

Hyperactivity of cathepsin B and other lysosomal enzymes in fibroblasts exposed to azithromycin, a dicationic macrolide antibiotic with exceptional tissue accumulation

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Abstract Azithromycin accumulates in lysosomes where it causes phospholipidosis. In homogenates prepared by sonication of fibroblasts incubated for 3 days with azithromycin (66 µM), the activities of sulfatase A, phospholipase A₁, *N*-acetyl-β-hexosaminidase and cathepsin B increased from 180 to 330%, but not those of 3 non-lysosomal enzymes. The level of cathepsin B mRNA was unaffected. The hyperactivity induced by azithromycin is non-reversible upon drug withdrawal, prevented by coincubation with cycloheximide, affects the V_{\max} but not the K_m , and is not reproduced with gentamicin, another drug also causing lysosomal phospholipidosis. The data therefore suggest that azithromycin increases the level of lysosomal enzymes by a mechanism distinct from the stimulation of gene expression but requiring protein synthesis, and is not in direct relation to the lysosomal phospholipidosis.

Key words: Cathepsin B; Lysosomal phospholipidosis; Azithromycin; Gentamicin; Lysosomal enzyme hyperactivity

1. Introduction

Azithromycin is a dicationic derivative of erythromycin A [1,2] which avidly accumulates in tissues [3,4] and in cultured mammalian cells, reaching intracellular to extracellular concentrations ratios of 100 and more [5]. Cell fractionation studies show that the bulk of the drug accumulated by cells localizes in the lysosomes [6]. Azithromycin induces a thesaurismosis characterized morphologically by conspicuous enlargement of the lysosomes which display the prominent deposition of pseudomyelinic structures, and, biochemically, by a marked increase of the lysosomal content in undegraded phospholipids [7,8]. In vitro, azithromycin is a potent inhibitor of the activity of lysosomal phospholipase A₁ (measured towards phosphatidylcholine included in negatively charged liposomes), presumably because the drug binds to the phospholipid bilayers preventing the enzyme from exerting fully its activity [9]. Somewhat paradoxically, however, we observed and report here that the activity of phospholipase A₁ is actually increased when assayed in the presence of detergents in lysates of fibroblasts preincubated with azithromycin. To investigate the specificity and significance of this effect, we have examined the influence of azithromycin on the activity of other lysosomal and non-lysosomal enzymes, and have compared azithromycin to another lysosomotropic antibiotic, gentamicin, which also induces a lysosomal phospholipidosis [10].

2. Material and methods

2.1. Cell culture

Embryonic fibroblasts from Wistar rats were cultivated and collected essentially as described earlier [11]. Cell proteins and phospholipids were assayed using standard techniques [12–14].

2.2. Drugs

Azithromycin was a gift from Pfizer (Pfizer Central Research, Groton, CT) and gentamicin from Schering-Plough Corp. (Kenilworth, NJ). These drugs were assayed by a disc-plate microbiological assay using *Bacillus subtilis* as test organism [15], as described earlier [16].

2.3. Enzyme assays

Lysosomal enzymes were assayed in the presence of detergent with the following substrates (obtained from Sigma Chemical Co. (St Louis, MO) or from Amersham (Amersham International plc, Bucks, UK)): cathepsin B (EC 3.4.22.1), 2-*N*-benzoyl-DL-arginine-2-naphthylamide [17]; *N*-acetyl-β-hexosaminidase (EC 3.2.1.30), *p*-nitrophenyl 2-acetamido-β-D-glucopyranoside [18]; arylsulfatase A (EC 3.1.6.1), *p*-nitrocatecholsulfate [19]; and phospholipase A₁ (EC 3.1.1.32), 1-3-phosphatidylcholine, 1-palmitoyl-2-[1-¹⁴C]oleoyl [20]. The plasma membrane enzyme 5'-nucleotidase (EC 3.1.3.5) was assayed with adenosine 5'-monophosphoric acid disodium salt [21]. Two cytosolic enzymes were also assayed, namely lactate dehydrogenase (EC 1.1.1.8), with pyruvic acid and nicotinamide adenine dinucleotide disodium salt (reduced form) [22], and phosphoglucosyltransferase (EC 5.4.2.2), with glucose 1-phosphate [23].

2.4. Northern blot analysis

Poly(A)⁺ RNA, prepared using the Micro-Fast Track mRNA isolation system (Invitrogen, San Diego, CA), was electrophoresed on 1.3% agarose-formaldehyde gel, transferred onto nitrocellulose paper (Schleicher and Schuell, Millipore, Brussels, Belgium), hybridized with ³²P-labeled probes and autoradiographed. The cathepsin B probe was obtained by polymerase chain reaction (PCR) amplification of a fragment of 481 bp (nucleotides 360–841) of the gene encoding rat cathepsin B inserted in pcrB5 (a plasmid kindly offered by Dr. Donald Steiner, University of Chicago, Illinois) with oligonucleotides CG1S (5'-ACACCATGTCAATGGCTCCCG3', nucleotides 360–380) and CG2AS (5'-GACTATACAATCAAGTCCAGC3', nucleotides 820–841). Control hybridization was performed on the same membrane with a rat actin probe obtained by reverse transcription (RT)-PCR amplification of rat embryo fibroblasts poly(A)⁺ RNA. The primers used were 5'-AAGAGAGGTATCTGACCCCTG3' (exon 2) and 5'-CCATCTCTGCTCGAAGTCTA3' (exon 3). The two PCR reactions were carried out using the Dynazyme (Finnzymes Oy, Espoo, Finland) and the PCR products were ³²P-labeled by random priming using the Megaprime DNA labelling system (Amersham International plc, Bucks, UK) before hybridization.

3. Results

Table 1 shows that a 3 day incubation with azithromycin (66.6 µM; 50 mg/l) induced a marked increase in the total cell phospholipids, together with an increased activity of all 4

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lysosomal enzymes assayed. In contrast, no change of activity was seen for two cytosolic enzymes, and a slight decrease in activity was noted for the plasma membrane-linked enzyme 5'-nucleotidase. Table 1 also shows that gentamicin, at a comparable cellular concentration, caused a phospholipidosis as marked as that of azithromycin, but no biologically significant change in the activities of two lysosomal enzymes and a decrease for the two others, especially phospholipase A₁.

Because cathepsin B showed the greatest increase in activity after incubation of the cells with azithromycin, all further studies were focused on this enzyme. We first checked that azithromycin did not increase the activity of cathepsin B when added directly to the incubation mixture. We actually noted a slight decrease in activity ($\approx 10\%$) of the largest achievable concentration (0.67 mM), without evidence of apparent change in K_m . In parallel, we also determined the kinetic parameters of *N*-acetyl- β -hexosaminidase in lysates of azithromycin-treated cells and found no significant change in K_m (≈ 0.4 mM) but a definitively increased V_{max} . In a second step, we examined the time dependence of the effect of azithromycin on cathepsin B activity and its reversibility, in relation to the drug intake and efflux on the one hand, and the development of the phospholipidosis, on the other. Fig. 1 shows that whereas the drug accumulated very quickly (reaching its maximal cellular level after only 1 day), the change in cathepsin B activity occurred only after 2 days, somewhat but not strictly in relation with the accumulation of phospholipids. Both the drug accumulation and the phospholipid overloading recessed after azithromycin withdrawal. In contrast, the activity of cathepsin B remained elevated and even increased still more during the washout period.

Cycloheximide, an inhibitor of protein synthesis in eucaryotic cells [24], completely abolished the effect of azithromycin on cathepsin B activity. Thus, as previously described [25], cycloheximide alone decreased the activity of cathepsin B to $\approx 20\%$ of the control values. Co-incubation with azithromycin did not affect this decrease.

We then directly evaluated by Northern blot analysis the amounts of cathepsin B mRNA present in fibroblasts after incubation with azithromycin. The Northern blot, prepared with poly(A)⁺ RNA from fibroblasts incubated for 2 days with azithromycin (50 mg/l; 66.6 μ M) or from control cells, was hybridized with a rat cathepsin B probe covering nucleotides 360–841. A unique band of approx. 2.2 kb was observed

(Fig. 2), the intensity of which was similar for control and treated cells. Control hybridization of the same preparation with a rat actin probe revealed a similar amount of hybridizable actin mRNA (band at 2.1 kb) under the two tested conditions.

4. Discussion

As demonstrated here, azithromycin causes conspicuous phospholipidosis in cultured cells. Other studies from our laboratory have shown that this phospholipidosis affects primarily the lysosomes [8] and can reasonably be ascribed (a) to the capacity of this antibiotic to accumulate in large amounts in these organelles [6] and (b) to its ability to bind to phospholipid bilayers and thereby to inhibit the activity of lysosomal phospholipase A₁ (and probably other phospholipases), as demonstrated by *in vitro* studies [9].

Paradoxically, however, we observe here that the incubation of fibroblasts with azithromycin increases the activity of lysosomal phospholipase A₁ that can be recovered from the cell lysates. The drug also increases the activity of other lysosomal hydrolases, i.e. a series of enzymes probably not involved in the development of the phospholipidosis, and most notably cathepsin B. Moreover, gentamicin, which also induces lysosomal phospholipidosis [10], does not cause an increase in lysosomal enzyme activities but rather a decrease in the activity of phospholipase A₁.

Since azithromycin causes no increase in the activities of non-lysosomal enzymes, it is probably safe to assume that we are not dealing here with an unspecific stimulation of protein synthesis. The experiments with cycloheximide nevertheless demonstrate that protein synthesis, or a process directly dependent on protein synthesis, must be operative for azithromycin to exert its stimulatory effect. Yet, the Northern blot experiments also indicate that the concentration of mRNA of cathepsin B is unchanged in spite of its increased activity, ruling out a direct or indirect effect of azithromycin on gene expression.

The slow onset in the change of activity of cathepsin B, compared to the rate of intracellular accumulation of azithromycin, and its lack of reversibility in spite of the efflux of the drug show that the hyperactivity caused by azithromycin is not merely and solely dependent upon the presence of drug in lysosomes or somewhere else in cells in close contact with the

Table 1
Activity of lysosomal (upper panel) or non-lysosomal (lower panel) enzymes in cell homogenates

| | Azithromycin (50 mg/l, 3 days) | Gentamicin (1 mg/ml, 5 days) |
|---|---------------------------------|--------------------------------|
| Drug cellular concentration | 22.2 \pm 0.5 ^a | 25.9 \pm 2.5 |
| Total cellular phospholipids | 159.0 \pm 5.0 ^{b,d} | 145.9 \pm 0.2 ^d |
| Lysosomal | | |
| Cathepsin B | 329.5 \pm 11.8 ^{b,c} | 131.1 \pm 20.7 ^{NS} |
| <i>N</i> -Acetyl- β -hexosaminidase | 204.4 \pm 10.7 ^c | 118.1 \pm 4.9 ^d |
| Sulfatase A | 182.5 \pm 1.4 ^c | 79.7 \pm 2.0 ^d |
| Phospholipase A ₁ | 190.2 \pm 11.9 ^c | 40.9 \pm 1.3 ^c |
| Non-lysosomal | | |
| Lactate dehydrogenase | 97.7 \pm 5.5 ^{NS} | ND |
| Phosphoglucosmutase | 102.0 \pm 7.5 ^{NS} | ND |
| 5'-Nucleotidase | 70.4 \pm 12.0 ^c | ND |

^aIn μ g/mg protein, corresponding to 5.7 and 11.2 mM, respectively.

^bIn % of control values (mean \pm S.D. of 3 independent experiments).

^c $p < 0.001$; ^d $0.001 < p < 0.01$; ^e $0.01 < p < 0.05$; NS, not significantly different from controls; ND, not determined.

enzyme. A direct effect of the drug on the enzymes also seems unlikely in view of the *in vitro* data which show that: (a) azithromycin does not significantly alter the kinetic properties of cathepsin B; (b) azithromycin is without effect on phospholipase A₁ when this enzyme is assayed *in vitro* on detergent-dispersed phospholipid micelles [8]; and (c) azithromycin is actually a potent inhibitor of phospholipase A₁ when the activity is assayed towards a substrate included in organized membrane bilayers [9].

Finally, the effect of cycloheximide on azithromycin-induced hyperactivity of cathepsin B is opposite to what we described earlier for leupeptin, an inhibitor of cysteine

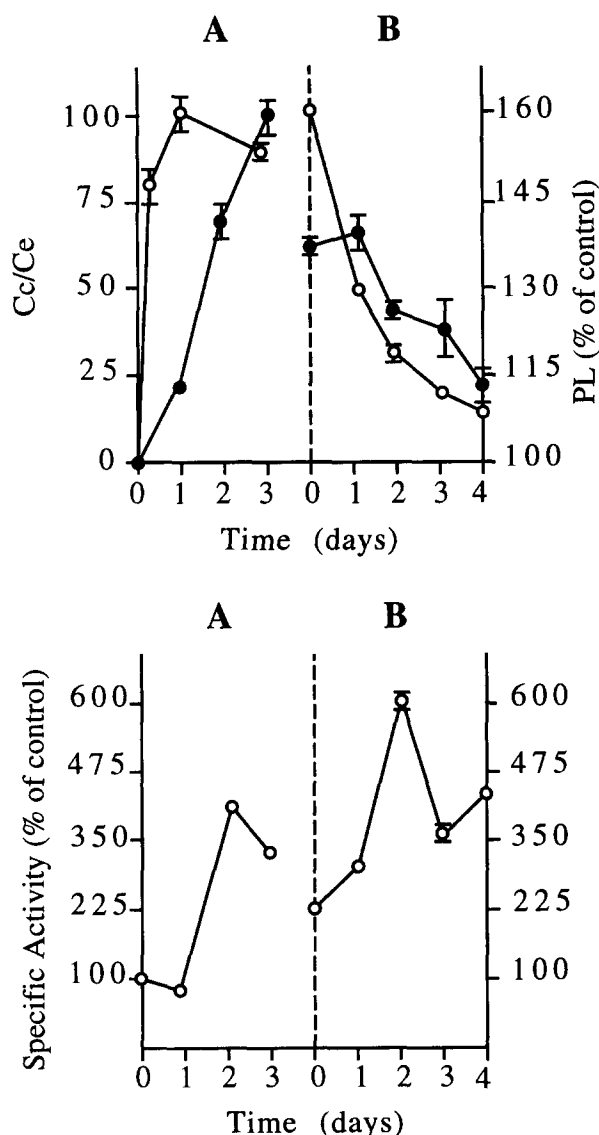


Fig. 1. Time evolution of the cellular to extracellular drug concentration ratio (Cc/Ce; open symbols; upper panel; this ratio was determined as described in [6]), the content of total cellular phospholipids (PL; closed symbols; upper panel) and the activity of cathepsin B (lower panel) in homogenates of rat fibroblasts incubated for 3 days with azithromycin (50 mg/l; 66.6 μ M) (A), and during a subsequent reincubation in drug-free medium (B). Phospholipid content and enzyme activity are expressed in % of control values. Data in (A,B) were obtained from 2 independent experiments, explaining the differences between the values recorded at day 3 in (A) and noted for day 0 in (B). Results are given as mean \pm S.D. ($n = 3$).

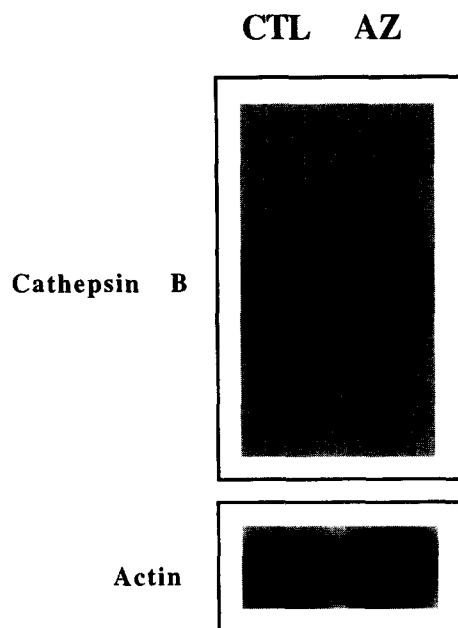


Fig. 2. Northern blot analysis of total mRNA of rat embryo fibroblasts incubated for 2 days in the absence (CTL) or presence of azithromycin (Az; 50 mg/l). Lanes contained 2 μ g of poly(A)⁺ RNA. The membrane was hybridized with a probe covering nucleotides 360–841 of the cathepsin B coding sequence (upper panel). Control hybridization was performed on the same membrane with a rat actin probe (lower panel).

proteinases. The latter also causes an increase of lysosomal enzyme activities in cells cultivated in its presence but by impairing their breakdown [25], and its effect is therefore not antagonized by cycloheximide. This difference of behavior therefore suggests that azithromycin exerts its effect not in the lysosomes but probably at some step during the processing and packaging of the lysosomal enzymes, which is protein synthesis-dependent. Because azithromycin probably accumulates in cells by proton-trapping (its accumulation is abolished if cells are co-incubated with the proton ionophore monensin, [8]), it is tempting to speculate that the drug will partly reach the Golgi vesicles and/or the so-called 'acidified-prelysosomal compartment' through which lysosomal enzymes are channelled to lysosomes [26]. In contrast, gentamicin, which is a very polar drug (see structural formula and biophysical properties in [27]), enters by endocytosis, and probably has only very limited access, if any, to that compartment.

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