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# Response of Immobilized Hepatocytes in a Perfusion System to Anoxia/Reoxygenation: Effect of Cyclosporine A Pretreatment

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## Summary

**The present study was designed to investigate the ameliorative effect of cyclosporine A (CsA) pretreatment on an anoxia/reoxygenation injury model by using immobilized perfused hepatocytes. Rats received an i.p. injection of two successive doses of CsA (5 mg/kg/day). Twenty-four hours later hepatocytes were isolated from CsA-treated and control rats. After hepatocyte isolation, immobilization, perfusion, induction of anoxia/reoxygenation, the structural and functional integrity of the hepatocytes was followed in a perfusion medium by measuring the leakage of lactate dehydrogenase (LD) and the time course of urea biosynthesis. CsA pretreatment reduced the initial rate of urea synthesis during normoxia but reduced the drop in the relative percentage rate of urea synthesis during the period of anoxia. LD leakage was increased threefold by anoxia and sevenfold by reoxygenation in cells of untreated animals. After CsA pretreatment *in vivo*, hepatocytes showed no increase in LD leakage into the medium. These findings demonstrate that the perfused immobilized hepatocytes can be used as a cellular model to assess the effects of liver insults such as anoxia/reoxygenation injury and that CsA modulates the injury. The mechanisms of CsA beneficial effects at the experimental level remain to be elucidated.**

**Key words**
Immobilized hepatocytes – Anoxia/reoxygenation – Cyclosporine A

## Introduction

Oxygen supply to the liver is critical in several pathophysiological conditions. An important situation is the ischaemia/reperfusion injury which is involved in the pathogenesis of various liver problems that occur during a number of pathological conditions such as hepatic surgery, shock and liver transplantation. Various experimental animal models (*in vivo*) and cellular models (*in vitro*) have been applied to study the liver injury produced by ischaemia/reperfusion (Jaeschke and Farhood 1990, Sakr *et al.* 1991, Wu *et al.* 1992). Although the results of various investigations are significant, it is difficult to interpret them due to the differences between various models employed and interferences of exogenous and endogenous factors. In our laboratory, the possibility of working with the model of isolated immobilized and perfused hepatocytes which excludes other types of cells, such as endothelial cells, macrophages, Kupfer and Ito cells, offers several advantages over those studies using classical cellular models, the whole perfused liver or the whole organism. This attractive model of homogeneous populations of immobilized perfused hepatocytes was used recently and effectively to study the role played by oxygen free radicals in reoxygenation injury of both rat and human hepatocytes where the importance of anoxia/reoxygenation injury in liver surgery is controversial (Caraceni *et al.* 1994).

We have used the model of perfused hepatocytes in a number of studies, including the physiological and biochemical events that follow anoxia/reoxygenation employing conventional and phosphorus-31 NMR spectroscopical methods (Gasbarrini *et al.* 1992a,b, Farghali *et al.* 1992). In addition, the potential ameliorative effect of the polypeptide cyclosporine A (CsA) against various hepatic injuries both *in vivo* and *in vitro* was investigated by using immobilized perfused hepatocytes (Farghali *et al.* 1991). Currently, CsA is the most widely used immunosuppressive agent for prevention of allograft rejection after transplantation and for the treatment of a number of autoimmune diseases (Calne *et al.* 1978, Griffeth *et al.* 1986, Tindall *et al.* 1987, Starzl *et al.* 1989). CsA has been demonstrated to possess some adverse effects such as nephrotoxicity and moderate hepatotoxicity (Inselmann *et al.* 1990). However, it was reported that CsA was found to be hepatoprotective against a number of chemical and non-chemical insults (Lie *et al.* 1991, Mazzaferro *et al.* 1990, Shimizu *et al.* 1994, Farghali *et al.* 1991, Hermansky *et al.* 1990). In a recent study, we have reported significant ameliorative effects of CsA pretreatment *in vivo* on acute carbon tetrachloride intoxication (Farghali *et al.* 1995).

Therefore, the present report was directed to the study of the effect of CsA pretreatment on anoxia/reoxygenation injury model by using the immobilized perfused hepatocyte model. The integrity of the plasma membrane was followed in the perfusion medium by measuring the leakage of lactate dehydrogenase into the bioreactor perfusion medium. Furthermore, the time course of urea biosynthesis by isolated immobilized perfused liver cells during the experiment was investigated as indicative of the metabolic competence of hepatocytes.

## Methods

*Animals and treatment*

Male Wistar rats weighing 250–300 g received an i.p. injection of two successive doses of CsA (CONSUPREN Galena a.s., 5 mg/kg) with a 24-hour interval. Twenty-four hours later (on the third day after the beginning of this experiment) hepatocytes were isolated. Control rats were treated intraperitoneally with sterile saline solution instead of CsA (control), or with CsA but without subjecting the cells to anoxia/reperfusion (CsA control). The hepatocytes were isolated, examined histologically, immobilized and perfused in a bioreactor model for further metabolic and functional activity evaluation.

*Methods of hepatocyte isolation, immobilization, perfusion, induction of anoxia/reoxygenation and the biochemical analysis*

The method of isolation, counting, immobilization and perfusion of hepatocytes with the RPMI 1640 medium was basically the same as described in our earlier reports (Farghali *et al.* 1992, 1994, Hynie *et al.* 1994). After thorough washing to remove unattached cells and cell debris, the cells in the bioreactor were allowed to stabilize for about 1 hour. Thereafter, the cells were perfused for another hour in a recirculating system and under normal oxygenation with carbogen to measure the rate of urea synthesis under normoxic condition. Anoxia was initiated by replacing carbogen with nitrogen and taking care that ambient atmosphere would not gain access to the perfusion medium by sealing the medium reservoir. Anoxia was continued for 2 hours. Then, reoxygenation with carbogen was resumed for a further hour. Urea biosynthesis was followed in the perfusate of the hepatocyte system every 10-30 min by taking the appropriate volume of the perfusate after cell stabilization during the four hours of normoxia, anoxia and reoxygenation, respectively. Urea estimations were carried out by using the appropriate Sigma kit. Control experiments were performed on cells obtained from saline-treated rats. In some experiments, we conducted other type of controls, where cells in the bioreactor were under normoxia for type of the entire 4 hours to evaluate the hepatocytes stability as indicated by lactate dehydrogenase leakage and their functionality by urea synthesis. The perfusate samples belonging to various types of experiments were also used for lactate dehydrogenase concentration measurement using an ultraviolet analytical method and Sigma kits. Consequently, the plasma membrane integrity of the hepatocytes in the bioreactor during various phases of normoxia or anoxia/reoxygenation was monitored.

The amount of urea synthesized under various experimental conditions was calculated in mg for 100 ml of the perfusate and was always normalized to a cell count of $1.6 \times 10^8$ cells in one experiment. The rate of the urea biosynthesis was also calculated and expressed in mg/$1.6 \times 10^8$ cells/h under various treatments.

## Results

The mean viability of isolated hepatocytes as measured by trypan blue exclusion after CsA administration was $88.5 \pm 1.8$ %, which is a little higher than the viability of the hepatocytes obtained from saline-treated animals which was $86 \pm 1.6$ %. The time course of cumulative urea biosynthesis is shown in Fig. 1. Urea production by immobilized hepatocytes was evident a few minutes after perfusion and increased steadily as time progressed. The time course of cumulative urea synthesis as obtained from cells under normoxia during 4 hours exhibits a steady increase, while anoxia reduced the cumulative increase for both untreated cells and CsA-pretreated cells.

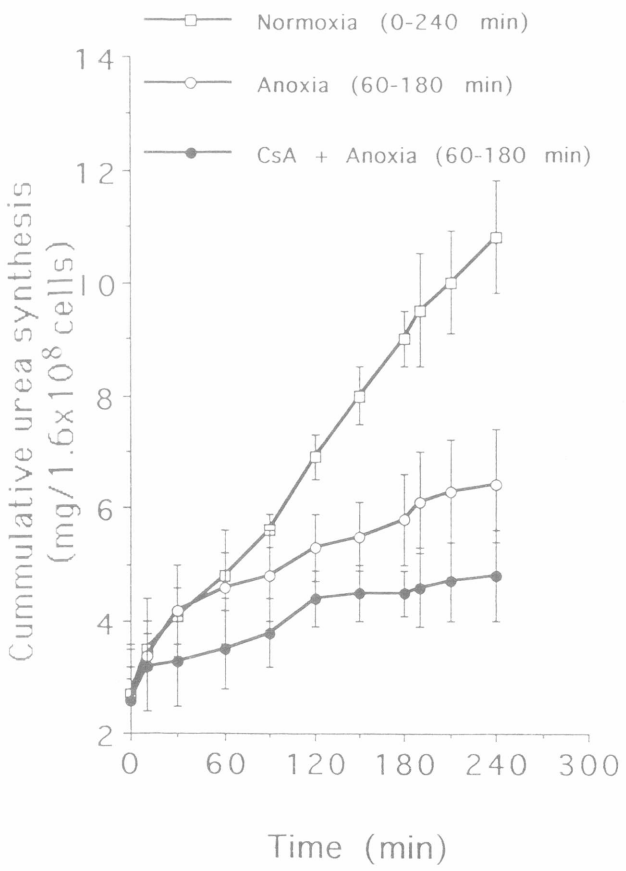


**Fig. 1**
*The time course of cumulative urea synthesis by immobilized hepatocytes obtained at different phases of perfusion of three experimental groups; a) control cells were subjected to continuous normoxia (0–240 min); b) control cells were subjected to normoxia (0–60 min), anoxia (60–180 min) and reoxygenation (180–240 min); c) hepatocytes obtained from CsA-pretreated rats were subjected to normoxia (0–60 min), anoxia (60–180 min) and reoxygenation (180–240 min). Data represent means ± S.E.M, n = 5–8.*

CsA-pretreated cells, however, exhibit lower values than those cells obtained from saline/treated rats (normoxia or anoxia 60–180 min). When the rates of urea synthesis by perfused immobilized hepatocytes at phases of normoxia, anoxia and reoxygenation or at continuous normoxia were calculated, significant differences were observed. As is shown in Fig. 2, the rate of urea synthesis of hepatocytes under normoxic conditions was constant during the 4-hour period. In experiments where hepatocytes were exposed to a second phase of anoxia, there was a significant difference between CsA-pretreated and untreated cells during the first phase of normoxia. There was a twofold higher urea synthesis rate of untreated cells. A two hours' period of anoxia reduced the rate of urea production by untreated cells to 25 % of the original normoxic control values while the same period of anoxia reduced urea synthesis rate by CsA-pretreated cells to only 60 % of the normoxic value. However, both absolute rate estimates were the same. After reoxygenation, the urea synthesis was non-significantly increased or decreased by untreated and CsA-treated hepatocytes, respectively.

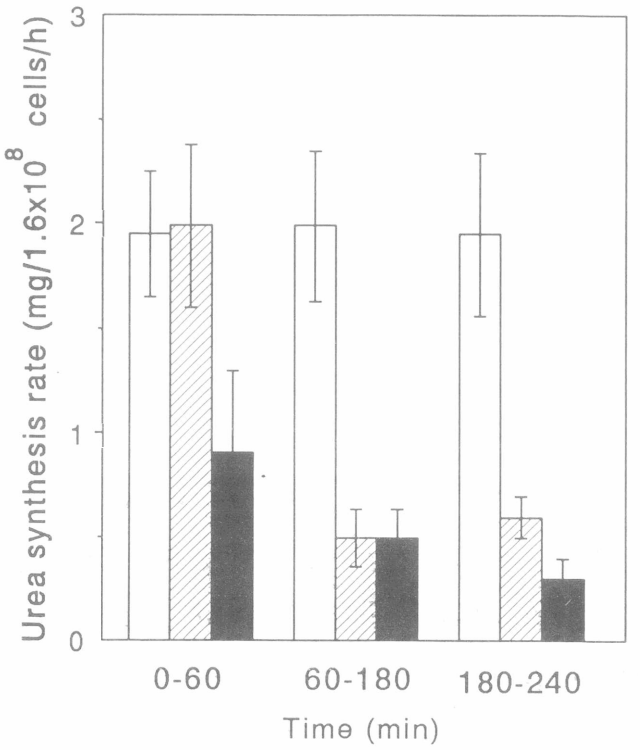


**Fig. 2**
*The rate of urea synthesis by immobilized hepatocytes obtained at different phases of perfusion of three experimental groups; a) control cells were subjected to continuous normoxia (0–240 min, open columns); b) control cells were subjected to normoxia (0–60 min), anoxia (60–180 min) and reoxygenation (180–240 min, hatched columns); c) hepatocytes obtained from CsA-pretreated rats were subjected to normoxia (0–60 min), anoxia (60–180 min) and reoxygenation (180–240 min, dark columns). Data represent means ± S.E.M, n = 5–8.*

The effect of CsA treatment on the leakage of lactate dehydrogenase under various conditions is presented in Fig. 3. It may be deduced from this figure that plasma membrane integrity of hepatocytes, as measured by LD leakage, was stable during oxygenation in control cells until the end of the experiment, i.e. during 4 hours of normoxia. Otherwise, the latter figure illustrates different profile of lactate dehydrogenase leakage during normoxia, anoxia and reoxygenation in control and CsA-pretreated cells subjected to anoxia. Lactate dehydrogenase leakage of the cells was stable during oxygenation and was minimal under CsA treatment. LD leakage was

increased to threefold by anoxia and to sevenfold by reoxygenation in cells of untreated animals. After CsA pretreatment *in vivo*, the hepatocytes showed no increase in LD leakage into the medium. LD values as obtained from the cellular perfusate subjected to anoxia/reoxygenation after CsA pretreatment did not differ from the control values, obtained from cells during four hours of normoxia. In other words, CsA pretreatment kept LD leakage to a minimum in all the three experimental phases of normoxia, anoxia and reoxygenation as assessed by measuring the cumulative amount of LD released into the medium.

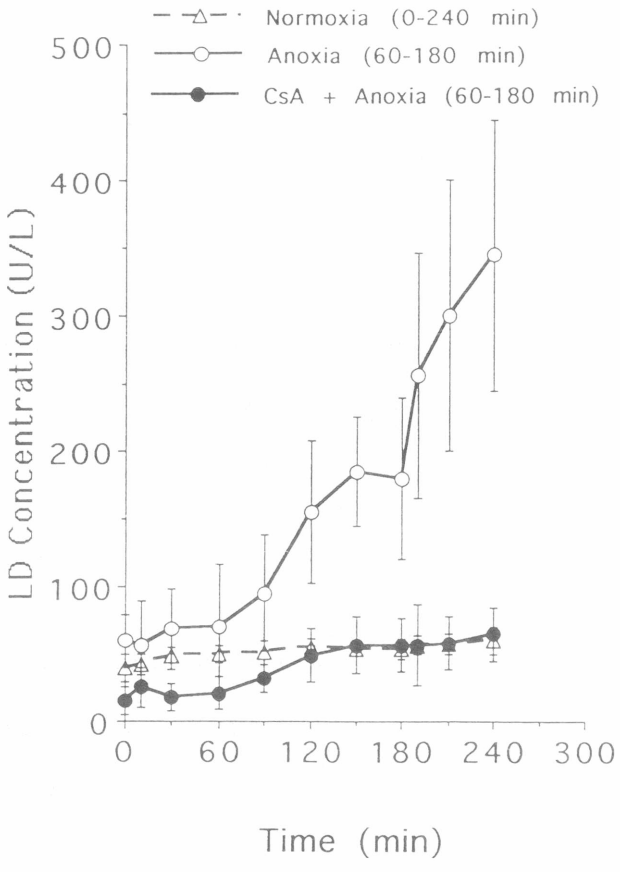


**Fig. 3**
*The time course of the cumulative leakage of lactate dehydrogenase into the perfusion medium from perfused immobilized hepatocytes in various experimental groups (means ± S.E.M, n = 5–8).*

## Discussion

In previous reports, we used the perfused hepatocyte model to investigate the effect of anoxia on intracellular ATP, $Na^+$, $Ca^{2+}$, $Mg^{2+}$ and cytotoxicity, and how fasting enhances the damaging effect of anoxia in rat hepatocytes (Gasbarrini *et al.* 1992a,b, Farghali *et al.* 1992). After two hours of anoxia β-ATP was reduced to 15 % of the normoxic value, while the inorganic phosphate increased. Using the same model, the present study demonstrates the effects of anoxia/reoxygenation on isolated perfused hepatocytes under control conditions and *in vivo* CsA pretreatment, as evaluated by ureagenesis and lactate dehydrogenase leakage.

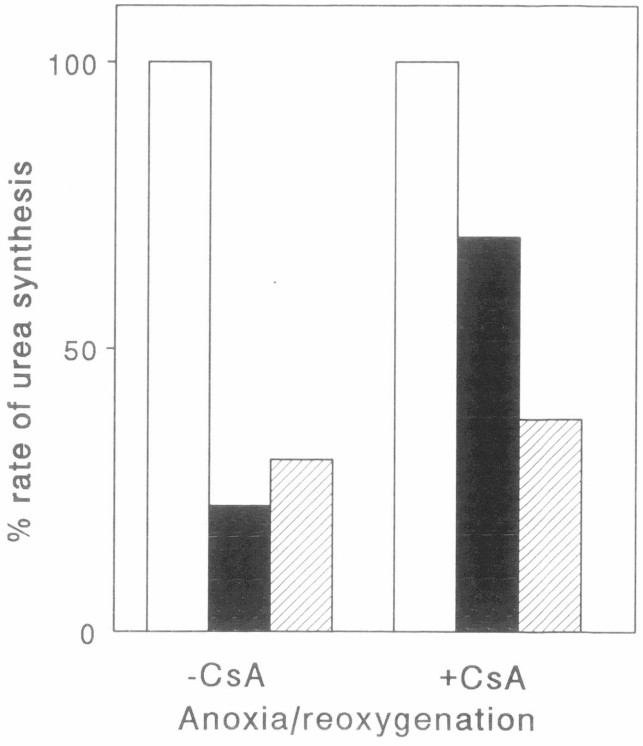


**Fig. 4**
*The relative percentage rate of urea synthesis by perfused immobilized hepatocytes. The relevant initial rate at normoxia was taken from Fig. 2 and considered as 100 % for two experimental groups. Groups 1 and 2 show the relative percentage rate at phases of normoxia (open columns), anoxia (black columns) and reoxygenation (hatched columns) for control and CsA-pretreated hepatocytes, respectively.*

It is well known that most ureagenesis occurs in the liver (Ampola 1994). Urea biosynthesis is a highly energy demanding process, where four high-energy phosphate equivalents are required for each turn of the cycle. Therefore, between a variety of approaches to assess cellular integrity, specifically metabolic competence, ureagenesis from amino acids in the medium was applied to the present investigation. In control experiments, the rate of urea synthesis by perfused hepatocytes after stabilization was constant during the four hours' study under normoxia and averaged 2±0.2 mg/h/1.6x$10^8$ cells. The reduction of ureagenesis during anoxia is not unexpected due to the limited supply of ATP. Interesting is the lowering effect of CsA pretreatment on the rate of urea synthesis

during normoxia. Our earlier reports (Farghali *et al.* 1991) and those of others indicated that CsA pretreatment maintains a higher hepatocyte energy compared to untreated hepatocytes in rats and that CsA treatment ameliorates the reduction of hepatic metabolism (measured in terms of the ATP content) normally observed after a fructose challenge in the liver with a portacaval shunt in dogs (Rossaro *et al.* 1991). However, the ATP content of hepatocytes, is not the determinant of urea biosynthesis. Urea production involves a highly regulated cycle in which any one of the five participating enzymes (carbamylphosphate synthetase, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase and arginase) could be the rate-limiting factor (Sierra-Santoyo *et al.* 1994). The effect of CsA pretreatment on the expression of these enzymes is not yet clear. Nevertheless, there is a significant change in the relative percentage rate of urea synthesis by hepatocytes during the anoxic period. The relative percentage rate of urea synthesis of control hepatocytes dropped to 25 % of its normoxic value, while the rate of urea synthesis of CsA-pretreated cells dropped to 60 % of its initial normoxic value (Fig. 4). During the next hour of reoxygenation, the relative percentage rates of urea synthesis were 30 % and 35 % of the normoxic values for both control and CsA-pretreated cells, respectively. At present it is not clear if this means an overall improvement in urea synthesis rate during anoxia.

Indeed, the ameliorative effect of CsA on anoxia/reoxygenation of injury hepatocytes was very significant through a test of the integrity of the plasma membrane as depicted by the retention of the cytosolic enzyme LD. Leakage of LD is the most popular test of plasma membrane integrity performed in cytotoxicity and cytoprotective studies (Fry and Hammond 1993). CsA abolished LD leak during anoxia and its dramatic release during reoxygenation. These results confirm the reports of other investigators who have found that CsA exhibited beneficial effects on various experimental models of ischaemia/reperfusion injuries of the liver *in vivo* (Kawano *et al.* 1989, Hayashi *et al.* 1988, 1989, 1991, Yamanoi *et al.* 1991, Goto *et al.* 1991, Kurokawa *et al.* 1992). A recent study also reported the beneficial effects of CsA on reoxygenation injury of the isolated perfused rat liver (Shimizu *et al.* 1994). The cytoprotective effect of CsA in the last study was demonstrated on the mitochondria but not on the plasma membrane. This discrepancy is expected to be due to variation in experimental models and the protocol, but this is not contrary to the final beneficial effect of CsA which is a common feature in various studies.

The precise mechanism of the protective effect of CsA in anoxia/reoxygenation injury has not yet been elucidated. We will present, however, some key points which may help to throw light on the mechanism(s) involved. We have reported (Gasbarrini *et al.* 1992a,b) that a large increase in $Ca^{2+}{}_i$ occurs in hepatocytes during anoxia. The origin of this $Ca^{2+}{}_i$ could be mitochondrial, from the endoplasmic reticulum or extracellular. It is an important initial step, but only one step in the cascade of events that leads to cell injury and even to damage if anoxia is prolonged (Shimizu *et al.* 1994). The latter authors suggested that the mitochondria are susceptible to reoxygenation, a type of injury that is due to permeability transition. They demonstrated that the reoxygenation of mitochondria causes the release of matrix proteins, impaired mitochondrial functional recovery, loss of their proton electrochemical gradient and membrane potential due to permeability transition. The release of matrix proteins is modulated by CsA and was described to be a CsA-sensitive pathway (Ibavboa *et al.* 1989). Therefore, we support the suggestion of Shimizu *et al.* (1994) who relate one of the mechanisms by which CsA reduces anoxia/reoxygenation injury to an alteration of permeability transition. Savage and Reed (1994) have recently reported that the release of both glutathione and calcium from mitochondria was inhibited by the addition of CsA which is a potent inhibitor of permeability transition. It was also found that CsA protected hepatocytes from prooxidant-induced injury by preventing mitochondrial calcium release ($Ca^{2+}$ cycling) and subsequent mitochondrial dysfunction (Kass *et al.* 1992). Although the physiological role of nitric oxide synthesis in the liver is not addressed in the present work and is beginning to be elucidated, an inhibitory effect of CsA on nitric oxide synthase activity was reported in other types of cells (Conde *et al.* 1995). We still do not know whether CsA also modulates NO production and whether NO has beneficial or harmful effects.

In conclusion, the present data demonstrate that the perfused immobilized hepatocytes can be used as a cellular model to assess the effects of liver insults such as anoxia/reoxygenation injury and that CsA modulates the injury. Though the mechanisms of CsA beneficial effects remain to be elucidated, some suggestions are presented. The ability of CsA to intercept certain hepatic injuries, beside the current use in organ transplantation and autoimmune diseases, implicates that the clinical indications of this drug may be potentially increased. Further studies are needed to define the mechanism(s) of CsA cytoprotective effects and the appropriate doses or blood levels needed for these effects.

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