharmacological inactivation of CB1 receptor in rats with biliary cirrhosis markedly decreased the proliferation of hepatic fibrogenic cells. Furthermore, hepatic TGF-β1, which was significantly suppressed by CB1 receptor blocking, served as a fibrogenic marker [14]. Consistent with these findings, the hepatic TGF-β1 level and collagen deposition in the cirrhotic rats in the present study were also inhibited by chronic CB1 receptor antagonism. Enhanced TGF-β1 expression had been reported in experimental and human with biliary cirrhosis [48,49]. Previous studies have shown that TGF-β1, which could induce and activate the PLA2 activity in human embryo lung fibroblasts, calf pulmonary artery endothelial cells and rat mesangial cells [50,51]. Thus, in the present study, the suppression of hepatic TGF-β1 expression and collagen deposition by chronic CB1 receptor antagonism might result in the inhibition of hepatic PLA2 activity and a corresponding production of vasoconstrictive eicosanoids in cirrhotic rats. Moreover, the inhibition of hepatic TGF-β1 protein expression and collagen deposition also indicated a suppression of liver fibrogenesis by CB1 receptor inactivation in cirrhotic rats. Therefore it is possible that both the decreased production of vasoconstrictive eicosanoids and inhibition of the liver fibrogenesis contributed to the decrease in IHR and corresponding PVP in cirrhotic rats in the present study.

Among multiple PLA2 isofoms, sPLA2 and cPLA2 are likely to be the major phopholipases involved in agonist-stimulated AA release [19,20]. However, previous studies in cPLA2-deficient mice [47] demonstrated that cPLA2 is a crucial enzyme in determining the amount and rate of production of eicosanoids. In addition, it had been reported that the activation of cPLA2 was highly dependent on a rise in intracellular calcium concentration, which leads to the translocation of the enzyme from the cytosol to nucleus, allowing it access to its substrate [39]. Consistent with previous findings [52,53], in the present study, the protein levels of cPLA2 and sPLA2 were significantly increased in cirrhotic livers compared with normal livers. However, chronic blocking of CB1 receptors only significantly inhibited the up-regulated hepatic cPLA2 protein, rather than hepatic sPLA2 protein, in cirrhotic livers (Figure 3). It had been reported that activation of the CB1 receptor may decrease intracellular calcium concentration and stimulate the activation of cPLA2 [41–43,53]. It is possible that the up-regulation of hepatic CB1 receptors and increased circulating endocannabinoids in cirrhosis may activate hepatic cPLA2 to release hepatic eicosanoids, resulting in the increase in IHR found in the present study.

In conclusion, the activated endocannabinoid system increased the resistance of the hepatic microcirculation by stimulating the cPLA2 system and COX and LOX activities, resulting in the subsequent release of vasoconstrictive eicosanoids (TXA2 and Cys-LTs) in cirrhotic rat livers. The up-regulation of hepatic CB1 receptors may, in part, play an important role in the pathogenesis of increased IHR in rats with biliary cirrhosis. Chronic cannabinoid CB1 receptor blockade significantly decreased IHR in cirrhotic rats. This effect was, at least partly, mediated by the inhibition of hepatic TGF-β1 activity associated with the decreased collagen deposition and the suppression of the PLA2/eicosanoid cascade in cirrhotic rat livers.

ACKNOWLEDGMENTS

This study was supported by the National Science Council (grant number NSC94-2314-B-075-002), and by the Taipei Veterans General Hospital, Taipei, Taiwan (grant number VGH94-225).

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Received 14 September 2006/22 November 2006; accepted 18 December 2006
Published as Immediate Publication 18 December 2006, doi:10.1042/CS20060260