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Application of specific cell permeable cathepsin G inhibitors resulted in reduced antigen processing in primary dendritic cells

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ABSTRACT

The serine protease cathepsin G (CatG) is expressed in primary antigen-presenting cells and regulates autoantigen processing in CatG pre-loaded fibroblasts. To further investigate the function of CatG in the major histocompatibility complex (MHC) class II loading compartments, a specific, cell permeable CatG-inhibitor is needed. In this study, several CatG-inhibitors were tested for their ability to penetrate the cell membrane of peripheral blood mononuclear cells (PBMC). We find that the commercially available reversible CatG-specific inhibitor I (CatG inhibitor) and the irreversible Suc-Val-Pro-Phe^{P} $(OPh)_2$ (Suc-VPF) are both cell permeable and specifically inhibit intracellular CatG in the PBMC. Furthermore, selective inhibition of CatG resulted in reduced tetanus toxin C-fragment (TTC) and hemagglutinin (HA) processing and presentation to CD4$^+$ T cells. We conclude that these CatG inhibitors can be used for both antigen-processing studies and for modulation of T cell response *in situ* and *in vivo*.

1. Introduction

Processing of antigens in the major histocompatibility complex (MHC) class II compartments is critical for the formation of potent T cell epitopes that will activate the corresponding CD4$^+$ T cells. Previous studies have demonstrated that the serine protease cathepsin G (CatG) regulates the processing of autoantigens *in vitro* (Burster et al., 2004, 2005). Furthermore, the cysteine proteases (CatB, C, F, H, L, S, V, X, and AEP) (Riese et al., 1996; Rudensky and Beers, 2006; Watts et al., 2005; Yasuda et al., 2004; Zavasnik-Bergant and Turk, 2006), and aspartyl cathepsins (CatD, and E) (Bennett et al., 1992; Chain et al., 2005; Nishioku et al., 2002) are active proteases of the endocytic compartments. CatS is crucial for antigen processing and generating the CLIP fragment (Riese et al., 1996; Villadangos et al., 1997) that occupies the binding groove of the MHC class II molecule to prevent premature peptide binding. MHC class II molecules loaded with antigenic peptides travel from this compartment to the cell surface for inspection by CD4$^+$ T cells (Bryant and Ploegh, 2004).

The broad serine protease inhibitor phenylmethane sulfonyl fluoride (PMSF, James, 1978), which is unstable and toxic, reacts with the active site serine of its target protease. Selective inhibition of CatG can be performed using the reversible, competitive, and non-peptide-based CatG-specific inhibitor I (CatG inhibitor); however, it is not clear whether this inhibitor possesses the beneficial characteristics common to other inhibitors, such as high potency and the ability to cross cell membranes, which allows for investigation of CatG function in intact cells. Recently, a set of diaryl phosphonic-type irreversible CatG inhibitors were synthesized and their inhibitory activity was described in a series of enzymatic studies (Oleksyszyn and Powers, 1991; Sienczyk et al., 2008).

Presentation of the multiple sclerosis-associated autoantigen, myelin basic protein (MBP), in CatG-preloaded fibroblasts, which themselves do not express CatG, reduced activation of MBP84-98-specific T cells, in contrast to control cells, thereby demonstrating an essential role of CatG in processing this autoantigen *in situ* (Stoeckle et al., 2009). For determination of antigen processing in primary B cells or dendritic cells (DC), respectively, the use of a specific, cell permeable CatG inhibitor is necessary. With such an inhibitor it will not only be possible to investigate antigen-processing in antigen-presenting cells (APC), but also possible to manipulate the immune response.

This study determines that the CatG inhibitor and diaryl phosphonic-type inhibitor Suc-Val-Pro-Phe^{P} $(OPh)_2$ (Suc-VPF, Oleksyszyn and Powers, 1991) are both cell permeable and can specifically inhibit CatG in primary human peripheral blood

Abbreviations: AEP, asparagine endoprotease; APC, antigen-presenting cells; Cat, cathepsin; mDC, myeloid dendritic cells; HA, hemagglutinin; MBP, myelin basic protein; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; Suc-VPF, Suc-Val-Pro-Phe^{P} $(OPh)_2$; TTC, tetanus toxin C-fragment.

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mononuclear cells (PBMC). Decreased T cell activation was observed when mDC were treated with either the CatG inhibitor or Suc-VPF in a functional T cell assay using tetanus toxin C-fragment (TTC) or hemagglutinin (HA) as antigens. Therefore, these CatG-inhibitors are suitable for studies on the intracellular processing and presentation of antigens.

2. Materials and methods

2.1. Cells and inhibitors

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy HCMV-seronegative blood donors by density gradient centrifugation. Myeloid dendritic cells (mDC, $CD1c^+$) and B cells ($CD19^+$) were positively selected using the appropriate magnetic cell separation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. Cells were treated with the CatG-specific inhibitor I (**CatG inhibitor**; Calbiochem, Merck, Darmstadt, Germany), Suc-Val-Pro-Phe^P $(OPh)_2$ (**Suc-VPF**; Jozef Oleksyszyn, University of Wroclaw, Oleksyszyn and Powers, 1991), Cbz-Phg(4-guanidine)^P $(OPh)_2$ (**compound 4**), Cbz-Phg (4-guanidine)^P $(OC_6H_4\text{-}4\text{-}S\text{-}Me)_2$ (**compound 12**), Ac-Phe-Val-Thr-Phg(4-guanidine)^P $(OC_6H_4\text{-}4\text{-}S\text{-}Me)_2$ (**compound 19**), [Phe(4-guanidine)]^{5}SFTI-1 (**peptide 4**), [Nphe^{5,12}, Npip^{8,9},Nleu^{10}]SFTI-1 (**peptide 5**; Nphe, Npip and Nleu N substituted glycine by benzylamine piperonylamine and isobutanolamine; Adam Lesner, Faculty of Chemistry, University of Gdansk, Gdansk, Poland, Łęgowska et al., 2009a, 2009b; Sienczyk et al., 2008) in final concentrations of 1, 5, or 10 μM and cultured in complete RPMI 1640 medium (10% FCS and 70 μg/ml gentamycin) for 4 h or the indicated time points. Cells were washed four times with PBS and lysed for further experiments. The use of human cells for *in vitro* studies was in accordance with IRB regulations.

2.2. Active site label

The PBMC were lysed (10 mM Tris [pH 7.5], 150 mM NaCl, and 0.5% NP-40), adjusted for equal total protein (quantified by the Bradford Assay), and 20–30 μg of the cell lysate was incubated with PBS (pH 7.4) in the presence of DAP22c (2 μM, Oleksyszyn and Powers, 1991). Alternatively, 5 μg of cell lysate were incubated with reaction buffer (50 mM citrate [pH 5.0] and 50 mM DTT) in the presence of DCG-04 (10 μM; probe kindly donated by M. Bogyo, Stanford University, Palo Alto, CA, USA (Greenbaum et al., 2000)) for 1 h at room temperature. Samples were resolved on a 12% SDS-PAGE gel, blotted, and visualized using streptavidin-HRP (Vectastain, Burlingame, CA, USA).

2.3. T cell proliferation assay

Primary human B cells and mDC1 (3×10^5 cells) were preincubated with either the CatG inhibitor (Calbiochem), peptide 4, Suc-Val-Pro-Phe^P $(OPh)_2$ (Suc-VPF), or DMSO (Calbiochem) at a final concentration of 10 μM. $CD4^+$ T cells (9×10^5) were negatively selected using a magnetic cell separation kit (Miltenyi Biotec), transferred, along with 10 μg/ml tetanus toxin C-fragment (TTC; kindly provided by S. Rosinger, Ulm University, Ulm, Germany), or 5 μg/ml hemagglutinin (HA, H3N2, A/Wyoming/3/03; Immune Technology, NY, USA) to the assay and cultured for 3 days. Supernatants from the T cell assay were collected and the cytokine production (IFN-γ or IL-17) was measured using ELISA, following the protocols provided by the company's manual (R&D Systems, Wiesbaden, Germany).

2.4. In vitro processing of antigens

TTC was reduced with 50 mM DTT in 50 mM NH_4HCO_3 for 10 min at 60 °C and alkylated for 25 min at room temperature using 22 mM iodacetamide (Sigma, Taufkirchen, Germany), followed by an additional incubation with 5 mM DTT for 25 min at room temperature. The crude material was then purified by HPLC. 0.3 μg/μl TTC or 0.02 μg/μl HA was incubated with 2 ng/μl CatG (Sigma), 2 ng/μl CatD, 2 ng/μl CatS, or 2 ng/μl CatL (R&D Systems) and incubated for 1.5 h or 2 h at 37 °C in reaction buffer (0.1 M citrate pH 5.0, 2.5 mM DDT). The digestion pattern was resolved by reversed-phase HPLC using a C8 column 125 × 2 (Grom, Herrenberg, Germany). Silver staining was performed by using SilverQuest staining kit (Invitrogen, Karlsruhe, Germany).

2.5. Statistical analysis

Data depict the mean ± S.E.M. Statistical analysis was performed using the two-tailed Student's *t*-test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Inhibition of CatG activity in PBMC

To examine the intracellular inhibition of CatG by selected CatG inhibitors, peripheral blood mononuclear cells (PBMC) were isolated from healthy donors and incubated with various concentrations of one of the following inhibitors: the commercially available CatG-specific inhibitor I (CatG inhibitor); Cbz-Phg(4-guanidine)^P $(OPh)_2$ (compound 4); Cbz-Phg (4-guanidine)^P $(OC_6H_4\text{-}4\text{-}S\text{-}Me)_2$ (compound 12); Ac-Phe-Val-Thr-Phg(4-guanidine)^P $(OC_6H_4\text{-}4\text{-}S\text{-}Me)_2$ (compound 19); [Phe(4-guanidine)]^{5}SFTI-1 (peptide 4); [Nphe^{5,12}, Npip^{8,9},Nleu^{10}]SFTI-1 (peptide 5; Nphe, Npip and Nleu N substituted glycine by benzylamine piperonylamine and isobutanolamine); or Suc-Val-Pro-Phe^P $(OPh)_2$ (Suc-VPF). Cells were harvested and equivalent protein content was incubated with the serine activity-based probe DAP. This inhibitor detects active CatG by forming a covalent bond to the active center of the enzyme. Since DAP contains biotin, protease activity can be determined via streptavidin-HRP blot. CatG was inhibited specifically by the CatG inhibitor and Suc-VPF (Fig. 1A). Neither peptide 4 nor peptide 5 inhibited intracellular CatG, at a concentration of 10 μM. However, CatG was inhibited by compound 4, compound 12, peptide 4, and peptide 5 at concentrations of 100 μM, while compound 19 was not inhibitory (Supplementary data). DCG-04, which labels active cysteine cathepsins, was used as a control to confirm that no other cathepsins were inhibited by these inhibitors (Fig. 1B).

The PBMC were then incubated with 10 μM of the CatG inhibitor, peptide 4, Suc-VPF, or PMSF at different time points. CatG was inhibited in the PBMC within 0.5 h of CatG-inhibitor and Suc-VPF treatment (Fig. 2A and B). PMSF inhibited both CatG and an unidentified serine protease migrating around 35 kDa. Although CatG was not inhibited in the PBMC after 0.5 h, 1 h, or 4 h of incubation with peptide 4, activity was reduced beginning after 1 day. Active cysteine cathepsins were not affected by the CatG inhibitors, as shown in Fig. 2C and D. Of note, reduction of the ~35 kDa serine protease was observed on day 3 of cell culture after inhibition with the CatG inhibitor, peptide 4, and Suc-VPF.

3.2. Inhibition of CatG reduces the presentation of antigens to $CD4^+$ T cells

In an initial experiment, TTC was investigated as a substrate for CatG and found to be completely degraded by CatG (Fig. 3A). Based on these results, the presentation of tetanus toxin C-fragment (TTC)