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Extracellular catalase activity protects cysteine cathepsins from inactivation by hydrogen peroxide

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Abstract The resistance of secreted cysteine cathepsins to peroxide inactivation was evaluated using as model THP-1 cells. Differentiated cells released mostly cathepsin B, but also cathepsins H, K, and L, with a maximum of endopeptidase activity at day 6. Addition of non-cytotoxic concentrations of H_2O_2 did not affect mRNA expression levels and activity of cathepsins, while the catalase activity remained also unchanged, consistently with RT-PCR analysis. Conversely inhibition of extracellular catalase led to a striking inactivation of secreted cysteine cathepsins by H_2O_2. This report suggests that catalase may participate in the protection of extracellular cysteine proteases against peroxidation.

Keywords: Cathepsin; Cysteine protease; Inflammation; Oxidation; Proteolysis; THP-1 cell

1. Introduction

Macrophages play a crucial role by triggering a cascade of reactions under oxidative challenges. They initiate cellular responses, including secretion of proinflammatory cytokines and release of cytotoxic reactive oxygen species (ROS) [1]. H_2O_2, which is a potent marker of oxidative stress [2], promotes tissue injuries and elevated concentrations may induce death of phagocytic cells [3–5]. Nevertheless human macrophages can survive to exposure to oxidants, as a result of the presence of glutathione peroxidase or catalase, which is primarily localized in the peroxisome [6].

During inflammations, oxidant–antioxidant and protease–antiprotease systems are altered, and together participate in remodeling of extra cellular matrix (ECM) components [7]. Among these proteases, cysteine cathepsins (CPs) are located mainly within the acidic compartments of cells, but are also found extracellularly as soluble enzymes or bound to the plasma membrane [8]. In addition to their basic maintenance duties (intracellular protein degradation and turnover), CPs fulfill specific functions in a variety of biological and pathophysiological processes, including MHC-II antigen presentation, prohormone processing, tumor invasion, or osteoporosis (see for review [9,10]). Moreover remodeling and degradation of constitutive elements of the ECM, the basement membrane and elastin fibers depends on their potent collagenolytic and elastinolytic activities besides serine proteases and MMPs [11,12].

However a relatively weak attention has been given so far to the role of oxidants to control enzymatic activities of cathepsins [13,14]. Nucleophilic active site cysteine is a key residue of enzyme function and is highly sensitive to oxidative reagents [15]. Accordingly inactivation of cysteine cathepsins by H_2O_2 is time- and dose-dependent [16] as reported for caspases and papain [17,18]. Nevertheless active CPs were found in bronchoalveolar lavage fluids (BALFs) from patients suffering of acute and chronic inflammations (see for review [19,20]) in spite of an unfavorable oxidizing environment. To tentatively understand these apparently contrasting observations we have used as model a myelomonocytic cell line (THP-1 cells) [21] to assess the presence of an extra cellular anti-oxidant system able to counterbalance deleterious effects of exogenous H_2O_2 and to provide some protection to thiol-dependent proteases. Extra cellular activities of CPs and catalase were examined in the presence or not of non-cytotoxic concentrations of H_2O_2. Finally the ability of catalase to protect secreted cysteine cathepsins from peroxidation was considered in the presence of catalase inhibitors.

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Abbreviations: AMC, 7-amino-4-methyl coumarin; 3-AT, 3-amino-l, 2, 4-triazole; BALF, bronchoalveolar lavage fluid; BCA, bicinchoninic acid; CA-074, *N*-(L-3-*trans*-propylcarbamoyl oxirane-2-carbonyl)-L-isoleucyl-L-proline; CP, cysteine protease; DTT, DL-dithiothreitol; E-64, L-3-carboxy-*trans*-2.3-epoxypropionyl-leucylamido-(4-guanidino) butane; ECM, extra cellular matrix; FBS, fetal bovine serum; GM-CSF, granulocyte–macrophage colony-stimulating factor; MDM, monocyte-derived macrophage; MMTS, methylmethanethiosulfonate; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; Z, benzyloxycarbonyl

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2. Material and methods

2.1. Enzymes and reagents

DL-Dithiothreitol (DTT) came from Bachem (Weil am Rhein, Germany). H_2O_2, L-3-carboxy-*trans*-2.3-epoxypropionyl-leucylamido-(4-guanidino) butane (E-64), *N*-(L-3-*trans*-propylcarbamoyl oxirane-2-carbonyl)-L-isoleucyl-L-proline (CA-074), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, 3-AT, KCN, EDTA and methylmethanethiosulfonate (MMTS) were from Sigma–Aldrich (Saint-Quentin Fallavier, France). Cathepsin K was kindly provided by Dr. Dieter Brömme (University of British Columbia, Vancouver, Canada). Cathepsins B and L were supplied by Calbiochem (VWR International, Libourne, France). Z-Phe-Arg-AMC and Z-Arg-Arg-AMC were purchased from Bachem. All other reagents were of analytical grade.

2.2. Cell culture

Human THP-1 cells (LGC Promochem, Molsheim, France) were subcultured and maintained in suspension, at 37 °C, in 5% CO2, with RPMI-1640 Glutamax I (Invitrogen, Cergy-Pontoise, France), containing 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), 0.05 mM 2-β-mercaptoethanol, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Monocytes were differentiated into macrophages by addition of 162 nM phorbol myristate acetate (PMA) (Sigma–Aldrich); after 3 days, FBS was substituted by 4% Ultroser G (Biosepra, Cergy-Pontoise, France), and the growth medium was changed every 2 days.

Following cell lysis (50 mM Tris–HCl buffer, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1% Protease Inhibitor Cocktail 100X Sigma–Aldrich) and centrifugation (14 000 × *g*, 15 min, 4 °C), protein concentration was determined using bicinchoninic acid (BCA) Protein Assay Kit (Interchim, Montlucon, France).

2.3. RT-PCR analysis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen S.A., Courtaboeuf, France). Reverse transcription was performed on 500 ng of total RNA, using the SuperScript III Reverse Transcriptase (Invitrogen). Primers were: 5′-TCATACGATCTGGGCATGAA-3′ (sense strand) and 5′-AGGTTCTGGGCACTGAGAGA-3′ (antisense strand) for cathepsin S; 5′-TTCTGCTGCTACCTGTGGTG-3′ (sense strand) and 5′-CCAGGTGGTTCATAGCCACT-3′ (antisense strand) for cathepsin K; 5′-ACAGTGGACCAAGTGGAAGG-3′ (sense strand) and 5′-TGGGCTTACGGTTTTGAAAG-3′ (antisense strand) for cathepsin L; 5′-GCCTGCAAGCTTCGATGCAC-3′ (sense strand) and 5′-GCAATTCTGAGGCTCTGACC-3′ (antisense strand) for cathepsin H; 5′-GCCTGCAAGCTTCGATGCAC-3′ (sense strand) and 5′-CTATTGGAGACGCTGTAGGA-3′ (antisense strand) for cathepsin B; 5′-CGTGCTGAATGAGGAACAGA-3′ (sense strand) and 5′-TTGTCCAGAAGAGCCTGGAT-3′ (antisense strand) for catalase and 5′-GGCGGCACCACCATGTACCCT-3′ (sense strand) and 5′-AGGGGCCGGACTCGTCATACT-3′ (antisense strand) for β-actin. Standard PCR was carried out in a 50 μl reaction mixture containing 25 ng of reverse transcribed total RNA, 0.5 μM of each primers and 1 U of Taq DNA Polymerase (Invitrogen). Conditions were 95 °C for 5 min, followed by 40 cycles consisting of 94 °C for 45 s, 56 °C for 30 s, 72 °C for 45 s, and a final elongation step (72 °C, 5 min). The amplification products were identified by electrophoresis on a 1.5% agarose gel containing ethidium bromide and scanned with a Gel DocTM XR (Bio-Rad, Marnes-la Coquette, France).

2.4. Immunoblotting

Polyclonal anti-human cathepsins B, L, and H were supplied by Fitzgerald (Concord, USA), while anti-human cathepsin S was from Calbiochem. Anti-human cathepsin K was kindly provided by Dr. Dieter Bromme [22]. Polyclonal anti-human catalase antibody was provided by Calbiochem. The IgG-peroxidase conjugate was from Sigma–Aldrich. Prestained molecular masses was from Bio-Rad. Samples were subjected to a 12% SDS–PAGE gel under reducing conditions prior immunoblotting [23].

2.5. Extra cellular enzymatic activities

Aliquots of cell supernatants were collected every day. Proteolytic assays were carried out at 37 °C in 0.1 M sodium acetate buffer, pH 5.0, 2 mM DTT and 2 mM EDTA, using as substrate Z-Phe-Arg-AMC (50 μM; λ_{ex} = 350 nm, λ_{em} = 460 nm). Extra cellular cysteine cathepsins were titrated by E-64 [24]. Catalase activity was determined by the Amplex Red Catalase Assay Kit (Invitrogen).

2.6. Effect of hydrogen peroxide

After differentiation, the growth medium was substituted by fresh RPMI medium, supplemented by 4.5 g/l glucose, 10 mM HEPES, 4% Ultroser G (day 5), in the absence of sodium pyruvate. At day 6, H_2O_2 (0–200 μM) was added, and supernatants collected (0–24 h) for measurement of cysteine cathepsins activity using Z-Phe-Arg-AMC as substrate (50 μM). Cytotoxic effects of H_2O_2 were examined using the "Cell Titer 96® AQueous One Solution Cell Proliferation Assay" (Promega, Madison, WI, USA) (triplicate tests, with n = 2/well). Alternatively catalase inhibitors, i.e KCN (50μM)or 3-AT (20 mM) [5,25], were preincubated for 30 min at 37 °C, before addition of H_2O_2 (200 μM).

3. Results and discussion

3.1. Secretion of active cysteine cathepsins by PMA-differentiated THP-1 cells

In a previous study, Whatling et al. [26] conducted a real-time polymerase chain reaction analysis with human primary monocytes and macrophages and with myelomonocytic THP-1 cells. They showed that changes in gene expression was nearly identical, and thus advocated that THP-1 cells, which have been widely used as models for oxidation studies, reflect most of macrophage characteristics. Under our experimental conditions, following addition of PMA (40.5 nM, day 0), THP-1 cells were differentiated at day 3. mRNA expressions of β-actin (used as control) and cysteine cathepsins B, H, K, L, and S were analyzed by PCR after reverse transcription of total RNA isolated from cell lysates. mRNA expression levels of cathepsins B, L, H and S remained stable from day 0 to day 8, suggesting that their transcription is not activated during the differentiation of THP-1 cells. However it has been reported that phorbol esters could modify the stability of some cysteine cathepsins mRNA; hence such changes in stability may represent a mode of post-transcriptional regulation for cysteine cathepsins: accordingly the half-life of cathepsin B mRNA is noticeably increased during differentiation of HL60 cells into macrophages [27]. In contrast to cathepsins B, L, H and S, mRNA expression level of cathepsin K increased from day 0 to day 6 before declining (data not shown). Despite it has been reported that transcription factors of the ETS family as well as interferon gamma and interleukins-6 and -13 regulate the expression of cathepsin K in macrophages [28], other factors, including NF-κB that possess a binding site in the promoter, may be also involved (see for review [29]).

Western blot analysis indicated that secreted cysteine cathepsins were detected only 72 h after the addition of PMA (Fig. 1A). While mature cathepsins L and K were early identified (day 3), both the single-chain and the two-chain forms of cathepsin B were heavily stained at day 6. According to the apparent steadiness of mRNA expression levels, the increased secretion of cysteine cathepsins probably relies on an altered intracellular trafficking and distribution of cysteine cathepsins rather than a transcriptional induction during the differentiation of THP-1 monocyte-derived macrophages [12]. Contrary to that observed with alveolar macrophages and activated smooth muscle cells [30,31], no secreted mature cathepsin S was detected by immunostaining. Analysis of the overall endopeptidase activity of cathepsins was performed

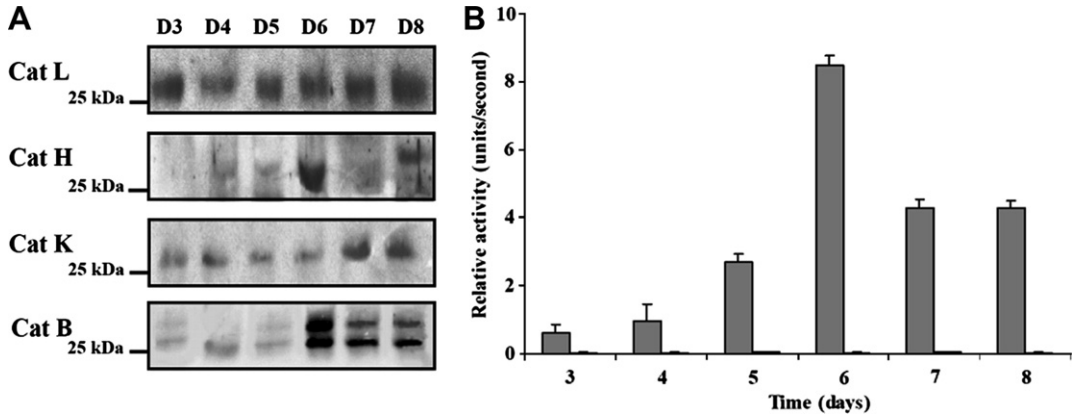

Fig. 1. Secretion and enzymatic activity of cysteine cathepsins. (A) Following addition of PMA (162 nM), THP-1 cell-free supernatants were collected (days 3–8) and subjected to a 12% SDS–PAGE gel under reducing conditions, prior immunoblot analysis as reported in Section 2. (B) The proteolytic activity of cysteine cathepsins (grey) from cell supernatants was measured by monitoring of the fluorescence release (λ_{ex} = 350 nm, λ_{em} = 460 nm), in the presence of Z-Phe-Arg-AMC (50 μM) (n = 6). Controls (black) were carried out in the presence of E-64 (100 μM).

using as substrate Z-Phe-Arg-AMC, a broad-spectrum substrate of cysteine cathepsins, which is preferentially cleaved by cathepsins B and L. No CP activity was monitored in cell supernatants before differentiation. However proteolytic activity was found in supernatants of differentiated THP-1 cells. It maximal potency towards Z-Phe-Arg-AMC was reached at day 6 and was totally impaired by E-64 (Fig. 1B). Of major concern, THP-1 cells express a H+-ATPase on their plasma membrane that is 3/4-fold up-regulated during monocyte differentiation [32], and thus may favor the activity and stability of extracellular cathepsins by reducing the pH of the pericellular environment, as demonstrated for monocyte-derived macrophages (MDMs) [33]. After titration by E-64, an active site concentration of ~140 nM (2×10^6 cells/well) was found at day 6. Eighty-five percent of the activity was inhibited by CA-074, indicating that cathepsin B is the most abundant cysteine cathepsin secreted by differentiated THP-1 cells.

3.2. Effect of hydrogen peroxide on secreted cysteine cathepsins

Trials were performed in a growth medium that was depleted in sodium pyruvate, a potent peroxide scavenger [34], and containing a serum substitute (Ultroser G) to get rid of most of proteins, including circulating cathepsin inhibitors (cystatins, kininogens) that are present in FBS. Deleterious effects of H_2O_2 (0–200μM, addition at day 6) on differentiated THP-1 cells were first considered (Cell Titer 96® AQueous One Solution Cell Proliferation Assay, Promega) at various interval times during 24 h (triplicate trials with n = 2/well). The cellular viability of treated cells remained identical (97 ± 6%) compared to controls in the absence of peroxide. Present results confirm that, under our experimental conditions, H_2O_2 had no cytotoxic effects on differentiated THP-1 cells [35], whereas macrophages could go through apoptosis in the presence of harsher amounts [4]. In addition, protein concentrations of cell lysates and their supernatants (days 6 and 7) were not modified

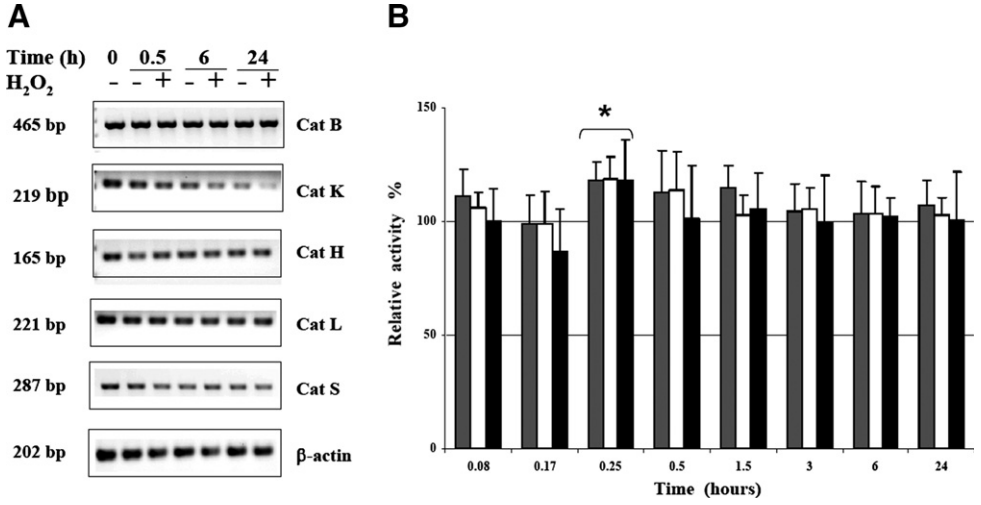

Fig. 2. Effect of hydrogen peroxide on cysteine cathepsins expression and extra cellular activity. (A) Following addition of H_2O_2 (200 μM) to cell medium (day 6), mRNA expressions of cysteine cathepsins B, H, K, L, and S were analyzed by RT-PCR (0 to 24 h). β-Actin was used as control. (B) The proteolytic activity of cysteine cathepsins was determined as reported in Fig. 1, in the presence of various concentrations of hydrogen peroxide (5 μM: grey, 50 μM: white, 200 μM: black). Proteolytic activities were expressed as normalized values (%), using CP activities in the absence of H_2O_2 as reference. Average values were represented as the means + S.E.M. Differences were determined by ANOVA "one-way" test, and were considered significant at $P < 0.05$ (n = 12).

by addition of H_2O_2 (0–200 μM). Despite a slight decrease was observed for cathepsin K (Fig. 2A), mRNA expression levels of cathepsins B, H, L, and S remained stable, suggesting that 200 μM H_2O_2 did not affect dramatically the overall transcriptional level of cathepsin genes. Surprisingly, apart from a weak disparity at $t = 15$ min ($n = 12$; $P \leqslant 0.05$), no significant variation of enzymatic activity towards Z-Phe-Arg-AMC was observed after addition of H_2O_2 (Fig. 2B), notwithstanding the H_2O_2-dependent sensitivity of cysteine cathepsins to oxidation [16]. Interestingly CPs, most probably secreted by macrophages, were found partly active in human inflammatory BALFs in spite of a deleterious oxidizing environment [20,23,36]. Taken together these apparently contrasting data, one could hypothesize that a potent antioxidant system may take place against damaging effects of peroxidation on CP activities.

3.3. Extra cellular catalase activity of THP-1 cells

Similar extra cellular peroxidase activity (Fig. 3A) and mRNA expression level of catalase (Fig. 3B) were found in both monocytes and differentiated cells, bearing that THP-1 cells release catalase constitutively. However the faintness of immunostaining supported also that the level of secreted catalase remained markedly lower than its intracellular level (data not shown). Addition of 200 μM H_2O_2 did not modify significantly both extra cellular catalase activity (Fig. 3C, ANOVA "one-way" test) and its transcriptional level (Fig. 3D), conversely to alveolar macrophages that secrete higher levels of catalase in response to exogenous H_2O_2 [35]. Cell supernatants were further preincubated in the presence of catalase inhibitors [5,25], KCN and 3-amino-l,2,4-triazole (3-AT) prior to addition of H_2O_2 in order to check if THP-1 extra cellular catalase activity may contribute to the protection of cysteine cathepsins towards peroxidation. 3-AT had no inhibitory effects on the peptidase activity of purified cathepsins B, L and K used as controls (Fig. 4A). Similarly under experimental concentrations (0 μM) potassium cyanide did not inhibit cathepsins, contrary to peptide-derived nitriles (see for review [11]).

Conversely CP activities of THP-1 supernatants were strongly impaired by H_2O_2, in the presence of both KCN (~75%, $P < 0.01$), and of 3-AT (~50%, $P < 0.015$), while no significant inactivation was observed in the lack of catalase inhibitors (Fig. 4B). While catalase is primarily located in peroxisomes, data support that a constitutive, basal level of extra cellular peroxidase activity of catalase may ensure a potent protection to secreted cysteine cathepsins against exogenous hydrogen peroxide, which are commonly found during inflammatory events and oxidative stress. Our findings also corroborate previous reports indicating that both freshly isolated macrophages and granulocyte–macrophage colony-stimulating factor (GM-CSF)-induced monocyte-derived macrophages used catalase-dependent pathways to consume H_2O_2 [35,37]. Most probably, this protective arsenal has to be reinforced by other, but not identified so far, anti-oxidant systems displaying complementary peroxidase activities.

In summary, the ability of extra cellular cathepsins to resist to peroxidation was studied by using as model the monocyte–macrophage THP-1 cell line. Under non-cytotoxic oxidative conditions, H_2O_2 does not display noxious effects on secreted cathepsins. This defensive effect is partly due to the peroxidase activity of extra cellular catalase, since its inhibition prior addition of H_2O_2 led to a significant reduction of CP activity. Regardless of a disturbed antioxidant/oxidant balance, the current report provides the first evidence that catalase may directly participate to the preservation of extra cellular cathepsin activities, which could be of biological relevance during inflammatory episodes.

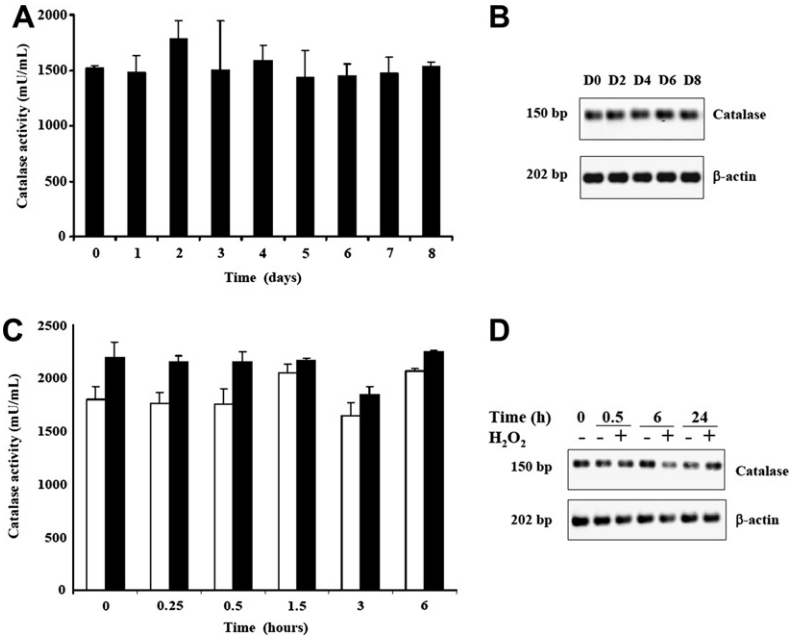

Fig. 3. Expression and secretion of catalase by differentiated THP-1 cells. (A) Catalase activity in cell supernatants was measured using the Amplex® Red Catalase Assay Kit ($n = 6$). (B) Analysis of mRNA expression of catalase by RT-PCR (control: β-actin). (C) Extra cellular catalase activity after addition (black bars) or not (white bars) of 200 μM hydrogen peroxide (0 = day 6) ($n = 6$). (D) mRNA expression of catalase (RT-PCR) after addition or not of hydrogen peroxide (control: β-actin).

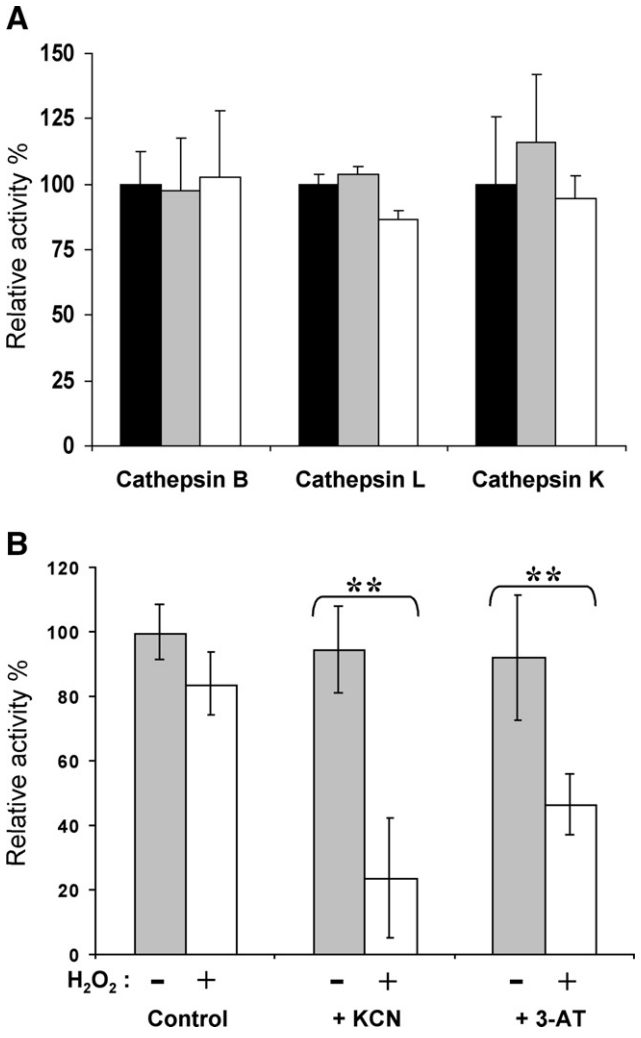

Fig. 4. Effect of catalase inhibitors on residual extra cellular cysteine cathepsins activity. (A) Human cathepsin B (2 nM) was incubated with KCN (50 μM) (grey bar) or 3-AT (20 mM) (white bar) for 30 min at 37 °C prior the residual enzymatic activity towards Z-Phe-Arg-AMC was measured (black bar: control without catalase inhibitor). The same procedure was repeated for cathepsins L (2 nM) and K (2 nM). Results are means ± S.E.M. (B) Cell supernatants (day 6) were first incubated 30 min at 37 °C in the presence of KCN (50 μM) or 3-AT (20 mM), followed by the addition of hydrogen peroxide (200 μM) for 30 min. Alternatively controls were performed in the absence of catalase inhibitors prior addition of H_2O_2. Residual proteolytic activities were measured in the activity buffer using Z-Phe-Arg-AMC (50 μM) as substrate. Average results were reported by means ± S.E.M. Differences were determined by ANOVA "one-way" test, and were considered significant at $P < 0.05$ ($n = 6$).

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