Chapter 5

Mercuric ions activate calcineurin and upregulate NFAT-dependent IL-4 promoter activity in mast cells

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Abstract

Mercuric ions induce abnormal immune responses in vivo. IL-4 is necessary for certain elements of Hg^{2+}-induced immunomodulation and its expression is upregulated in mast cells and lymphocytes following exposure to $HgCl_2$. In search for molecular mechanism of mercury-mediated IL-4 expression we investigated the effect of mercuric ions on IL-4 promoter activity in mouse mast cells. $HgCl_2$ upregulated IL-4 promoter activity in mast cells in a process, which required NFAT binding site of minimal IL-4 promoter and was sensitive to calcineurin (CaN) inhibitor FK506. Furthermore, Hg^{2+}-upregulated transcription driven by artificial NFAT-dependent promoter containing three NFAT sites and this transcription was sensitive to inhibitors of CaN and to dominant negative mutant of CaN. Finally, we observed that low concentrations of Hg^{2+} increased activity of purified rCaN in vitro. These observations are consistent with the hypothesis that Hg^{2+} ions increase activity of CaN that in turn upregulates NFAT, which binds to specific DNA motif present in IL-4 promoter resulting in IL-4 expression. Thus, Hg^{2+} ions activate CaN/NFAT signaling pathway in mast cells and this molecular mechanism could be important for mediating immunotoxic activities of mercuric compounds observed in vivo.

Introduction

Mercuric compounds are known to cause various adverse effects on immune system. Pathological changes observed in experimental animals exposed to Hg^{2+} ions include autoimmune glomerulonephritis (1), polyclonal arthritis (2), and induction of polyclonal IgE (3). Cellular and molecular mechanisms that are possibly involved in these pathological immune responses include Hg^{2+}-mediated changes in the expression of certain cytokines (4). One of the cytokines upregulated in vivo following administration of $HgCl_2$ is interleukin-4 (5, 6) necessary for induction of IgE and IgG1 (5) and upregulation of MHC II (7) in $HgCl_2$ treated animals. Hg^{2+}-mediated upregulation of IL-4 expression was reproduced in vitro in isolated lymphocytes and mast cells (8, 9).

Several molecular mechanisms explaining Hg^{2+}-mediated IL-4 expression in lymphocytes and mast cells have been proposed (10-12). Among molecules suspected of mediating the effect of Hg^{2+} on IL-4 expression are PKC, P type calcium channels, calcineurin (CaN), and C_Jun N-terminal kinase (10, 12). The possible engagement of CaN in Hg^{2+}-mediated cell responses is supported by observations that CaN inhibitor CsA inhibits Hg^{2+}-mediated IL-4 expression in lymphocytes in vitro and prevents development of Hg^{2+}- induced pathological immune response in vivo (13).

Antigen-mediated IL-4 expression in immune cells is predominantly controlled at the level of gene transcription by regulatory elements located in a proximal promoter (14-16). CaN a protein phosphatase capable of dephosphorylation nuclear transcription factor NFAT resulting in its translocation from cytoplasm to nucleus (17, 18) plays essential role in this regulatory mechanism (19-23). Therefore, we decided to investigate the role of CaN/NFAT signaling pathway in Hg^{2+} -mediated expression of IL-4 in mast cells. Data presented in this report support the critical role of CaN in Hg^{2+}-mediated IL-4 expression and show that Hg^{2+} directly interact with this phosphatase increasing its activity.

Results

It has been shown previously that Hg^{2+} ions mediate IL-4 secretion in mast cells and that this process requires de novo transcription of cytokine mRNA (8). To test if Hg^{2+} ions are able to upregulate IL-4 promoter activity, C57 cells or BMMC were transfected with DNA reporter construct -87 IL-4 pCAT, in which CAT expression is under control of minimal proximal promoter of IL-4. Following transfection cells were sensitized with DNP-specific IgE, and incubated with $HgCl_2$ or antigen (DNP-HSA). CAT concentrations in cell lysates were determined by ELISA and normalized for total protein concentrations.

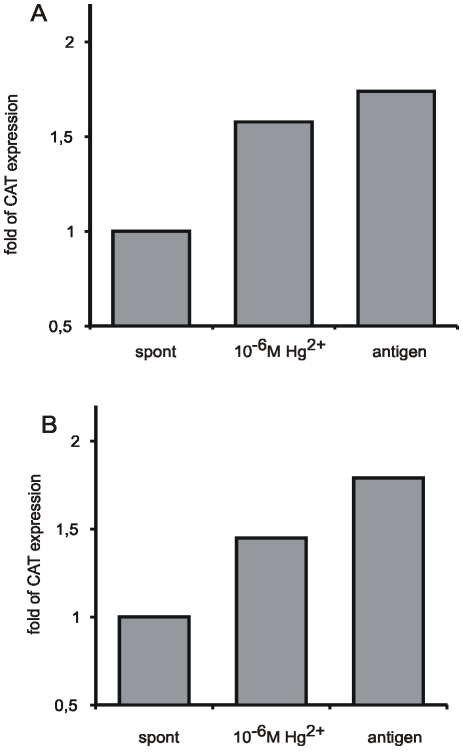

Figure 1 Mercuric ions increase IL-4 promoter activity in mast cells. BMMC (A) and C57.1 (B) mast cells were transfected with -87p IL-4 CAT, sensitized with IgE and incubated for 4 h with medium only (spont.), 10^{-6}M $HgCl_2$ (Hg^{2+}), or 50ng/ml DNP-HSA (antigen). Mast cells were collected by centrifugation, lysed and CAT concentration was determined by ELISA. CAT content was normalized for total protein concentrations and is expressed as fold increase compared to control. Bars represent the mean of 4 (A) and 2 (B) independent experiments each of them performed in duplicate.

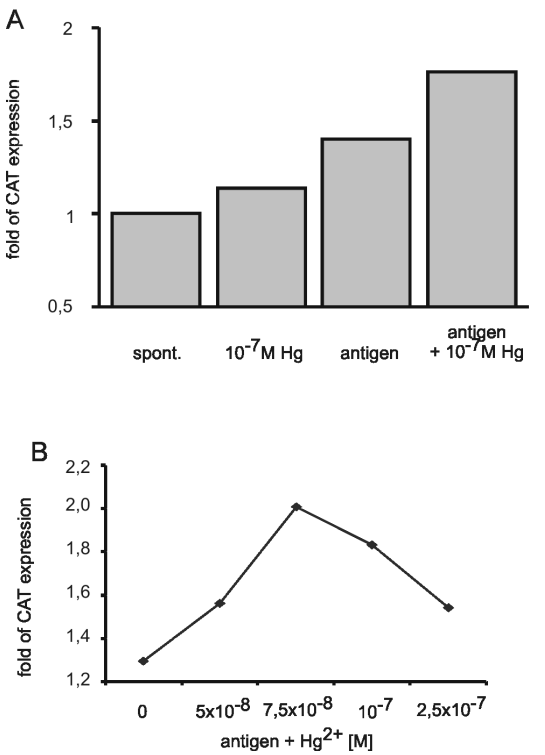

Figure 2 Mercuric ions enhance antigen-mediated IL-4 promoter activity in mast cells. A. C57.1 mast cells were transfected with -87 IL-4 pCAT sensitized with IgE and incubated for 4 h with medium only (spont), 10^{-7}M $HgCl_2$ (Hg^{2+}), 50ng/ml DNP-HSA (antigen), or combination of both 10^{-7}M $HgCl_2$ and 50ng/ml DNP-HSA (antigen + Hg^{2+}). B. C57.1 mast cells were transfected with -87 IL-4 pCAT sensitized with IgE and incubated for 4 h with a single dose of antigen (10 ng/ml DNP-HSA) and increasing concentrations of $HgCl_2$. In each experimental design (A and B) after incubation cells were collected by centrifugation, lysed and concentration of CAT in cell lysates was determined with ELISA. CAT content was normalized for total protein concentrations and is expressed as fold increase compared to control. Each point represents the mean of 2 independent experiments, each of them performed in duplicate.

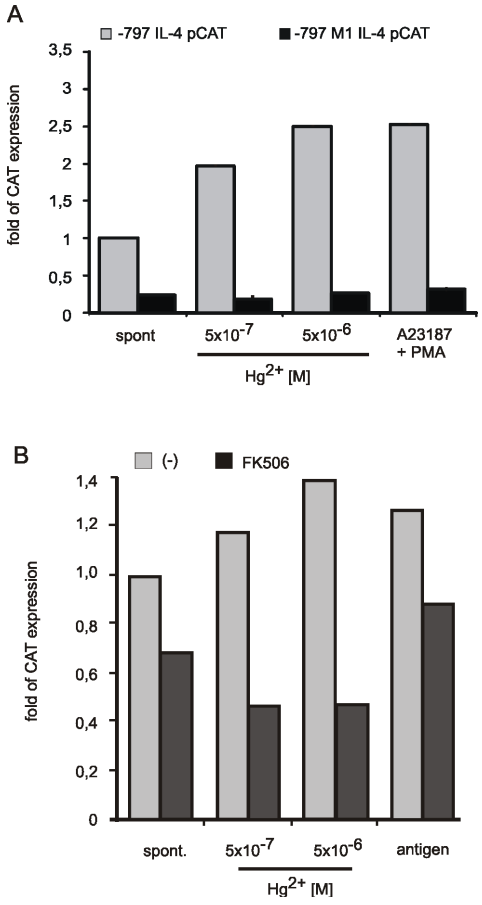

Figure 3 The effect of mercuric ions on IL-4 promoter activity depends on NFAT-binding site and CaN activity. A. C57.1 mast cells were transfected with -797 IL-4 pCAT or -797 M1 IL-4 pCAT , sensitized with IgE and incubated for 4 h with medium only (spont), 10^{-6}M $HgCl_2$ (Hg^{2+}) or combination of 10^{-6}M calcium ionophore and 50 ng/ml phorbol ester (A23187/PMA). B. C 57.1 mast cells were transfected with -87 IL-4 pCAT construct then sensitized with IgE and incubated for 4 h with medium only, $5x10^{-6}$M $HgCl_2$ (Hg^{2+}), or 50 ng/ml DNP-HSA (antigen) in the absence or presence of 1 μM FK506. In each experimental design (A and B) after incubation cells were collected by centrifugation, lysed and concentration of CAT was determined with ELISA. CAT content was normalized for total protein concentrations and is expressed as pg CAT per mg of total protein (A) or as fold increase compared to control (B). Bars represent data from one of two experiments performed in triplicate with similar results.

As seen in Fig. 1 A and B 10^{-9} M $HgCl_2$ significantly increased CAT expression in both BMMC and C57 mast cells. Subsequently we tested the effect of combination of Hg^{2+} ions and antigen on minimal IL-4 promoter activity in mast cells. Low concentrations of $HgCl_2$ (10^{-7} M) increased antigen-mediated activity of minimal IL-4 promoter (-87 to 5 bp) (Fig. 2 A) in mast cells. This effect of Hg^{2+} ions on antigen-mediated IL-4 promoter activity was dose-dependent with maximum enhancement observed for $7,5x10^{-8}$ M (Fig. 2 B). Thus, $HgCl_2$ alone or in combination with antigen/IgE upregulated IL-4 promoter activity in mast cells.

Next, we investigated whether Hg^{2+} -mediated IL-4 promoter activity requires intact NFAT binding site present in position -78 to -70 bp. BMMC were transfected with plasmids -797 IL-4 pCAT (wild type) and -797 M1 IL-4 pCAT, in which mutation in position -71 to –76 bp prevents NFAT binding to promoter. As seen in Fig. 3 A both ionophore 23187 / PMA and $HgCl_2$-induced reporter gene expression only in mast cells transfected with wild type but not with mutated IL-4 promoter DNA construct. Similar data were also obtained for antigen-stimulated BMMC (data not shown). These data suggest that NFAT binding site present in P1 NFAT/AP-1 composite site is required for Hg^{2+} ions-mediated IL-4 promoter activity. Availability of NFAT capable to bind to DNA depends on activity of CaN that dephosphorylates NFAT protein. Therefore we have investigated the effect of CaN inhibitor, FK506, on Hg^{2+} ions-mediated IL-4 promoter activity. Mast cells were transfected with -87 IL-4 pCAT promoter construct, and stimulated with $HgCl_2$ ($5x10^{-6}$ M) or antigen (50 ng/ml DNP HSA) in the absence or presence of 1 μM FK506. As seen in Fig. 3B, FK506 prevented both Hg^{2+} ions and antigen-stimulated increase in reporter gene expression. Thus, $HgCl_2$ upregulated IL-4 promoter activity in mast cells in a process that requires

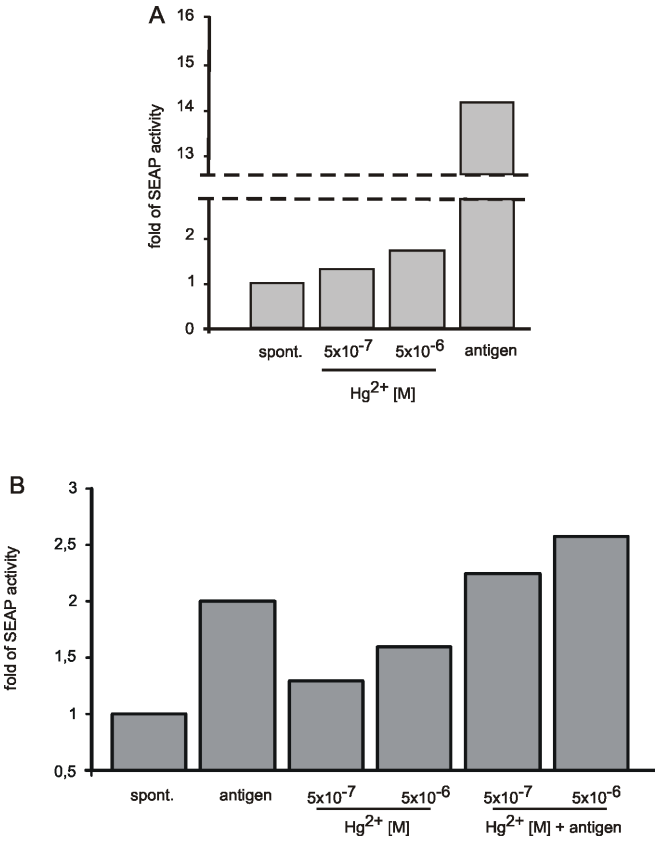

Figure 4 Mercuric ions increase NFAT-dependent SEAP expression in mast cells. (A) BMMC were transfected with pNFAT-SEAP, sensitized with IgE and stimulated with increasing concentrations of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen). (B) C57.1 mast cells were transfected with pNFAT-SEAP, sensitized with IgE and stimulated with increasing concentrations of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen) or combination of both as indicated. Each point represents the mean of 2 independent experiments, each of them performed in duplicate.

NFAT binding motif in IL-4 promoter and CaN activity. We have next decided to verify if Hg^{2+} ions induce CaN-dependent NFAT driven transcription in mast cells employing DNA construct pNFAT-SEAP, in which SEAP expression is controlled with an artificial NFAT-dependent promoter. Mast cells were transfected with pNFAT-SEAP promoter construct and stimulated with $HgCl_2$ ($5x10^{-6}$ M) or antigen (50 ng/ml DNP HSA) in the absence or presence of CaN inhibitors (1 μM FK506 or 1 μg/ml CsA). As seen in Fig. 4 A, incubation of BMMC cells in the presence of $HgCl_2$ resulted in an increased level of NFAT-dependent expression of reporter gene and this effect of $HgCl_2$ was dose-dependent. Similar results were obtained for C57.1 mast cells stimulated with $HgCl_2$ alone or in combination with antigen (Fig. 4 B). Therefore we next investigated the effect of CaN inhibitors on Hg-stimulated SEAP expression in mast cells. CaN inhibitors CsA and FK506 effectively blocked expression of SEAP in mast cells stimulated with $HgCl_2$, antigen and combination of both stimuli (Fig. 5A). To further verify if Hg^{2+}-induced transcription requires CaN activity, dominant negative variant of CaN A subunit was expressed in mast cells cotransfected with pNFAT-SEAP. Mast cells were cotransfected with pNFAT-SEAP and pDN-CLN-GFP or pEGFP-N1 (control) and stimulated with $HgCl_2$ antigen, and combination of both stimuli. As seen in Fig. 5 B expression of dominant negative variant of CaN resulted in abrogation of NFAT-dependent expression of SEAP in mast cells stimulated with $HgCl_2$, antigen, and combination of both stimuli. Thus, both pharmacological inhibition of CaN and expression of dominant negative mutant of this enzyme inhibited Hg^{2+} ions induced NFAT-dependent expression of reporter gene in mast cells.

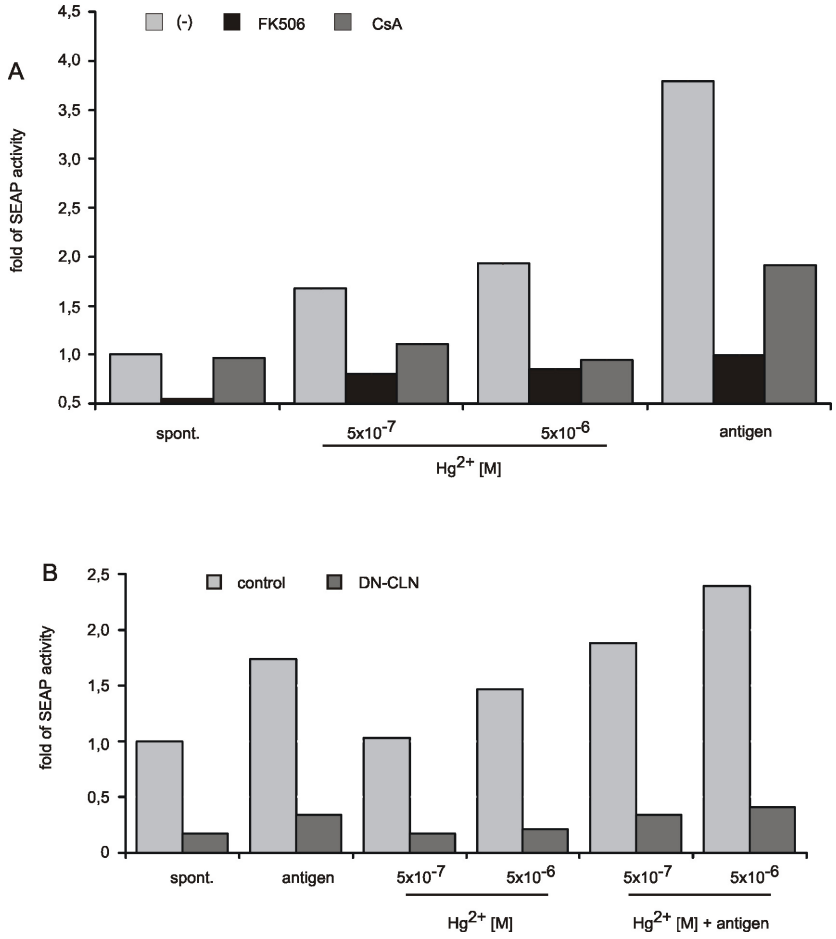

Figure 5 NFAT-driven SEAP transcription induced by mercuric ions in mast cells requires CaN activity (A) C57.1 mast cells were transfected with pNFAT-SEAP, sensitized with IgE and stimulated with increasing concentrations of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen) in the absence or presence of 1 μM FK506 or 1 μg/ml CsA. Supernatants were collected by centrifugation and SEAP activity concentration was determined with chemiluminescence-based assay. (B) C57.1 mast cells were cotransfected with pNFAT-SEAP and pDN-CLN-GFP (DN-CLN) or pEGFP-N2 (Control), sensitized with IgE and stimulated with indicated concentration of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen) or combination of both stimuli. Supernatants were collected by centrifugation and SEAP activity concentration was determined with chemiluminescence-based assay. Each point in A and B represents the mean of 2 independent experiments, each of them performed in triplicate.

It is known that Ni^{2+} and Mn^{2+} are able to increase CaN activity in vitro (37). Therefore we have next tested direct effect of Hg^{2+} on enzymatic activity of CaN in vitro. As seen in Fig. 6 addition of 10^{-8} M Hg^{2+} to the assay buffer has significantly increased activity of human rCaN. In contrast, higher concentrations of Hg^{2+} (10^{-5}M) completely inhibited this enzymatic activity. These effects of Hg^{2+} on the amount of released phosphate were observed at the presence of $5x10^{-4}$M Ca^{2+} and were absent in control reactions where either calmodulin or CaN was absent. We have next repeated this experiment using bovine CaN purified from brain rather than human recombinant enzyme and observed similar effect of Hg^{2+} on enzymatic activity (data not shown). Thus, low concentrations of Hg^{2+} increased enzymatic activity of CaN in vitro.

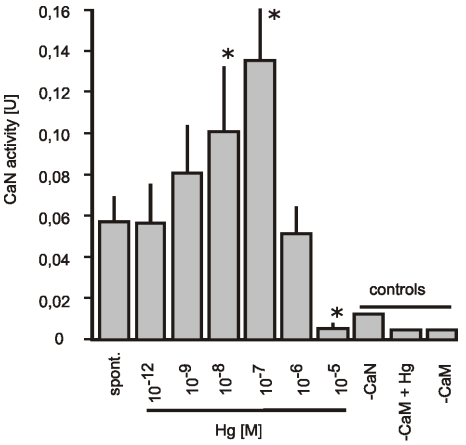

Figure 6 The effect of mercuric ions on CaN activity in vitro. Activity of human rCaN was measured in reaction performed for 10 min at 37 C° in the presence of 2.5 μM calmodulin, 500 μM CaCl2, and in the absence (spont.) or presence of indicated concentration of $HgCl_2$. The amount of PO43- was determined with malachite green., 1 Unit is defined as the enzymatic activity that releases 1 nmol PO43- per 1 min. Controls represent reaction performed without CaN (-CaN), without calmodulin (-CaM), and without calmodulin in the presence of 10-8M $HgCl_2$ (-CaM+Hg). Data represents results of 7 independent experiments, each of them performed in triplicates. Each bar represent means ± SEM. * - Statistically significant difference at P < 0.05.

Discussion

IL-4 expression is regulated at the level of transcription (14-16), and the sequence -87bp to +1 in IL-4 promoter, containing NFAT/AP1 composite binding site, is sufficient to induce transcription in antigen activated mast cells (26). We observed in two different types of murine mast cells that this DNA sequence mediated upregulation of transcription following incubation with Hg^{2+} ions (Fig.1 A and B). This observation is consistent with Hg^{2+}-mediated activation of IL-4 promoter sequences –726 and +26 observed in RBL-2H3 rat mast cell line (11). The effect of Hg^{2+} on -87bp to +1 IL-4 promoter activity was observed at concentration that mediates upregulation of IL-4 expression in mast cells (9) (8). Hg^{2+} ions have also mediated enhancement of antigen-driven IL-4 promoter activity in mast cells stimulated with optimal dose of antigen (Fig. 2 A and B) that might explain previously observed effect of Hg^{2+} ions on antigen-mediated IL-4 secretion (8). These observations are consistent with the hypothesis that Hg^{2+} increase IL-4 promoter activity resulting in de novo transcription and translation of IL-4 in mast cells.

Binding NFAT to IL-4 promoter is necessary for IL-4 expression in mast cells (26) and in T cells (29). NFAT/AP1 composite binding site present within the region –88 bp to –60 bp of IL-4 promoter has been shown to be critical for inducible IL-4 promoter activity in mast cells (26) and in T lymphocytes (14) activated with antigen and PMA/ ionomycine. Promoter construct with mutation within this NFAT binding sequence did not mediate transcription in mast cells activated with antigen or Hg^{2+} ions (Fig. 3 A) suggesting similar mechanisms of promoter activation. NFAT is regulated by CaN that controls shuttling of NFAT to nucleus (17, 18). We observed that CaN inhibitor FK506 suppressed Hg^{2+} ions-mediated and antigen-mediated IL-4 promoter activity in mast cells (Fig. 3 B). FK506 was earlier reported to inhibit IL-4 expression in activated mast cells (23, 30). This inhibitor has been also reported to inhibit activity of IL-2 promoter that, similarly to IL-4 promoter, contains NFAT/AP1 composite binding site critical for its activity (31). These facts and our results suggest that upregulation of IL-4 expression in mast cells exposed to Hg^{2+} is mediated by CaN/ NFAT signal transduction pathway. In agreement with this hypothesis exposure of

BMMC and C57.1 mast cells to Hg^{2+} ions alone or in combination with antigen resulted in induction of NFAT-dependent gene expression from pNFAT-SEAP reporter plasmid (Fig. 4A and B) that was effectively blocked with CaN inhibitors FK506 and CsA (Fig. 5A). Hypothesis that Hg^{2+} activate NFAT in mast cells and this process depends on CaN activity is further supported by the effect of co-expression of dominant negative variant of CaN that abrogated Hg^{2+}-mediated NFAT-dependent expression of reported gene (Fig.5B).

Other metal ions, namely nickel and vanadium, have previously been reported to activate CaN/NFAT signaling pathway in fibroblasts, possibly by generation of reactive oxygen species (32, 33). The exposure of mast cells to Hg^{2+} ions also results in generation of reactive oxygen species (34) and mercuric ions-mediated activation of NFAT driven transcription could involve reactive oxygen species. However, Hg^{2+} enter the cell and accumulate in different cellular compartments (35), where they could directly interact with CaN. In support of such hypothesis we observed that Hg^{2+} (Fig. 6) significantly increased CaN activity in vitro at concentrations lower than those needed to activate NFAT-dependent transcription in vivo (Fig.1 A and B). As far as we know, it is the first observation that Hg^{2+} ions are able to interfere with CaN activity. The difference in Hg^{2+} concentrations activating NFAT-driven transcription in vivo and CaN activity in vitro could be explained by the fact that only a fraction of internalized Hg^{2+} is present in cytoplasmic compartment (36) where it interacts not only with CaN but also with other proteins. Other divalent metal ions (Ni^{2+} and Mn^{2+}) are known to increase CaN activity in vitro but the mechanism of their action is not well understood (37). Activity of CaN depends on Fe^{2+} and Zn^{2+} present in the bimetal catalytic center (38, 39) and it is conceivable that other metal ions, including Hg^{2+}, could also interact with this domain of CaN molecule. However, it is not clear if purified CaN retains natural cofactors Fe^{2+} and Zn^{2+} in metal catalytic center (37). or is partially depleted of these ions (39). Thus it could not be ruled out that Hg^{2+} substitute for depleted metal ions. We have, however, observed similar effects (235 % compared to 221 % increase at 10^{-7} M Hg^{2+}, Fig 6 and data not shown) of Hg^{2+} with CaN preparations of different origin (recombinant human and purified bovine) and, more importantly, of different specific activities (1490 nmol PO43-/min/mg and 27.6 nmol PO43-/min/mg) that is consistent with hypothesis that Hg^{2+} are able to increase activity of CaN under optimal conditions.

In conclusion, our data support hypothesis that Hg^{2+} ions directly increase activity of CaN that in turn upregulates NFAT that binds to specific DNA motif present in IL-4 promoter resulting in IL-4 expression in mast cells. This molecular mechanism, possibly in cooperation with other signaling pathways (10-12), may be involved in initiation of Th2 immune response observed in humans (40) and experimental animals exposed to mercuric compounds (3, 5-7) partly explaining immunotoxic activities of mercuric compounds.

Acknowledgements

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Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), FCS, HEPES, L-glutamine, 2-ME, penicillin/streptomycin, BSA, dinitrophenyl-conjugated human serum albumin (DNP-HSA), PMA, leupeptin, aprotinin, TLCK, TPCK, and PMSF (Sigma Chemical Company, St. Louis, MO, USA), A23187 (Calbiochem, La Jolla, CA, USA), FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, pNFAT-SEAP and pEGFP-N1 (Clontech), human rCaN (specific activity 1492 nmol/min/mg), bovine CaN purified from brain (specific activity 27.6 nmol/min/mg), and BIOMOL GREEN Calcineurin Assay Kit (Biomol Research Laboratories, Inc. Plymouth Meeting, PA, USA), analytical grade $HgCl_2$ (POCH, Gliwice, Poland), were purchased from indicated manufacturers. Reporter gene constructs -87 IL-4 pCAT, -797 IL-4 pCAT, and -797 M1 IL-4 pCAT were obtained from Dr M. Brown, Emory University School of Medicine Atlanta, Georgia, USA. Expression vector pDN-CLN-GFP coding dominant negative variant of was obtained from Dr B. Kaminska, Nencki Institute of Experimental Biology, Warsaw, Poland. This plasmid codes for a mutant of subunit A of CaN with deleted catalytic domain fused to EGFP and its expression results in inhibition of CaN activity and NFAT-dependent transcription (Kaminska personal communication). Murine monoclonal anti-dinitrophenyl (DNP) – specific IgE was obtained from the culture of hybridoma Hi-DNP-ε-26.82 (24).

Culture and transfection of mast cells

Murine mast cells C57.1 (25) were cultured in DMEM supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 25mM HEPES, and 100 μg/ml penicillin/streptomycin (complete DMEM). Murine Bone Marrow Derived Mast Cells (BMMC) were obtained in 4-6 week culture of cells from bone marrow of female BALB/c mice (6 to 9 weeks old) in RPMI 1640 supplemented with 20% WEHI-3 conditioned medium as a source of IL-3, 10% FCS, 4 mM L-glutamine, 25mM HEPES, and 100 μg/ml penicillin/streptomycin (complete RPMI). Cells were cultured at 370C in a CO2 incubator. For transfection, mast cells were collected by centrifugation and 107 cells were suspended in 0.4 ml complete DMEM. Cell suspension was mixed with 50 μg plasmid DNA in a total volume of 0,4 ml and electroporated at 1000 μF and 0,25 kV using Bio-Rad Gene Pulser II. Following electroporation cells were transferred to culture flask, cell density adjusted to $2.5x10^5$ per ml and cultured for 16-18 h in a CO_2 incubator.

Sensitization and stimulation of mast cells

Transfected mast cells were collected by centrifugation, and suspended in complete DMEM or complete RPMI supplemented with 20% anti-DNP IgE containing supernatant to final density 10^6 cells/ml. Cells were incubated for 1,5 hour followed

by three washings with medium. Sensitized mast cells were suspended in media at density of 10^6 cells/ml and incubated in medium alone, in the presence of indicated concentrations of $HgCl_2$, antigen, or combination of both. In some experiments CsA or FK506 dissolved appropriately in DMSO or EtOH, or those solvents alone, were added at indicated concentrations to mast cell suspension 30 min before stimulation with $HgCl_2$ or antigen. Following 4 hours incubation mast cells and supernatants were collected by centrifugation and frozen for further analysis.

Gene reporter activity assays

Reporter gene constructs -87 IL-4 pCAT, and -797 IL-4 pCAT contain respectively sequences -87 to +5, and -797 to +5 of murine il-4 gene inserted upstream of open reading frame of chloramphenicol transferase (CAT) (14, 26) -797 M1 IL-4 pCAT differs from -797 IL-4 pCAT by mutation of sequence AATTTT in position -76 to -71 into sequence CTGCAG (26). Plasmid pNFAT-SEAP contains three tandem repeats of NFAT consensus sequence fused to a TATA like promoter region from Herpes simplex virus thymidine kinase promoter. To measure the expression of chloramphenicol acetylotranspherase (CAT) reporter gene mast cells were lysed in the presence of proteases inhibitors (aprotinin, leupeptin, TPCK, TLCK, PMSF) and CAT content was determined using ELISA (Roche) and normalized to total protein content measured with BCA (Pierce) assay. To measure the expression of secreted alkaline phosphatase (SEAP) reporter gene, enzymatic activity of SEAP released into supernatant was determined by chemiluminescence based assay (Great EscAPe SEAP Kit, Clontech) and luminometry.

Calcineurin activity assay

Activity of CaN was measured using BIOMOL GREEN Calcineurin Assay Kit, according to manufacturer's protocol. Briefly, recombinant human CaN and bovine calmodulin dissolved in assay buffer containing 100mM Tris, pH 7.5, 200mM NaCl, 12 mM MgCl2, 1mM DTT, 0.05% NP-40, and 1mMCaCl2 were mixed in selected wells of microtiter plate. Increasing concentrations of $HgCl_2$ dissolved in dH_2O or dH_2O were added to selected wells and the plate was incubated for 10 min. Next, 0.15 mM RII phosphopeptide (27) as a CaN substrate was added and plate was incubated for additional 10 min. Determination of released phosphate concentration was performed using malachite green (28). In some experiments human rCaN was replaced with bovine CaN purified from brain.

Statistical analysis

One way repeated measures ANOVA followed by Bonferroni t-test were used to determine statistical significance of observed differences.

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