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SHORT COMMUNICATION

Cyclosporin A Modifies Cytoplasmic Calcium Levels in Isolated Hepatocytes Exposed to Oxidative Stress Due to *tert*-Butyl Hydroperoxide

E. KMONÍČKOVÁ, L. KAMENÍKOVÁ, S. HYNIE, H. FARGHALI

Institute of Pharmacology, First Faculty of Medicine, Charles University, Prague, Czech Republic

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Summary

Within the framework of our studies on hypertension in various rat strains, we have examined the effect of cyclosporin A (CsA) on intracellular calcium signaling under conditions of oxidative stress. For these preliminary experiments, we have chosen isolated hepatocytes of normotensive rats as a model system for the study of the role of intracellular calcium. We used *tert*-butyl hydroperoxide (t-BHP, 1 mmol.l^{-1}) as an prooxidant agent. When compared to the controls, we found increased levels of cytosolic free calcium concentration (Ca^{2+}_i) during 120 min incubation. The preincubation of hepatocytes with CsA in the concentration of 0.5 μmol.l^{-1} did not change the physiological level of cytosolic calcium. However, a dual action of CsA on elevated Ca^{2+}_i was observed during oxidative injury of hepatocytes: while in the first period of incubation CsA increased Ca^{2+}_i, CsA reduced the effect of t-BHP on Ca^{2+}_i during the next period of incubation. This indicates the ability of CsA to modify oxidative stress, but further studies are necessary to explain these findings.

Key words

Rat hepatocytes • Hypertension • Ca^{2+}_i • Cyclosporin A

In the framework of our studies on hypertension in various rat strains, we have examined the effect of cyclosporin A (CsA) on intracellular calcium levels under conditions of oxidative stress. Although there is no doubt about the importance of calcium for normal cell functions, numerous reports have shown that excess of intracellular calcium is responsible for cell death. Since it is very difficult to study the role of intracellular free calcium in hypertension, we decided to use experimental model of isolated hepatocytes to demonstrate the effects of oxidative stress on intracellular calcium levels and the effect of cyclosporin A on this parameter. CsA in this study was used from two reasons, namely because this drug is known to induce hypertension (Cavarage *et al.* 1998). Secondly, it was demonstrated that it acts not only as an immunosuppressive agent but also as a drug which has beneficial effects on cells subjected to a variety of injurious conditions (Farghali *et al.* 1996) and was used as a useful agent for reducing cell damage (Gatewood *et al.* 1996).

Fax +420 2 293 894
E-mail: physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

Recent data have shown that the degree of CsA-induced hypertension correlates with the intensity of oxidative stress (Suzuki *et al.* 1995). Wolf *et al.* (1997) suggested that CsA induces oxidative stress in cultured hepatocytes. Based on these data, the aim of the present study was to investigate the participation of oxidative stress on some biochemical parameters in isolated hepatocytes and to study the effects of CsA on this pathological condition. Since the level of cytosolic free calcium concentration (Ca^{2+}_i) is a sensitive indicator of the physiological state of cells, we decided to study the Ca^{2+}_i levels in isolated hepatocytes of normotensive rats under the influence of oxidative stress. In our experimental setting we used *tert*-butyl hydroperoxide (t-BHP) as a widely utilized prooxidant, and CsA was used as a potentially toxic or protective agent.

The experimental animals were two-month-old male Wistar rats fed a standard laboratory diet and water *ad libitum* and maintained under standard light and temperature condition. Rats were anesthetized with pentobarbital (60 mg/kg) and perfusion of the liver was performed in two phases, and isolated hepatocytes were prepared by the standard collagenase perfusion method according to Moldeus *et al.* (1978). After separation, the cells were washed twice with the Krebs-Henseleit medium containing 1 mmol.l^{-1} Ca^{2+}; ultimately, the cell suspension was diluted to a final concentration of 0.5 x 10^6 cells/ml. The cell suspension prepared from the liver of each animal was divided into 4 aliquots which were used to test control values and after exposure to CsA, t-BHP and their combinations. The groups thus prepared were incubated at 37 °C for time periods indicated in Figure 1. We used 1 mmol.l^{-1} t-BHP in the presence or in the absence of CsA. This immunosuppressive drug was used at a concentration of 0.5 μmol.l^{-1} and was applied 30 min before the addition of t-BHP. Measurements of the level of Ca^{2+}_i were obtained from cells loaded with the fluorescent probe quin-2 by incubation of cells with 0.1 mmol.l^{-1} quin-2/AM for 15 min at 37 °C (Blackmore and Exton 1985). The viability of hepatocytes under these conditions was assessed by the Trypan blue exclusion procedure from samples used for Ca^{2+}_i estimation. Initial cell viability in each experiment was more than 90 % and the decrease at 60-min incubation did not drop below the value of 60 % in the t-BHP group (see below). The values are given as the means ± S.E.M. Statistical analysis was performed using Student's t-test with various poststatistical analyses. A probability level of 0.05 was considered as significant.

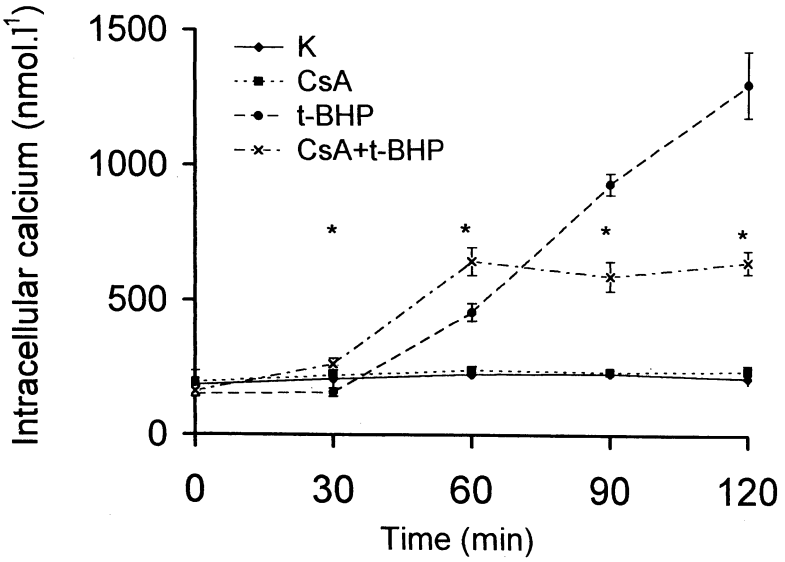

Fig. 1. *Time course of intracellular Ca^{2+} concentrations after incubation of hepatocytes with 1 mmol.l^{-1} tert-butyl hydroperoxide (t-BHP) and the effect of 0.5 μmol.l^{-1} CsA. Isolated hepatocytes were incubated in the Krebs-Henseleit medium and intracellular level of Ca^{2+} was measured as described above in the text. CsA was added 30 min before the addition of t-BHP, which was added at time 0 min. Values are means ± S.E.M. of 5-9 hepatocyte preparations from separate animals (smaller n was used in controls and CsA alone, where no changes were observed). The asterisks indicate significant difference (P<0.05) CsA + t-BHP group versus t-BHP group. ♦ t-BHP absent and CsA absent (= control) , ■ CsA present, ● t-BHP present, X both t-BHP and CsA present.*

Our results on the effect of prooxidant t-BHP and/or CsA on cytosolic Ca^{2+} in isolated rat hepatocytes are shown in Figure 1. After a lag period of 30 min, t-BHP induced a rapid increase of Ca^{2+}_i which lasted throughout the whole observation period up to 120 min. After preincubation of hepatocytes with CsA for 30 min, we observed a diverse response of hepatocytes to oxidative stress due to t-BHP. CsA enhanced the already

increased Ca^{2+}_i levels due to t-BHP after 30 and 60 min. In the following period (i.e. 60-120 min), the presence of CsA reduced the effect of t-BHP on calcium accumulation which became manifested as the leveling out of Ca^{2+}_i which remained below the concentration of 600 nmol.l^{-1}. The observed decrease of Ca^{2+}_i was significant compared to values in samples treated with t-BHP alone. The values below 600 nmol.l^{-1} are admitted to be the limiting concentrations which induce toxicity in hepatocytes (Orrenius *et al.* 1990). The exposure of isolated hepatocytes to CsA alone had no influence on the intracellular calcium concentrations during 30 min of preincubation and subsequent 120-min lasting incubation. To compare these data, we assessed the viability of hepatocytes (Trypan blue exclusion test) under the same conditions. Sixty minutes after the addition of t-BHP, the following values were obtained: control 85 %, CsA 76 %, t-BHP 60 %, CsA + t-BHP 65 %. The observed changes in free intracellular calcium levels in four experimental groups at various time intervals were substantially different from the changes in cell viability. This indicates the effects of tested drugs on Ca^{2+}_i levels. In conclusion, the main results seems to be due to the dual action of CsA on elevated Ca^{2+}_i in hepatocytes during oxidative injury induced by t-BHP.

In this study, we decided to use cyclosporin A (CsA), the most important immunosuppressive drug, as a model drug for the evaluation of its effect on intracellular levels of calcium (Ca^{2+}_i). Since the study of this parameter is very difficult in the intact cardiovascular system, we decided first to test the effect of this drug on isolated hepatocytes, where we already have some useful information about its actions on other biochemical parameters (Farghali and Mašek 1998). Over the last decade, many experimental problems were studied on animal models using more complex and powerful analytical methods. Recently, we have studied the possibility of various compounds to ameliorate oxidative injury induced by t-BMP in perfused hepatocytes *in vitro* (Kmoníčková *et al.* 1999, Farghali *et al.* 2000). In the present study we decided to observe the effects of oxidative stress on intracellular free calcium levels. We have chosen a model of isolated hepatocytes and started our testing in normotensive Wistar rats. Furthermore, we estimated the basic values of Ca^{2+}_i during 120-min incubation. We also observed the effect of oxidative stress on calcium levels and the modifying influence of CsA on this action in the indicated time intervals. Although CsA is known to induce hypertension as a side effect, it plays a protective role in cells and exerts an immunosuppressive effect (Farghali and Mašek 1998).

In the present pilot study, we have found a dual effect of CsA on the calcium free level in hepatocytes. In the early period of incubation, CsA seems to enhance the calcium accumulation in hepatocytes that is caused by a prooxidant drug t-BHP. A potentiating effect of CsA on the increase of Ca^{2+}_i induced by other types of drugs (e.g. hormones) was observed in aortic smooth muscle (Lo Russo *et al.* 1997) in the range of incubation times that we have also used. Up to now, however, the mechanism of action of CsA on intracellular calcium accumulation has not yet been elucidated.

In our experimental model, we also observed a second phase of CsA action on cytoplasmic Ca^{2+}_i in isolated hepatocytes. At longer time intervals, t-BHP increases intracellular Ca^{2+}_i levels to much higher values which are already known to be cytotoxic (Orrenius *et al.* 1990). In this second (protective) phase, CsA significantly reduces Ca^{2+}_i levels induced by this prooxidative agent. The explanation for this effect is not known, however, it seems that CsA may be used as a useful experimental tool for various studies where oxidative stress deteriorates cell functions. It is well known that CsA can inhibit mitochondrial permeability transition under conditions of oxidative stress (Broekemeier *et al.* 1992). During oxidative stress, the inner mitochondrial membrane changes its permeability and mitochondrial Ca^{2+} is released. This is followed by an opening of Ca^{2+}-dependent pores, which results in a collapse of the electrochemical potential for H^+, leakage of small molecules and proteins and a production of reactive oxygen species. CsA effectively inhibits the opening of transition permeability pores, and in this way it can protect the cells subjected to oxidative stress (Kristian and Siesjo 1998).

We consider our experiments as pilot studies performed on one tissue model (isolated rat hepatocytes) which require further elaboration and extension of these studies to other systems, especially to the cardiovascular system.

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Reprint requests

E. Kmoníčková, Ph.D., Institute of Pharmacology, First Faculty of Medicine,, Charles University, Albertov 4, 128 00 Prague 4, Czech Republic. e-mail: ekmon@lfl.cuni.cz