EFFECTS OF POLYMYXIN ANTIBIOTICS ON IODOHIPPURATE ACCUMULATION IN RABBIT RENAL CORTICAL SLICES

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The *in vitro* effects of polymyxin antibiotics on *o*-^{125}I-hippurate (OIH) accumulation in rabbit renal cortical slices were studied using incubation media with pH ranging from 6.9 to 7.9 and containing polymyxin B sulfate, colistin sulfate, sodium colistimethate and antibacterially inactive N-succinyl colistin in concentrations ranging from 1 to 2,000 μg base/ml. Polymyxin B, colistin and colistimethate depressed OIH accumulation significantly in concentrations $\geq$ 300 μg/ml. The effects on accumulation were clearly pH-dependent and most pronounced at alkaline pH. N-Succinyl colistin had only a marginal influence on accumulation, even in high concentrations. Colistimethate produced a significantly smaller decrease in accumulation at all pH values than both polymyxin B and colistin. The results suggest that the presence of free amino groups is necessary to obtain a decrease in accumulation and correlate with the known *in vivo* nephrotoxicity of these antibiotics.

The polymyxins are cyclic polypeptide antibiotics, effective against a wide range of Gram-negative bacteria. The most used members of this group in human therapy are polymyxin B, colistin, and colistimethate which is a methane sulfonate derivative of colistin. All polymyxins possess nephrotoxic properties, as demonstrated in animal experiments[1] and experiences from the human clinic.[2,3] The nephrotoxicity is characterized by a spectrum of tubular damages, from slight degenerative changes to frank tubular necrosis.[1,2,3] In the present study the effects of various polymyxins on the *o*-^{125}I-hippurate (OIH) accumulation in rabbit renal cortical slices have been assessed in an attempt to elucidate further the nephrotoxicity of these antibiotics.

Materials and Methods

Measurement of *o*-^{125}I-hippurate accumulation:

The technique employed is a modification of the method reported by CROSS and TAGGART,[4] and has been described in details elsewhere.[5] Rabbit renal cortical slices were incubated in Erlenmeyer vessels for 60 minutes at 25°C with 100% oxygen in the gas space, while being shaken at 100 cycles/minute. Each vessel contained 90~110 mg slices, 10 ml CROSS-TAGGART medium[4] and 20 μCi *o*-^{125}I-hippurate/liter corresponding to approximately 3 μmol/liter. The slices and aliquots of the incubation media were counted in a gamma scintillation counter and the OIH accumulation expressed as the slice to medium ratio *i.e.* the ratio of the counts in 1 g tissue to those in 1 ml incubation medium.

Polymyxin studies:

All the polymyxin substances employed were kindly provided by Dumex Ltd., Copenhagen, Denmark. N-succinyl colistin sodium was obtained from colistin base, which was dissolved in pyridine, and incubated at ambient temperature for 2 weeks after the addition of succinic anhydride, whereafter N-succinyl colistin was precipitated with ether. By this procedure approximately 98% of the free amino groups were blockaded and the antibacterial activity almost abolished. By disc assays with *Bordetella bronchiseptica*, N-succinyl colistin had an antibacterial activity equivalent to 0.02 μg colistin

base/mg substance, as compared to colistin sulfate having an activity of 633 μg base/mg substance. The calculated (antibacterially inactive) base content in N-succinyl colistin was 621 μg/mg substance.

Prior to incubation the following polymyxins were added to the incubation media in concentrations ranging from 1 to 2,000 μg base/ml: Polymyxin B sulfate (800 μg base/mg), colistin sulfate (633 μg base/mg), sodium colistimethate (456 μg base/mg) and N-succinyl colistin sodium (621 μg calculated base/mg). Hereafter pH was adjusted to 6.9, 7.4 and 7.9 by means of 2 N hydrochloric acid or 2 N sodium hydroxide and using a pH-meter. Incubation was then performed as described above. All experiments were done 6~8 times and each setup included control vessels without polymyxins. The OIH accumulation in the polymyxin-containing vessels was expressed in per cent of the accumulation in the controls.

STUDENT's *t*-test for paired and unpaired values was employed in statistical evaluation of the results and the 5% value chosen as the level of significance.

Results

The results are summarized in Table 1 and Fig. 1. The antibacterially active polymyxins inhibited OIH accumulation in high concentrations. The effects were clearly pH-dependent and most pronounced at alkaline pH.

Polymyxin B: The OIH accumulation was significantly depressed at all pH values by concentrations $\geq$300 μg/ml. At concentrations $\geq$300 μg/ml, elevation of pH from 6.9 to 7.4 and 7.9 significantly accentuated the decrease in accumulation produced by identical concentrations of polymyxin B. At pH 7.9 and 2,000 μg/ml accumulation was only 2.0% *i.e.* almost completely inhibited.

Colistin: Concentrations $\geq$300 μg/ml depressed accumulation distinctly at all pH values. At concentrations $\geq$500 μg/ml the effect was significantly more pronounced at pH 7.9 compared to pH 7.4 and 6.9. At pH 7.9, 2,000 μg/ml depressed accumulation to 4.6%. There was no significant difference between the accumulation profiles (dose response curves) of polymyxin B and colistin at pH 6.9 and 7.4. However, at pH 7.9 colistin depressed accumulation significantly less than polymyxin B at concentrations $\geq$300 μg/ml.

Colistimethate: Accumulation was moderately but significantly depressed by concentrations $\geq$300 μg/ml. The influence of pH variations was less pronounced than for polymyxin B and colistin and apparent only at concentrations $\geq$1,000 μg/ml; the differences, however, were insignificant. At pH 7.9, 2,000 μg/ml depressed accumulation to 46.4%. Colistimethate produced a significantly smaller decrease in accumulation at all pH values and concentrations $\geq$300 μg/ml than both polymyxin B and colistin.

N-Succinyl colistin: At pH 6.9 OIH ac-

Fig. 1. Effects of different concentrations of polymyxin B sulfate, colistin sulfate, colistimethate sodium and N-succinyl colistin sodium on ^{125}I-hippurate accumulation in rabbit renal cortical slices at various pH-values in the incubation media.

Results are expressed in per cent of the accumulation in antibiotic free controls.

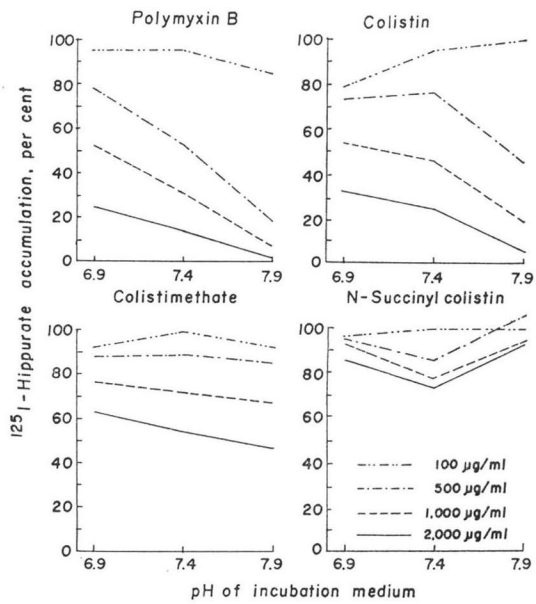

Table 1. Effects of polymyxin B sulfate, colistin sulfate, colistimethate sodium and N-succinyl colistin sodium on ^{125}I-hippurate accumulation in rabbit renal cortical slices at different pH-values in the incubation media. Results are expressed in per cent of the accumulation in antibiotic free controls (mean ±S. E.M.).

Polymyxin base μg/ml	^{125}I-Hippurate accumulation, per cent											
	Polymyxin B			Colistin			Colistimethate			N-Succinyl colistin		
	pH 6.9	pH 7.4	pH 7.9	pH 6.9	pH 7.4	pH 7.9	pH 6.9	pH 7.4	pH 7.9	pH 6.9	pH 7.4	pH 7.9
1	98.8 ±5.8	93.1 ±5.2	98.4 ±4.9	89.7 ±5.9	92.2 ±4.3	101.5 ±6.5	103.1 ±4.5	109.4 ±3.4	100.1 ±4.1	—	—	—
10	95.2 ±3.3	93.9 ±4.2	92.1 ±4.0	93.4 ±5.3	97.4 ±6.3	103.5 ±3.3	96.0 ±2.6	101.4 ±5.2	98.5 ±4.3	90.8 ±6.6	108.1 ±5.1	100.0 ±4.1
100	94.9 ±5.1	94.9 ±6.6	84.5 ±6.4*	79.1 ±5.0*	95.4 ±5.0	99.9 ±3.1	91.9 ±2.2	99.4 ±2.3	92.2 ±4.7	96.6 ±3.7	100.6 ±4.9	100.7 ±4.2
300	86.9 ±3.0*	82.3 ±5.9*	32.6 ±7.0*	77.5 ±5.0*	80.9 ±4.7*	76.7 ±3.3*	91.9 ±2.2	89.3 ±3.7*	84.6 ±5.6*	93.0 ±3.3	99.5 ±3.9*	100.0 ±2.0
500	77.8 ±0.8*	52.5 ±3.3*	18.2 ±4.9*	72.8 ±2.3*	75.9 ±4.4*	44.9 ±3.2*	88.3 ±4.3*	89.0 ±0.9*	85.3 ±5.5*	96.3 ±3.7	86.1 ±3.5*	106.7 ±8.6
1,000	52.2 ±1.8*	30.7 ±1.8*	7.2 ±2.1*	53.7 ±1.1*	45.4 ±5.3*	17.7 ±2.0*	76.4 ±4.3*	72.0 ±2.3*	66.8 ±5.5*	93.0 ±2.1	78.6 ±2.6*	94.8 ±5.0
2,000	24.2 ±1.0*	13.8 ±2.8*	2.0 ±0.4*	31.8 ±2.3*	23.9 ±3.5*	4.6 ±0.5*	63.4 ±8.3*	54.1 ±2.9*	46.4 ±3.5*	86.2 ±3.2*	74.3 ±2.9*	94.3 ±6.9

* $P<0.05$

cumulation was slightly depressed by 2,000 μg/ml. At pH 7.4 accumulation decreased moderately at concentrations ≥500 μg/ml. However, at pH 7.9 even high concentrations had no influence on accumulation, being 94.3% at 2,000 μg/ml. N-Succinyl colistin had a significantly smaller effect on accumulation than both polymyxin B, colistin and (to a lesser extent) colistimethate.

Discussion

The accumulation of *p*-aminohippurate and OIH is a metabolic characteristic of the proximal tubular cell[4], involving an active transport of *p*-aminohippurate across the peritubular membrane, followed by diffusion through the luminal membrane into the tubular fluid.[6] The *p*-aminohippurate transport system is energy requiring and closely connected with oxygen utilization[4,7] and is common to numerous organic acids, which compete with each other about the transport capacity.[8]

The mechanisms responsible for the polymyxin-induced effects on OIH accumulation are unclarified, but seem to imply the presence of free amino groups. Inactivation of the amino groups by N-succinylation almost eliminates the inhibitory influence on accumulation.

The antibacterial properties of the polymyxins are effected through interaction with phospholipids, especially phosphatidylethanolamine groups in bacterial membranes and subsequent damage to membrane function.[9] These antibiotics also interact with phospholipids in mammalian cell membranes.[9,10,11] Impairment of the tubular cell membrane might affect the function of the OIH receptor sites,[12] causing decreased influx of OIH, and might also increase membrane permeability and thereby the efflux of OIH.

The results demonstrate that the influence of polymyxins on OIH accumulation is pH-dependent and most pronounced at alkaline pH. An increase in pH reduces the number of protonated amino groups and thus facilitates diffusion into and through the cell membrane according to the theory of nonionic diffusion.[13] Furthermore the binding of polymyxins to phospholipids shows a pH optimum

around 7.5～8.0, suggesting that the antibacterial and toxic effects depend on a certain balance between protonated and unprotonated amino groups in the molecule.[9] In accordance with these considerations the influence of N-succinyl colistin, where the amino groups have been inactivated and which has almost no antibacterial properties, on OIH accumulation is marginal and on the whole pH-independent.

Polymyxin B and colistin, but not colistimethate, are bound to and inhibited by rabbit kidney homogenates, and subcellular fractions containing mitochondria and lysosomes are the most potent inhibitors[11]; also polymyxins bind strongly to nucleic acids.[9] It is usually assumed that polymyxins penetrate poorly into cells[9]. However, polymyxin B accumulate in high concentrations in the kidney[14] where it is retained for considerable time and most likely part of this is situated intracellularly, where it through binding to important structures might impair metabolic processes involved with OIH accumulation.

Mutual substrate competition between polymyxins and OIH seems unlikely in view of previous studies[5] and the observation that urinary excretion of these drugs is unaffected by probenecid.[15,17] The proximal tubular cells also possess a transport system for organic bases,[8] but the affinity of polymyxins to this system is unknown.

When equimolar base concentrations were compared, polymyxin B and colistin had almost identical and much greater depressive effects on OIH accumulation than colistimethate. The *in vitro* results thus correlate with the recognized *in vivo* nephrotoxicity of these drugs.[17,18] The degree of tissue binding, toxicity and antibacterial activity are closely related to the number of free amino groups.[19,20] Polymyxin B and colistin each contains 5 free amino groups per molecule. In colistimethate 3 to 4 of these have been combined with methane sulfonate (Dumex Ltd., personal communication), causing a reduction in tissue binding[20] as well as in nephrotoxicity[17,18] and antibacterial activity.[19]

The high tissue binding[13,20] and consequent inactivation of the antibacterial effect, and the lack of suitable assays have impeded exact pharmacokinetic studies of the polymyxins. Colistimethate is probably eliminated mainly by glomerular filtration[15], although the extent of transtubular transport is unclarified. Ionic diffusion trapping has apparently no influence on the urinary excretion, which is unaffected by variations in urinary pH.[15] However, the protective effect of D-glucarates suggests that the degree of protonation plays some kind of a role in colistimethate nephrotoxicity.[21]

References

1) Moyer, J. H.; L. C. Mills & E. M. Yow: Toxicity of polymyxin B. I. Animal studies with particular reference to renal function. Arch. Intern. Med. 92: 238～247, 1953
2) Koch-Weser, J.; V. W. Sidel, E. B. Federman, P. Kanarek, D. C. Finer & A. E. Eaton: Adverse effects of sodium colistimethate. Manifestations and specific reaction rates during 317 courses of therapy. Ann. Intern. Med. 72: 857～868, 1970
3) Ryan, K. J.; L. I. Schainuck, R. O. Hickman & G. E. Striker: Colistimethate toxicity. Report of a fatal case in a previously healthy child. J. Amer. Med. Ass. 207: 2099～2101, 1969
4) Cross, R. J. & J. V. Taggart: Renal tubular transport: Accumulation of *p*-aminohippurate by rabbit kidney slices. Am. J. Physiol. 161: 181～190, 1950
5) Dahlager, J. & N. Milman: Aminoglycoside nephrotoxicity. I. Effects of aminoglycoside antibiotics on iodohippurate accumulation in rabbit renal cortical slices. J. Antibiotics 30: 597～603, 1977
6) Tune, B. M.; M. B. Burg & C. S. Patlak: Characteristics of *p*-aminohippurate transport in proximal renal tubules. Am. J. Physiol. 217: 1057～1063, 1969
7) Maxild, J. & J. Møller: Metabolic studies on renal transport of *p*-aminohippurate *in vitro*. Biochem. Biophys. Acta 184: 613～624, 1969
8) Lant, R. F.: Renal excretion and nephrotoxicity of drugs. pp. 591～614. *In* D. Black (*ed*), Renal disease. Blackwell Scientific Publications, London, 1972
9) Feingold, D. S.; C. C. Hsuchen & I. J. Sud: Basis for the selectivity of action of the polymyxin antibiotics on cell membranes. Ann. N.Y. Acad. Sci. 235: 480～491, 1974
10) Hasselbarth, A.: Effect of polymyxin B on the replication of poliovirus in cell cultures. Med. Microbiol. Immunol. 157: 239～243, 1972
11) Kunin, C. M.: Binding of antibiotics to tissue homogenates. J. Infect. Dis. 121: 55～64, 1970
12) Berner, W. & R. Kinne: Transport of *p*-aminohippuric acid by plasma membrane vesicles isolated from

rat kidney cortex. Pflügers Arch. 361: 269~277, 1976
13) WEINER, J. M. & G. H. MUDGE: Renal tubular mechanisms for excretion of organic acids and bases. Am. J. Med. 36: 743~762, 1964
14) JACOBSON, M.; A. KOCH, R. KUNTZMAN & J. BURCHALL: The distribution and binding of tritiated polymyxin B in the mouse. J. Pharmacol. Exp. Ther. 183: 433~439, 1972
15) AL-KHAYYAT, A. A. & A. L. ARONSON: Pharmacologic and toxicologic studies with the polymyxins. II. Comparative pharmacologic studies of the sulfate and methanesulfonate salts of polymyxin B and colistin in dogs. Chemotherapy 19: 82~97, 1973
16) CALDWELL, A. D. S.; A. J. MARTIN & D. J. TRIGGER: Comparative study of the effect of three antibiotics on renal function. Brit. J. Pharmacol. 37: 283~293, 1969
17) PEDERSEN, M. F.; J. F. PEDERSEN & P. O. MADSEN: A clinical and experimental comparative study of sodium colistimethate and polymyxin B sulfate. Invest. Urol. 9: 234~237, 1971
18) VINNICOMBE, J. & T. A. STAMEY: The relative nephrotoxicities of polymyxin B sulfate, sodium sulfomethyl-polymyxin B, sodium sulfomethyl-colistin (colymycin), and neomycin sulfate. Invest. Urol. 6: 505~519, 1969
19) NORD, N. M. & P. D. HOEPRICH: Polymyxin B and colistin. A critical comparison. New Eng. J. Med. 270: 1030~1035, 1964
20) KUNIN, C. M. & A. BUGG: Recovery of tissue bound polymyxin B and colistimethate. Proc. Soc. Exp. Biol. Med. 137: 786~790, 1971
21) FURUNO, K.; A. KUNIO & S. SUZUKI: Effect of D-glucarates on basic antibiotic-induced renal damage in rats. J. Antibiotics 29: 187~194, 1976