

Studies on the nephrotoxicity of *Cortinarius orellanus* (Fr.) Fr.: The Effect of Dipyriddylen on Renal Epithelial Cell Cultures

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Summary. – The effect of 2,2'- and 4,4'-dipyridyl on the morphology and the activities of apical membrane enzymes of renal epithelial cell cultures LLC-PK₁ and MDCK has been investigated. Morphological effects on confluent monolayers of LLC-PK₁ and MDCK cells could be observed, when 2,2'- and 4,4'-dipyridyl was added to the culture medium at a final concentration of 10^{-3} mol/l after 24 h incubation. The activities of γ -glutamyltranspeptidase (γ -GT), alkaline phosphatase (AP) and leucine-aminopeptidase (LAP) in LLC-PK₁ cells are affected by both 2,2'- and 4,4'-dipyridyl, whereas γ -GT activity in MDCK cells is only influenced by 4,4'-dipyridyl. The data presented are in good accord with results obtained on rat kidneys after intoxication with *Cortinarius orellanus* (Fr.) Fr.

Introduction

Many cases of mushroom poisoning with *Cortinarius orellanus* (Fr.) Fr. have been described, which cause renal failure and, in the most severe forms, may end lethally. It is now well established, that the nephrotoxicity of *Cortinarius orellanus* (Fr.) Fr. is caused by its main toxic component orellanine, which has been identified as a bis-N-oxide of 3,3', 4,4'-tetrahydroxy-2,2'-dipyridyl (ANTKOWIAK & GESSNER, 1979).

In rats, a single dose of 2 g mushroom per kg body weight caused renal failure after 48 h (PRAT, 1982). Severe damage of the proximal tubular epithelium of rat kidney, increased vacuolisation of tubular cells and a loss of microvilli could be observed, accompanied by a decrease of the activities of the luminal membrane enzymes γ -glutamyltranspeptidase and alkaline phosphatase and of other renal enzymes.

Recently, the toxicity of 2,2'- and 4,4'-dipyridyl on rats was investigated (GROCE & KIMBROUGH, 1982), where it was observed morphologically, that both drugs cause renal damage.

Since 2,2'- and 4,4'-dipyridyl are molecules very similar to orellanine, it must be suggested, that these compounds may act on renal cells in a similar way.

Renal epithelial cell cultures offer new advantages to study kidney specific cell functions, e. g. transepithelial transport and its control by hormones or drugs (HANDLER & al., 1980; HORSTER, 1980). In addition, renal cell cultures are a valuable tool for studying the specific effects of toxins, from which is known, that the kidney is the main target organ.

In recent years, two established renal cell lines have been most widely used, the MDCK and the LLC-PK₁ cell line (HANDLER & al., 1980). MDCK cells are distal tubular cells (RINDLER & al., 1979), where LLC-PK₁ cells are suggested to be of proximal tubular origin (RABITO & AUSIELLO, 1980; MISFELDT & SANDERS, 1981).

Both renal cell lines retain the morphological properties of tubular epithelial cells, they build up monolayers of differentiated and polarized cells and form tight junctions (CEREJIDO & al., 1978). Furthermore, the cells exhibit on their apical surface high activities of the renal brush border membrane enzymes γ -glutamyltranspeptidase (γ -GT), alkaline phosphatase (AP) and leucine-aminopeptidase (LAP). These enzymes play an essential role in the utilization and reabsorption of peptides, polysaccharides and other molecules by splitting them into reabsorbable compounds.

In the present study, the effect of 2,2'- and 4,4'-dipyridyl on the morphology and the activities of γ -GT, AP and LAP of confluent monolayers of MDCK and LLC-PK₁ cells has been investigated.

Materials and Methods

Cell Culture: MDCK and LLC-PK₁ cells were grown as monolayers in culture flasks (NUNC) in a 50 : 50 mixture of Dulbecco's modified Eagle's Medium (DMEM) and Ham's F-12 Medium, supplemented with 10% fetal calf serum and penicillin (100 U/ml) and streptomycin (100 μ g/ml). Culture flasks were incubated in a humidified 5% CO₂/95% air mixture at 37° C.

2,2'- and 4,4'-dipyridyl (SIGMA) was each added to confluent monolayer cultures in a final concentration of 10⁻⁵, 10⁻⁴ and 10⁻³ mol/l. Cultures were further incubated for 24 h.

Enzyme Assays: Activities of the luminal membrane enzymes γ -GT, AP and LAP were assayed in confluent monolayers in the culture flasks "in situ". Buffer/substrate solutions were layered over the cells and incubated at 37° C.

γ -GT activity was assayed according to GLOSSMANN & NEVILLE (1972) at pH 8.25 using γ -glutamyl-p-nitroanilide as substrate, AP activity was determined according to WALTER & SCHÜTT (1974) with 4-nitrophenylphosphate as substrate at pH 9.8 and LAP according to APPEL (1974) with L-leucine-p-nitroanilide as substrate at pH 7.2. Protein was determined by the method of LOWRY & al (1951) with bovine serum albumine as a standard.

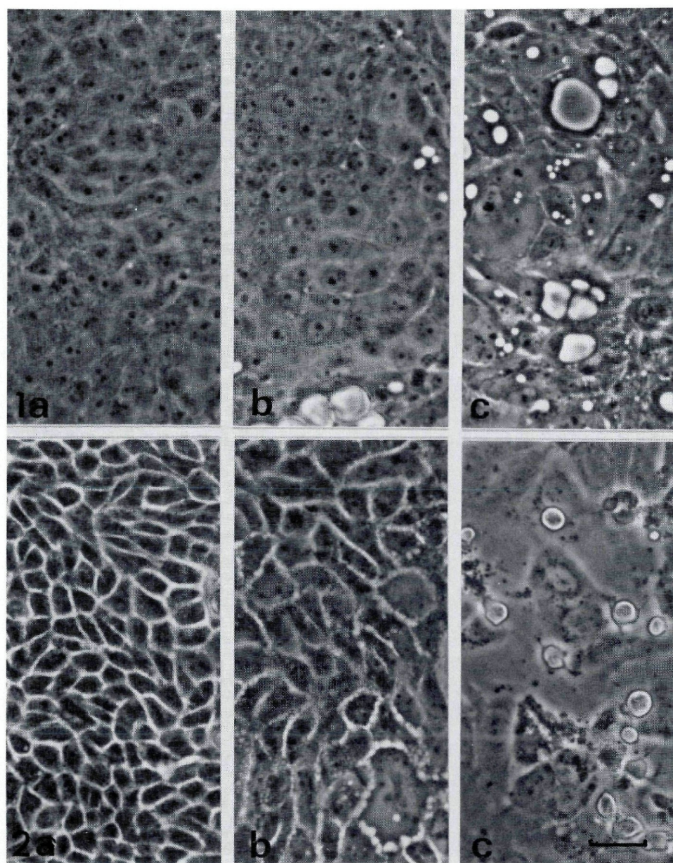


Fig. 1. Effect of 2,2'- and 4,4'-dipyridyl on the morphology of LLC-PK₁ monolayer: 1 a) control culture, 1 b) culture after 24 h incubation in the presence of 10^{-3} mol/l 2,2'-dipyridyl and 1 c) incubated with 4,4'-dipyridyl (10^{-3} mol/l, 24 h).

Fig. 2. Appropriate figures of MDCK cultures: 2 a) control, 2 b) 2,2'-dipyridyl and 2 c) 4,4'-dipyridyl 10^{-3} mol/l, 24 h. Phase contrast micrographs, magnification 175 \times , bar = 50 μ m.

Absolute data of control enzyme activities of luminal membrane enzymes of LLC-PK₁ and MDCK cultures and total protein content per culture flasks of control cultures. Values are expressed as mean \pm SD of three separate experiments.

LLC-PK ₁ γ -glutamyltranspeptidase	160.1 \pm 10.5 mU/mg
alkaline phosphatase	15.3 \pm 1.1 mU/mg
leucine-aminopeptidase	6.0 \pm 0.2 mU/mg
MDCK γ -glutamyltranspeptidase	36.5 \pm 7.4 mU/mg
total protein per culture flask	1.40 \pm 0.13 mg

Results

1. Morphology

Both renal epithelial cell lines, LLC-PK₁ and MDCK, are affected by 2,2'- and 4,4'-dipyridyl. As shown in fig. 1 b, 2,2'-dipyridyl (10^{-3} mol/l) has only small effects on the morphology of LLC-PK₁ monolayer when compared to 4,4'-dipyridyl (fig. 1 c). Here, severe damage of the confluent monolayer can be observed 24 h after incubation with the drug. Numerous cells are floating in the medium and, after washing, the remaining monolayer can be seen in fig. 1 c. The confluent monolayer is disrupted and the cell-cell contact as well as the contact of cells with the substratum is strongly disturbed. In fig. 2 the experiments with MDCK cells are shown. 2,2'-dipyridyl in a final concentration of 10^{-3} mol/l causes disturbances in the tight junctions, resulting in an enlargement of the intercellular space (fig. 2 b). The most severe damage of MDCK monolayer can be obtained 24 h after incubation with 4,4'-dipyridyl (10^{-3} mol/l) (fig. 2 c). Again, cells are floating in the culture medium and only few, strongly damaged cells remain on the bottom of the culture flask.

These effects are well documented by measuring the protein content of the remaining monolayers in the culture flasks (fig. 3). As can be seen, only 4,4'-dipyridyl has significant effects on the protein content of both renal cell lines. These results are in good accord when compared to the micrographs in fig. 1 c and 2 c.

2,2'- and 4,4'-dipyridyl showed no effects on the morphology and protein content in culture flask when applied in lower concentrations (10^{-5} and 10^{-4} mol/l) to confluent LLC-PK₁ and MDCK monolayers.

2. Enzyme Activities

The activities of γ -glutamyltranspeptidase (γ -GT), alkaline phosphatase (AP) and leucine-aminopeptidase (LAP) in LLC-PK₁

cells are affected by both 2,2'- and 4,4'-dipyridyl (fig. 4). A straight concentration dependency can be observed, where 10^{-3} mol/l 2,2'- and 4,4'-dipyridyl cause decreases of the enzyme activities to nearly 60% to 70% of control values. In MDCK cells, only γ -GT activity was determined, because only traces of AP and LAP can be measured, which depends on the distal origin of this particular cell line. The results are presented in fig. 5. Here, only 4,4'-dipyridyl in a concentration of 10^{-3} mol/l showed a remarkable effect on γ -GT activity, causing a decrease to nearly 50% of the control value.

Discussion

In the present study, the effect of 2,2'- and 4,4'-dipyridyl on the morphology and the activities of apical membrane enzymes of renal epithelial cell cultures LLC-PK₁ and MDCK has been investigated.

The morphologic alterations found are a severe damage of confluent monolayers of LLC-PK₁ and MDCK cells, caused by both, 2,2'- and 4,4'-dipyridyl and are very similar to the damage of renal tubular epithelium found after intoxication of rats with *Cortinaris orellanus* (Fr.) (PRAST, 1982) and with 2,2'- and 4,4'-dipyridyl (GROCE & KIMBROUGH, 1982). The renal failure observed after intoxication with orellanine may in this view be explainable by this tubular cell injury.

Physiological observations revealed increased fractional sodium and potassium excretion in rats 24 h after orellanine intoxication (PRAST, 1981), which can be seen as disturbances of renal tubular integrity and an impairment of active transport systems in the kidney.

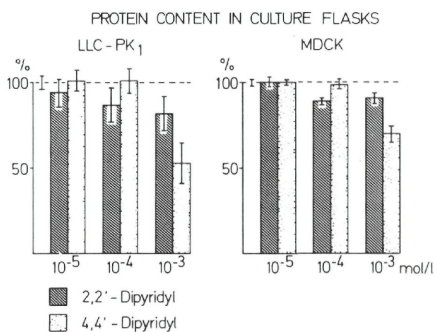
Single morphological alterations, which can be seen 24 h after orellanine intoxication, resulting in a vacuolisation of tubular cells and a decrease of luminal membranes caused by a loss of microvilli are more pronounced and distributed over the renal cortex 48 h and 72 h after intoxication. Additionally, severe decreases of luminal

Fig. 3. Effect of 2,2'- and 4,4'-dipyridyl on protein content of LLC-PK₁ and MDCK cultures. Values are expressed as percentage of untreated cultures (for absolute values see).

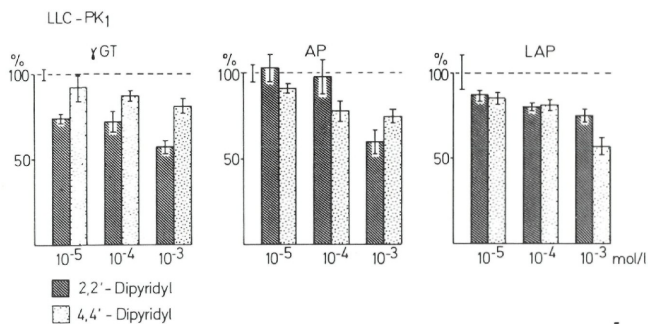
Fig. 4. Effect of 2,2'- and 4,4'-dipyridyl on apical membrane enzyme activities of LLC-PK₁ cells. Enzyme activities are expressed as percentage of control values obtained from untreated cultures (for absolute data see table 1). Each value given represents mean \pm SD of three separate experiments.

Fig. 5: Effect of 2,2'- and 4,4'-dipyridyl on γ -GT activity of MDCK cells. Results are expressed as in fig. 4.

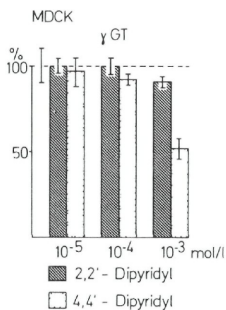
Fig. 6. Effect of orellanine on luminal membrane enzyme activities in rat kidney. Values are expressed as percentage of control enzyme activities obtained from normal kidneys, 36 h and 72 h after intoxication. (from PRAST, 1982)



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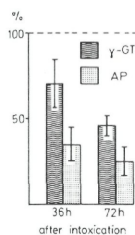


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membrane enzyme activities in rat kidney cortex have been found (fig. 6) (PRAST, 1982). These data obtained on rat kidneys after orellanine intoxication are in good accord with the observations obtained with renal epithelial cell cultures in the present study. Both, 2,2'- and 4,4'-dipyridyl cause decreases of the apical membrane enzymes γ -GT, AP and LAP in LLC-PK₁ cells in a dose dependent manner (fig. 4), and only 4,4'-dipyridyl showed significant effects on γ -GT activity in MDCK cells (fig. 5).

These results suggest, that similarities in the mechanisms of toxicity of orellanine and 2,2'- and 4,4'-dipyridyl on renal cells must exist.

The different effects of 2,2'- and 4,4'-dipyridyl on morphology and apical membrane enzymes of "proximal" LLC-PK₁ and "distal" MDCK cells, may be seen in some yet unknown differences in the cellular uptake and toxicity, respectively, of these two drugs on the different renal cell types. Further studies will be necessary, to exactly point out these differences mentioned, using the model system of renal cell cultures, where no superior regulatory mechanisms like in an animal model exist. In addition, renal epithelial cell cultures are a valuable tool for studying the toxic mechanisms of nephrotoxins at the cellular level.

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