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Repression of T-Cell Function by Thionamides Is Mediated by Inhibition of the Activator Protein-1/Nuclear Factor of Activated T-Cells Pathway and Is Associated with a Common Structure

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ABSTRACT

Treatment of hyperthyroidism by thionamides is associated with immunomodulatory effects, but the mechanism of thionamide-induced immunosuppression is unclear. Here we show that thionamides directly inhibit interleukin-2 cytokine expression, proliferation, and the activation (CD69 expression) of primary human T lymphocytes. Inhibition of immune function was associated with a repression of DNA binding of the cooperatively acting immunoregulatory transcription factors activator protein 1 (AP-1) and nuclear factor of activated T-cells (NFAT). Likewise, thionamides block the GTPase p21Ras, the mitogen-activated protein kinases, and impair the calcineurin/calmodulin-dependent NFAT dephosphorylation and nuclear translocation. The potency of inhibition correlated with the chemical reactivity of the thionamide-associated sulfur group. Taken together, our data demonstrate that thio-derivates with a common heterocyclic thioureylene-structure mediate a direct suppression of immune functions in T-cells via inhibition of the AP-1/NFAT pathway. Our observations may also explain the clinical and pathological resolution of some secondary, calcineurin, and mitogen-activated protein kinase-associated diseases upon thionamide treatment in hyperthyroid patients. This offers a new therapeutic basis for the development and application of heterocyclic thio-derivates.

Thyroid dysfunction is a widespread disease commonly caused by autoimmune chronic lymphocytic thyroiditis and is characterized by increased levels of serum thyroid-stimulating hormone (Hollowell et al., 2002). Antithyroid thionamide treatment inhibits thyroid hormone synthesis (Orgiazzi and Millot, 1994), but its use is associated with agranulocytosis, aplastic anemia, granulocytopenia, and general immune suppression by lactoperoxidase inhibition, diminished antigen presentation, reduced release of proinflammatory mediators, T-cell abnormalities, and decreased IL-2 receptor expression (Volpé, 2001; Bandyopadhyay et al., 2002; Pearce, 2004). In addition, hyperthyroid patients with active myocardial damage or cardiomyopathy described a complete remission upon antithyroid treatment with thionamides (Martí et al., 1995; Hardiman et al., 1997; Khandwala, 2004).

Calcineurin and MAP kinases have both been implicated in the regulation of immune functions, cardiotoxicity, and cardiohypertrophy (Rao et al., 1997; Molkentin, 2000; Rincón et al., 2000). Calcineurin is a serine/threonine-specific phosphatase that is activated by sustained elevations in intracellular calcium (Rao et al., 1997). Activation of calcineurin is associated with cell death and with the induction of the nuclear factor of activated T-cell (NFAT) transcription factor family that is involved in immune regulation or cardiac hypertrophy (Rao et al., 1997; Molkentin, 2000). MAP kinases (MAPKs)

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ABBREVIATIONS: IL, interleukin; AP-1, activator protein 1; CaMKII, calmodulin kinase II; CD, cluster of differentiation; JNK, c-Jun NH_2-terminal kinase; LAT, linker for activation of T-cells; MAP, mitogen activated protein; MAPK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T-cells; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; LDH, lactate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay; RBD, Ras binding domain; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

are composed of the stress-activated protein kinases p38 and c-Jun N-terminal kinases (JNKs), whereas the extracellular signal-regulated kinases (ERKs) respond to mitogenic stimuli (Whitmarsh and Davis, 1996). Signaling is initiated at the plasma membrane in conjunction with the small G proteins and couples with the phosphorylation of diverse effector proteins and the activation of the transcription factor activator protein 1 (AP-1) (Pastor et al., 1995; Whitmarsh and Davis, 1996). NFAT-calcineurin signaling is tightly regulated in coordination with the MAPKs (Rao et al., 1997; Molkentin, 2000; Macián et al., 2001).

Animal experiments and several clinical trials demonstrate that thiourea, its aliphatic derivates, and other heterocyclic thioureylenes such as thiobarbiturates and drugs of the sulfonylurea class notably possess antithyroid activity (Farwell and Braverman, 1996). It is interesting that these heterocyclic thio-derivates are structurally related to the thionamides and inhibit innate and adaptive immune responses. Direct effects on the activation, proliferation, and differentiation of granulocytes, macrophages, and T-lymphocytes have been described previously (Correa-Sales et al., 1997; Nishina et al., 1998; Salman et al., 1998). We proposed a dual mechanism of immune suppression by theses agents associated with impaired GTPase function and inhibition of calcineurin phosphatase activity (Humar et al., 2004a,b).

Because the cellular functions of these agents are common, we asked whether thionamides might impair immune cells by a common structure-dependent mechanism. Here we demonstrate that thionamides show similarities in affecting cellular signaling compared with other heterocyclic thio-derivates (Humar et al., 2004a,b). In general, our work leads to the understanding of how thioureylenes, embedded within a mononuclear heterocycle, exert immunomodulatory effects. These compounds affect ubiquitously expressed proteins that are involved in numerous aspects of cellular responses, and thus, their use might result in substantial side effects. We suggest a general evaluation of the immunosuppressive potential of heterocyclic thiol-derived pharmaceuticals before clinical use. On the other hand, new therapeutic applications of clinically approved pharmaceuticals or the development of new agents on a heterocyclic thioureylene basis as key building blocks in medicinal chemistry may evolve.

Materials and Methods

Isolation and Treatment of Human Primary T Lymphocytes. T-lymphocytes from whole blood were enriched by immunomagnetic cell sorting with anti-CD3 microbeads according to the recommendations of the manufacturer (Miltenyi Biotech, Auburn, CA). Cells were suspended in RPMI 1640, supplemented with 10 mM HEPES, pH 7.3, 50 μM β-mercaptoethanol, and 2 mM L-glutamine, and pretreated with methimazole (Sigma, St. Louis, MO), propylthiouracil (Sigma), and carbimazole (LKT Laboratories, St. Paul, MN) as indicated. Activation was induced by cross-linking of the CD3/CD28 receptors using T-cell Expander Dynabeads (Dynal Biotech, Lake Success, NY) or 1 μg/ml ionomycin (Sigma).

IL-2 and CD69 Expression. IL-2 secretion of T cells was analyzed by the human IL-2 Quantikine Immunoassay (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cell suspension (200 μl; 2×10^6 cells) were incubated with or without thionamides 2 h before activation of the cells by cross-linking of the CD3/CD28 receptor (10^6 T-cell Expander Dynabeads). After 15 h, supernatants were diluted 1:5 in the Quantikine Immunoassay assay diluent before determination of the IL-2 concentration.

T-cell activation was measured by fluorescence-associated cell sorting using the Fastimmune Assay System (BD Biosciences, San Jose, CA). Aliquots (50 μl) of heparinized whole blood were incubated with thionamides for 2 h as indicated before cells were activated by 10^6 T-cell Expander Dynabeads. After 15 h, CD3-CD69-CD4 Fastimmune assay reagent was added to each reaction for 45 min. Erythrocytes were lysed in 450 μl of FACS lysing solution (BD Biosciences). Thereafter, residual cells were fixed in 500 μl of cell fix solution (BD Biosciences). The CD3-CD69-CD4-associated fluorescence was measured by a fluorescence-activated cell sorter (FACSCalibur; BD Biosciences).

T-Cell Proliferation and LDH-Release Assay. T-cell growth was determined by a colorimetric XTT-cellular proliferation assay according to the description of the manufacturer (Roche Applied Science, Indianapolis, IN). Primary human T lymphocytes (5×10^5) were incubated with various concentrations of thionamides. After 2 h, cells were stimulated by 2.5×10^5 T-cell Expander Dynabeads for 15 h. Thereafter, the XTT-labeling mixture was added for 2 h. Formazan dye formation was determined at $OD_{490\ nm}$ by a spectrophotometer (Spectramax PLUS; Molecular Devices, Sunnyvale, CA). Data were expressed as percentage of induction by absorbance compared with activated cells (100%).

Cytotoxicity of thionamides was determined by the Cytotoxicity Detection Kit (Roche Applied Science) according to the instructions of the manufacturer. T-cells (2×10^6) were incubated with or without thionamides for 17 h. Cells were centrifuged and LDH enzyme activity was measured in cell culture supernatants. Data are displayed as LDH release in a percentage compared with total LDH release after cellular lysis by sonication (100%).

AP-1- and NFAT-Dependent Luciferase Reporter Gene Expression. Jurkat cells (2×10^5) were transiently transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer using 2 μg of pAP1-TA-Luc (Clontech, Mountain View, CA). Fifteen hours before analysis of reporter gene expression, transfection reactions were pooled, equally redistributed at 10^5 cells into individual wells, and incubated with thionamides and 1 μg/ml ionomycin plus 15 ng/ml PMA. Alternatively, the stably transfected Jurkat cell line C4-NFAT was used as described previously (Humar et al., 2004b). Luciferase reporter gene expression was measured in harvested cells that were lysed in 100 μl of luciferase reporter lysis buffer (Promega, Madison, WI) using a Microlumat Plus LB 96P luminometer (Berthold Technologies, Bad Wildbad, Germany). Protein levels were normalized by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Nuclear Protein Extraction and Electrophoretic Mobility Shift Assays. Preparation of nuclear cell extracts and electrophoretic mobility shift assays were performed as described previously (Humar et al., 2004a,b). Used oligonucleotide sequences were 5′-CGCTTGATGAGTCAGCCGGAA-3′ for AP-1 and 5′-TTTCTCATGGAAAGATGACATA-3′ for NFAT. Binding reactions were carried out at room temperature for 30 min in a volume of 20 μl containing 5 μg of nuclear cell extract, 22 mM HEPES, pH 7.9, 70 mM KCl, 50 μM EDTA, 2.2 mM dithiothreitol, 2% glycerol, 4% Ficoll, 0.1% Nonidet P-40, 30 μM phenylmethylsulfonyl fluoride, 20 μg of bovine serum albumin, 2 μg of poly(dI-dC), and 1.75 pmol of ^{32}P-end-labeled oligonucleotides (5 μCi/pmol). The resulting DNA complexes were displayed by electrophoresis on 4% nondenaturing polyacrylamide gels and autoradiography.

ELISA-Based AP-1 Transcription Factor Activity Assay. Nuclear extracts (2 μg per assay) were analyzed by the TransAM AP-1 family transcription factor activation assay according to the recommendations of the manufacturer (Active Motif, Carlsbad, CA). AP-1 heterodimeric complexes were captured by binding to a consensus 5′-TGAGTCA-3′ oligonucleotide, immobilized on a 96-well plate. Bound c-Jun and c-Fos were quantified by coincubation of specific antibodies and spectrophotometric acquisition at optical density of 450 nm using a horseradish peroxidase conjugate. Data were ex-

pressed as a percentage of induction by absorbance compared with activated cells (100%).

Western Blotting. Primary human $CD3^+$ T lymphocytes were preincubated with various concentrations (0.1–5 mM) of thionamides for 2 h. Cell stimulation was induced by either CD3 or CD28 receptor stimulation using 0.5 T-cell Expander Dynabeads per cell (Dynal Biotech) or 1 μg/ml ionomycin at the indicated time points. Reactions were terminated by the addition of SDS sample buffer and boiling. Immunoblots of total cellular extracts were analyzed by antibodies specific for p38, p38(Thr180/Tyr182), p42/44, p42/44(Thr202/Tyr204), LAT, LAT(Tyr171), LAT(Tyr191), Raf(Ser259), calmodulin kinase II (CamKII), or CaMKII(Thr286) (New England Biolabs, Ipswich, MA) and enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Antibodies specific for calcineurin and NFATc2 (clone 4G6-G5) were from BD Biosciences, antibodies for c-Jun(Ser73), pan-phosphotyrosine (clone 4G10), calmodulin, and Ras (clone RAS10) were from Upstate (Lake Placid, NY), and antibodies for Raf-1 were from Calbiochem (San Diego, CA). Antibodies directed against the TATA binding protein TBP18 were obtained from Abcam (Cambridge, MA). Antibody dilutions and Western blot protocols were used according to the recommendations of the manufacturer.

Kinase Activity Assay. JNK activity was determined by the JNK activity immunoassay kit (Calbiochem). For immunoprecipitation, 200 μg of cell lysate was incubated with 2 μl of a JNK-specific rabbit polyclonal antibody at 4°C and rotated for 5 h, then for 10 h after the addition of 50 μl of protein A Sepharose slurry. Kinase reactions were performed at 30°C for 30 min in 50 μl of kinase assay buffer (25 mM Tris, pH 7.5, 5 mM $C_3H_7O_6PNa_2$, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 μM Na_3VO_4, and 200 μM ATP) containing 2 μg of recombinant c-Jun protein as a substrate. Reactions were terminated by the addition of SDS sample buffer and boiling.

Analysis of G-Proteins. Ras-Raf binding was analyzed by the Ras activation assay kit (Biomol, Plymouth Meeting, PA). T-cells (2×10^7) were harvested and lysed in 300 μl of ice-cold extraction buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1% Igepal CA-630, and 2% glycerol). Where indicated, 100 μM GTPγS was added at 30°C for 30 min. Reactions were terminated by 60 mM Mg_2Cl. After centrifugation at 14,000*g*, cell lysate supernatants were incubated with 10 μg of Raf-1 RBD agarose or 20 μg of H-Ras V12-GST Sepharose (Cytoskeleton, Denver, CO) on a wheel at 4°C for 30 min. Protein beads were washed three times with 1 ml of extraction buffer, then resuspended and boiled in 40 μl of 2× SDS sample buffer.

Statistical Analysis. Data are shown as the median ± S.E.M. Statistical analysis was performed using a one-way analysis of variance followed by a Holm Sidak post hoc test. *P* values less than 0.05 were considered significant.

Results

Thionamides Inhibited IL-2 Synthesis, T-Cell Proliferation, and Expression of CD69. Effects of thioureylenes on functional responses of T-lymphocytes were analyzed. Active thioureylene pharmaceuticals in clinical use were investigated, including the thionamides carbimazole, methimazole, and propylthiouracil (Fig. 1).

A functional antigenic T-cell response is mediated by an autocrine, IL-2-dependent mitotic pathway, which results in the clonal expansion of specific T-cells (Nelson, 2002). To analyze whether thionamides interfere with this mitotic pathway, IL-2 synthesis of T-cells was determined (Fig. 2A). T-cell receptor stimulation by CD3/CD28 antibody beads resulted in a marked IL-2 release into T-lymphocyte cell culture supernatants. Carbimazole and propylthiouracil significantly inhibited CD3/CD28-induced IL-2 synthesis. In contrast, methimazole showed no effect on cytokine production.

Direct effects of thionamides on the proliferation of T lymphocytes were quantified by an XTT cellular proliferation assay (Fig. 2B). In the absence of thionamides, the conversion of XTT-labeling reagent to formazan was 20 to 40% compared with CD3/CD28-stimulated T-cells (positive control; 100%). Incubation of T-cells with carbimazole or propylthiouracil repressed CD3/CD28-induced cellular growth, as demonstrated by a reduced formation of the formazan dye. In contrast, methimazole did not significantly affect the conversion of XTT to formazan. Microscopic analysis revealed that CD3/CD28-dependent clonal expansion of T lymphocytes was suppressed at 500 μM carbimazole or 2 mM propylthiouracil (data not shown). An LDH-release assay indicated no thionamide-mediated cytotoxicity (Fig. 2C).

Next, CD69 expression of T-cells was analyzed (Fig. 3). In whole blood, $CD3^+$ cells were gated and analyzed for CD69 expression of a subpopulation containing $CD3^+CD4^+$ T-helper cells and a $CD3^+CD4^-$ subpopulation that contained ≥93% $CD8^+$ cytotoxic T-cells. CD3/CD28 receptor costimulation resulted in the up-regulation of the activation marker CD69 (Fig. 3C), demonstrating that approximately 30.9% of $CD3^+$ T-cells were activated. Carbimazole and propylthiouracil strongly inhibited the cell surface expression of CD69, implicating that T-cell activation is impaired (Fig. 3, D and E). In contrast, treatment with methimazole had no effect (Fig. 3F). Data displayed were obtained at concentrations of 5 mM carbimazole and propylthiouracil, which completely inhibited CD69 expression. However, a difference in CD69 cell surface density primarily affecting the population of highly CD69 expressing T-cells was already observed at 100 μM (carbimazole) or 500 μM (propylthiouracil) (data not shown). In summary, both carbimazole and propylthiouracil repress mediators and markers of essential T-cell immune functions.

Thionamides Inhibited the Cooperatively Acting Transcription Factors AP-1 and NFAT. The IL-2 gene is

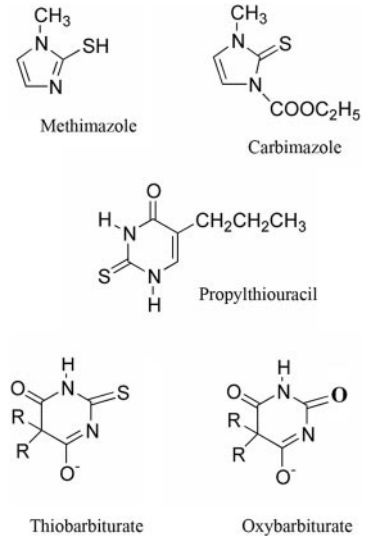

Fig. 1. Structure of heterocyclic thioureylene derivates in clinical use. Heterocyclic thio-derivates containing a thionamide group include the antithyroid drugs methimazole, carbimazole, and propylthiouracil. Thiobarbiturates with a pyrimidine nucleus are used for anesthesia and treatment of intracranial hypertension. Oxybarbiturates are structural analogs of thiobarbiturates with an oxygen substituent.

cooperatively regulated by the transcription factors AP-1 and NFAT (Macián et al., 2001). CD69 expression is also induced via AP-1, and sensitivity to cyclosporin A treatment implicates participation of NFAT (Castellanos et al., 1997, 2002). To evaluate whether thionamides specifically repress AP-1 and NFAT-dependent gene expression, luciferase reporter genes under control of AP-1 or NFAT were transfected into Jurkat T-cells (Fig. 4). Upon PMA and ionomycin treatment, transfected Jurkat cells demonstrated an AP-1- or NFAT-controlled luciferase reporter gene expression that was dose-dependently suppressed when cells were coincubated with propylthiouracil or carbimazole (Fig. 4). Partial and complete inhibition were observed at 2 and 4 mM propylthiouracil. Carbimazole totally blocked luciferase reporter gene expression at 500 μM. In contrast, methimazole treatment resulted in no significant change of luciferase reporter gene expression (Fig. 4).

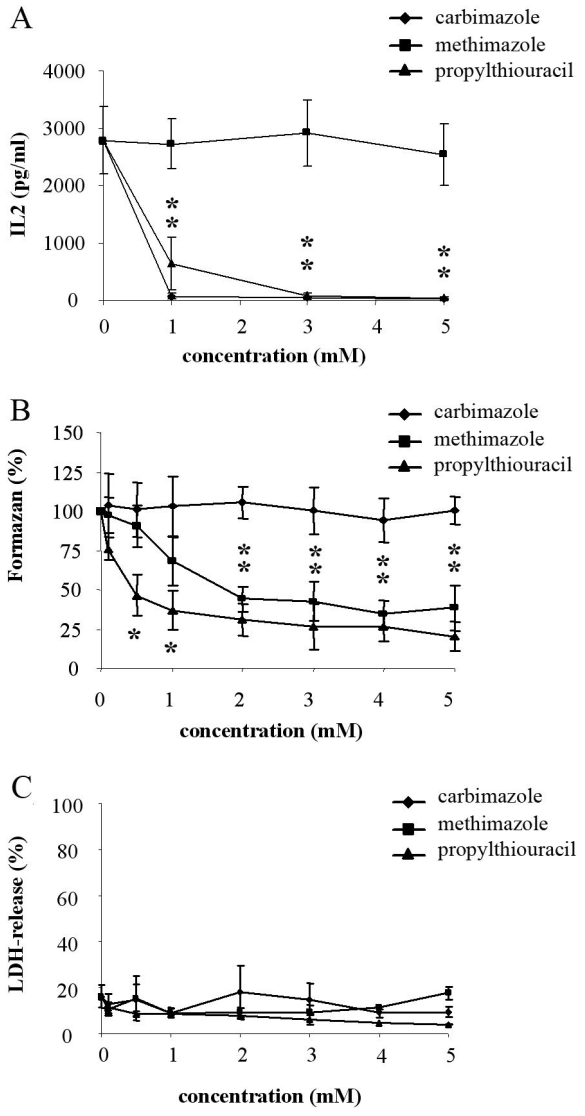

Fig. 2. Propylthiouracil and carbimazole inhibit IL-2 synthesis and proliferation of $CD3^+$ T lymphocytes. The effect of thionamides on CD3/CD28 receptor-induced interleukin-2 production in T-cells isolated from the blood of healthy donors is shown in A. T-cells were pretreated with 0.1 to 5 mM carbimazole, methimazole, or propylthiouracil for 2 h and subsequently stimulated with 10^6 T-cell Expander Dynabeads for 15 h. The cell culture supernatant was collected and analyzed for the concentration of interleukin-2 by enzyme-linked immunosorbent assay. In B, the effect of thionamides on CD3/CD28 receptor-induced T-cell proliferation is shown. T-cells (5×10^5) were pretreated with thionamides at the indicated concentrations and stimulated with 2.5×10^5 T-cell Expander Dynabeads for 15 h. XTT-labeling reagent was added for 2 h before the formation of the formazan dye was determined. In C, cytotoxicity of thionamides was measured by an LDH-release assay. T-cells were incubated with thionamides for 17 h. Cell culture supernatants were analyzed for LDH enzyme activity. *, $P < 0.001$ compared with CD3/CD28 stimulation alone. Data represent the median ± S.E.M. of six experiments.

To further analyze how thionamides interact with these transcription factors, protein/DNA binding studies were performed (Fig. 5). AP-1 is composed of a mixture of heterodimeric complexes of proteins, including c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2 (Foletta, 1996). Because only Jun proteins form transcriptionally active dimers with other AP-1 family members, and c-Jun/c-Jun homodimers or c-Jun/c-Fos heterodimers belong to the most common dimers found in AP-1 signaling pathways, DNA binding of c-Jun and c-Fos was analyzed by an ELISA-based transcription factor activity assay (Fig. 5A). c-Jun or c-Fos displayed minor DNA binding to a (5′TGACGTCA3′) DNA consensus sequence in the noninduced state. However, the DNA-binding activity could be induced four to five times upon CD3/CD28 T-cell receptor stimulation. Propylthiouracil and carbimazole dose-dependently inhibited DNA-binding of c-Jun or c-Fos to their correspondent DNA consensus sequence. In contrast, the presence of methimazole did not significantly affect DNA binding. Similar results were obtained when nuclear extracts of CD3/CD28-activated T-cells were tested by electrophoretic mobility shift assay (Fig. 5 B).

Protein-DNA binding studies were also used to analyze the association of NFAT with its oligonucleotide binding site. However, an inducible DNA binding of NFATc1 could not be demonstrated, although NFATc1 has been shown to be expressed at high levels in peripheral lymphoid tissue and T lymphocytes (Rao et al., 1997). Samples displayed high background and low induction when analyzing T-cell nuclear extracts (data not shown).

Thionamides Impaired MAP Kinase Signaling. The reason for reduced AP-1/DNA-binding was analyzed. The induction of the MAP kinases ERK1/2, p38, and JNK is a critical event in transcriptional activation of AP-1 family members and their transactivational activity (Whitmarsh and Davis, 1996). Likewise, the activity of these MAP kinases is determined by their own phosphorylation status. Upon CD3/CD28 T-cell receptor activation (Fig. 6A) or protein kinase C (PKC) and calcium mobilization (Fig. 6B), ERK1/2 and p38 were readily phosphorylated. JNK1/2, however, could not be detected by Western blot in total cellular lysates (data not shown), because it is expressed at low levels in naive T-cells. For this reason, the c-Jun NH_2-terminal kinase activity of immunoprecipitated JNK1/2 was determined in vitro using recombinant c-Jun protein as a substrate. The thionamides propylthiouracil and carbimazole inhibited the phosphorylation of p38 and ERK1/2 in a dose-dependent manner (Fig. 6A). Likewise, phosphorylation of c-Jun was also inhibited using JNK immunoprecipitates from propylthiouracil or carbimazole-treated cells (Fig. 6A). Methimazole had no influence on ERK1/2 and p38 phosphorylation or JNK kinase activity (Fig. 6A). PKC and calcium-induced pathways were not affected by propylthiouracil, because propylthiouracil had no effect on MAP kinase phosphorylation and only a minor impact on c-Jun NH_2-terminal kinase ac-

tivity after administration of phorbol ester (PMA) and ionomycin (Fig. 6B). In contrast, PKC and calcium mobilization are clearly involved in carbimazole-mediated effects, arguing for a distinct cellular mechanism that evidently depends on the chemical structure of the monocyclic nucleus (Fig. 6B).

Repression of MAP Kinases Was Due to Inhibition of the Small G-Protein p21Ras. The reason for reduced MAP kinase activity was analyzed. As a central molecular switch, p21Ras couples the T-cell receptor to an amplificatory kinase cascade involving the serine/threonine kinase Raf-1 and the MAP kinase pathway (Pastor et al., 1995). Thus, p21Ras controls a signaling system that stimulates the transcription factor AP-1 but also synergizes with the calcium/calcineurin-dependent NFAT activation (Pastor et al., 1995). Upon CD3/CD28 T-cell receptor stimulation, inactive RasGDP was converted to active RasGTP as demonstrated by its ability to complex with its binding partner Raf-1 (Fig. 7A). The presence of methimazole had no effect

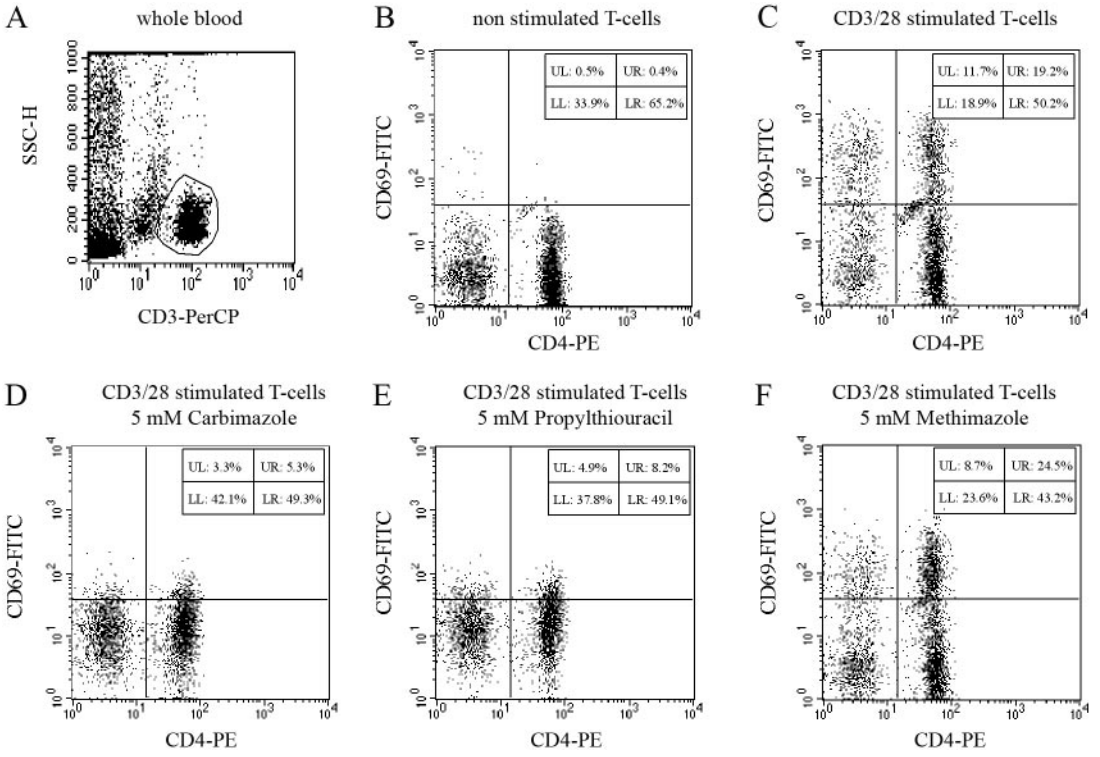

Fig. 3. Propylthiouracil and carbimazole inhibit cell surface expression of the T-cell activation marker CD69. The effect of thionamides on the CD3/CD28 receptor-induced CD69 expression was analyzed by FACS in whole blood. Blood samples were pretreated with 5 mM carbimazole, methimazole, or propylthiouracil 2 h before stimulation with 10^6 T-cell Expander Dynabeads for 15 h. Cells were analyzed by FACS using anti-CD3-CD69-CD4 immune complexes. $CD3^+$ T-cells were gated by CD3-PerCP staining and morphology (A) and subsequently analyzed for CD4/CD69 expression (B–F). Representatives of three independent experiments.

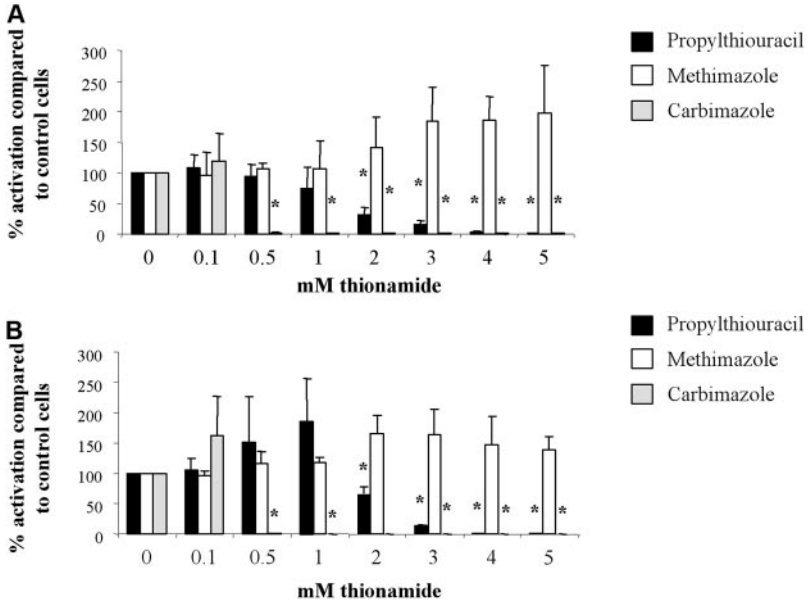

Fig. 4. Propylthiouracil and carbimazole inhibit AP-1 and NFAT-dependent gene expression. Luciferase reporter gene expression is shown. To analyze AP-1-dependent reporter gene expression, Jurkat cells were transfected with 2 μg of pAP-1-TA-Luc (A). To analyze NFAT-dependent reporter gene expression, the stably transfected Jurket cell clone C4-NFAT was used (B). Cells were incubated with 0 to 5 mM propylthiouracil, carbimazole, or methimazole and 15 ng/ml PMA plus 1 μg/ml ionomycin for 15 h to induce transcription factors. Lysates were analyzed for luciferase reporter gene activity, and the results were normalized to protein levels. Results are displayed as a percentage of relative light units compared with stimulated, transfected cells in the absence of thionamides. Statistics represent the mean ± S.E.M. of four independent experiments. *, $P < 0.001$ versus positive controls (stimulation in the absence of thionamides).

on the Ras/Raf-1 interaction. However, propylthiouracil and carbimazole repressed the ability of Ras to bind the serine/threonine kinase Raf-1.

The small G-protein p21Ras is activated by a cascade of multiple T-cell receptor-associated proteins whose function is tightly regulated by tyrosine phosphorylation (Cantrell, 1996). The tyrosine phosphorylation of LAT serves as a docking site for binding of the Grb2/SOS complex, translocating it to the plasma membrane where it catalyzes the Ras GTP/GDP exchange (Wange, 2000). However, antiphosphotyrosine Western blots displayed no marked changes in tyrosine phosphorylation upon thionamide treatment, as shown in total cell lysates (Fig. 7B). Likewise, the tyrosine phosphorylation of LAT (Fig. 7C) and Akt-dependent serine 209 phosphorylation of Raf-1 (data not shown) remained unchanged in the presence of thionamides. Thus, regulatory protein phosphorylations at the T-cell synapse do not participate in the repression of ras/Raf-1 interaction as depicted in Fig. 7A.

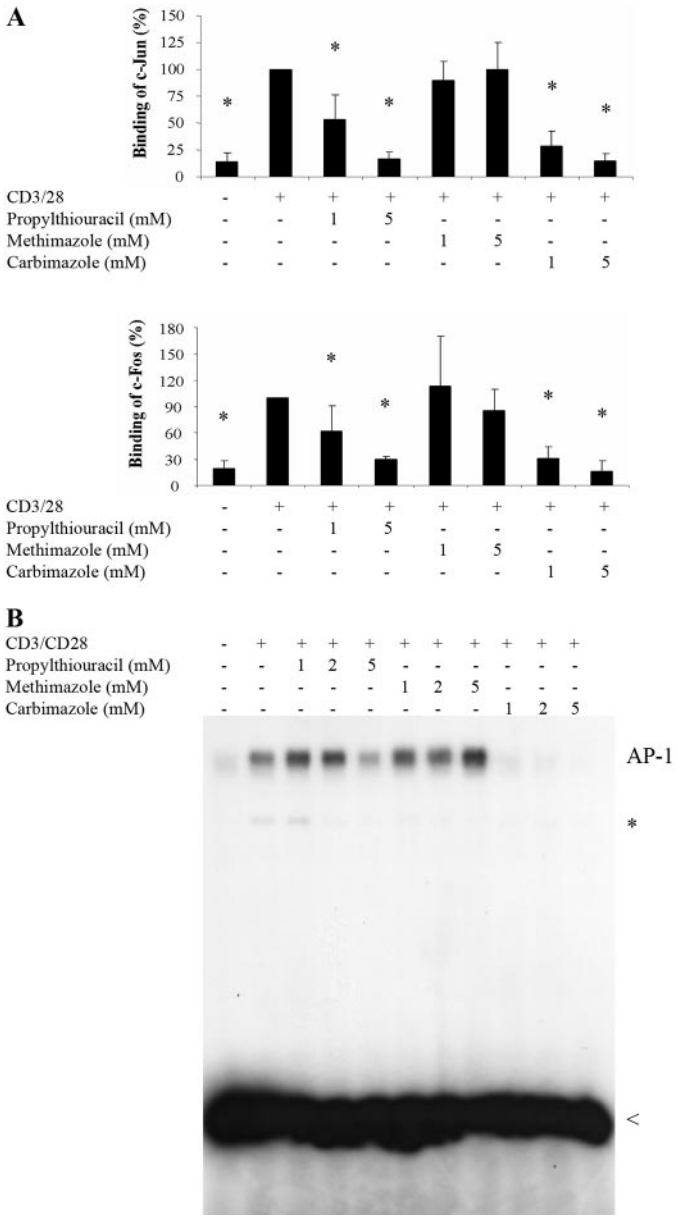

Fig. 5. Propylthiouracil and carbimazole inhibit DNA binding of AP-1. DNA interaction of AP-1 was analyzed by an ELISA-based transcription factor activity assay (A). $CD3^+$ T-cells were treated for 2 h with thionamides at the indicated concentrations and subsequently stimulated by CD3/CD28 T-cell receptor cross-linking for 1 h (10^7 T-cell Expander Dynabeads per assay). Nuclear extracts were prepared, and 2 μg of nuclear proteins were analyzed for DNA binding of c-Jun or c-Fos using a plate-immobilized oligonucleotide that contains a 5′-TGAGTCA-3′ AP-1 binding site. Bound c-Jun (A, top graph) or c-Fos (A, bottom graph) were recognized by specific antibodies and secondary horseradish peroxidase immune complexes for colorimetric readout. Spectrophotometric data were expressed as a percentage of induction by absorbance compared with activated cells (100%). Error bars indicate S.E.M. of six independent experiments. *, $P < 0.001$ versus positive controls was considered as significant. Binding of total AP-1 complexes was analyzed by electrophoretic mobility shift assay (B). Nuclear extracts from peripheral human T lymphocytes were used. Top blot, AP-1/DNA complex and nonspecific binding activity of the probe (*). Bottom blot. unbound oligonucleotide (<). Representative data of four independent experiments.

On the other hand, thioureylenes might directly prevent Ras/Raf-1 binding by interacting with the cysteine-rich binding domain of Raf-1. However, the constitutively active H-ras (G12V), which has no intrinsic GTPase activity, actively bound Raf-1 even when cell lysates of propylthiouracil- or carbimazole-treated lymphocytes were used to pull down Raf-1 (Fig. 7D, top blots). Thus, the thionamide-mediated inhibition of p21Ras is not mediated by a direct interference of Ras/Raf-1 binding. However, the nucleotide-related heterocyclic structure of thionamides suggests a direct interaction with the guanosine nucleotide exchange. Indeed, GTPγS-activated cell lysates showed a repression of Ras/Raf-1 binding upon carbimazole treatment (Fig. 7D, bottom blots). Activating p21Ras in cell lysates with GTPγS abolishes the necessity of regulatory events associated with the T-cell receptor and its associated proteins. Thus, p21Ras must directly be repressed by carbimazole. In contrast, propylthiouracil showed no effect on Ras/Raf-1 binding upon GTPγS stimulation, arguing for a different mechanism of Ras regulation.

Carbimazole Inhibited NFAT Dephosphorylation by Interfering with Calmodulin. The proximal mechanism for inhibition of NFAT was analyzed. Reagents containing reactive sulfur might lead to inactivation of calcineurin/calmodulin (King, 1986; Humar et al., 2004b), a protein complex that regulates NFAT dephosphorylation, its nuclear translocation, and the subsequent transcriptional activation. Therefore, the effect of thionamides on the calcineurin/calmodulin complex was analyzed (Fig. 8). Protein levels of calcineurin A, calcineurin B, and calmodulin were left unchanged upon calcium mobilization and coincubation with various thionamides (Fig. 8A). Because CD3/CD28 receptor stimulation activated only a small fraction of cells (10–30%) and thus induced only a partial dephosphorylation of NFATc2 (data not shown), calcium mobilization was induced by ionomycin. This resulted in a complete dephosphorylation of NFATc2 (Fig. 8A). Dephosphorylation of NFATc2 could be blocked by cyclosporin A and FK506, implicating a calcineurin-dependent mechanism (data not shown). We were surprised to find that only carbimazole significantly inhibited NFATc2 dephosphorylation as shown in whole-cell lysates, whereas propylthiouracil inhibited NFAT-dependent reporter gene expression (Fig. 4) but not the dephosphorylation of NFAT (Fig. 8A). NFAT dephosphorylation results in unmasking its nuclear localization sequence and its subsequent nuclear shuttling (Rao et al., 1997). Therefore, nuclear extracts of ionomycin-treated T-lymphocytes were analyzed for the presence of NFAT (Fig. 8B). NFATc2 nuclear trans-

location was comparable with its phosphorylation status: carbimazole treatment prevented nuclear entry of NFAT, whereas methimazole or propylthiouracil prevented neither dephosphorylation (Fig. 8A) nor the nuclear translocation (Fig. 8B). In parallel, we observed that carbimazole inhibited the autophosphorylation of CaMKII in an NFAT dephosphorylation-like pattern (Fig. 8A). Reduced levels of phospho-CaMKII (Thr286) originated from reduced intrinsic kinase activity but not from a decrease of whole protein.

As a consequence of reduced NFAT dephosphorylation and nuclear translocation, carbimazole inhibited the association of NFAT with the corresponding DNA binding site (Fig. 8C). The presence of propylthiouracil also prevented NFAT/DNA binding, however independent of NFAT dephosphorylation or nuclear shuttling of the transcription factor. This observation argues for different pharmacological mechanisms of carbimazole and propylthiouracil.

Discussion

Our results demonstrate that heterocyclic thio-derivates with a mononuclear structure inhibit essential immune functions of T-cells by blocking immunorelevant transcription factors and its regulated genes. The mechanism of immunosuppression is dependent on the chemical structure of the reagents. Thioureylenes with a pyrimidine-like nucleus, such as propylthiouracil and the thiobarbiturates, show high resemblance in dose-response and mechanistic pattern (Humar et al., 2004a) and inhibit the function of MAP kinases and its proximal regulatory protein p21Ras.

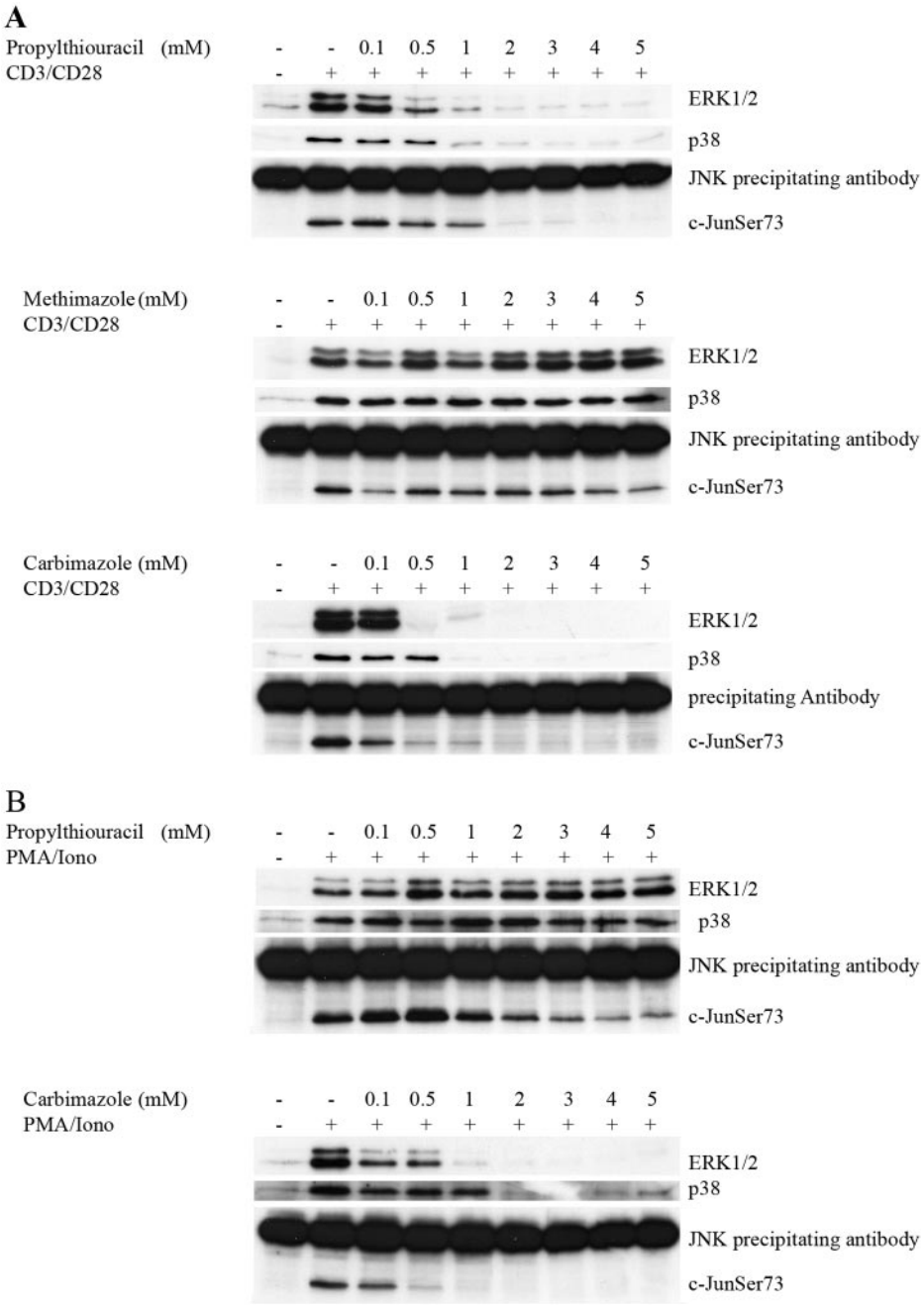

Fig. 6. Propylthiouracil and carbimazole inhibit the phosphorylation of p42/44 ERK, p38 MAP kinases, and JNK kinase activity. Western blot experiments are shown. T-cells were either left untreated (lane 1) or preincubated with 0.1 to 5 mM thionamide for 2 h (lanes 3–9). p42/44 ERK or p38 MAP kinase phosphorylation was induced by 10^6 CD3/CD28 T-cell Expander Dynabeads (A) or 15 ng/ml PMA plus 1 μg/ml ionomycin (B) for the last 10 min of the experiment (lanes 2–9). JNK kinase activity was determined in vitro by an immune complex kinase assay using 2 μg of recombinant c-Jun as substrate. The positions of phospho-ERK1/2, phospho-p38, precipitating JNK antibody, and phosphorylated c-jun substrate are indicated. Representatives of three independent experiments are shown.

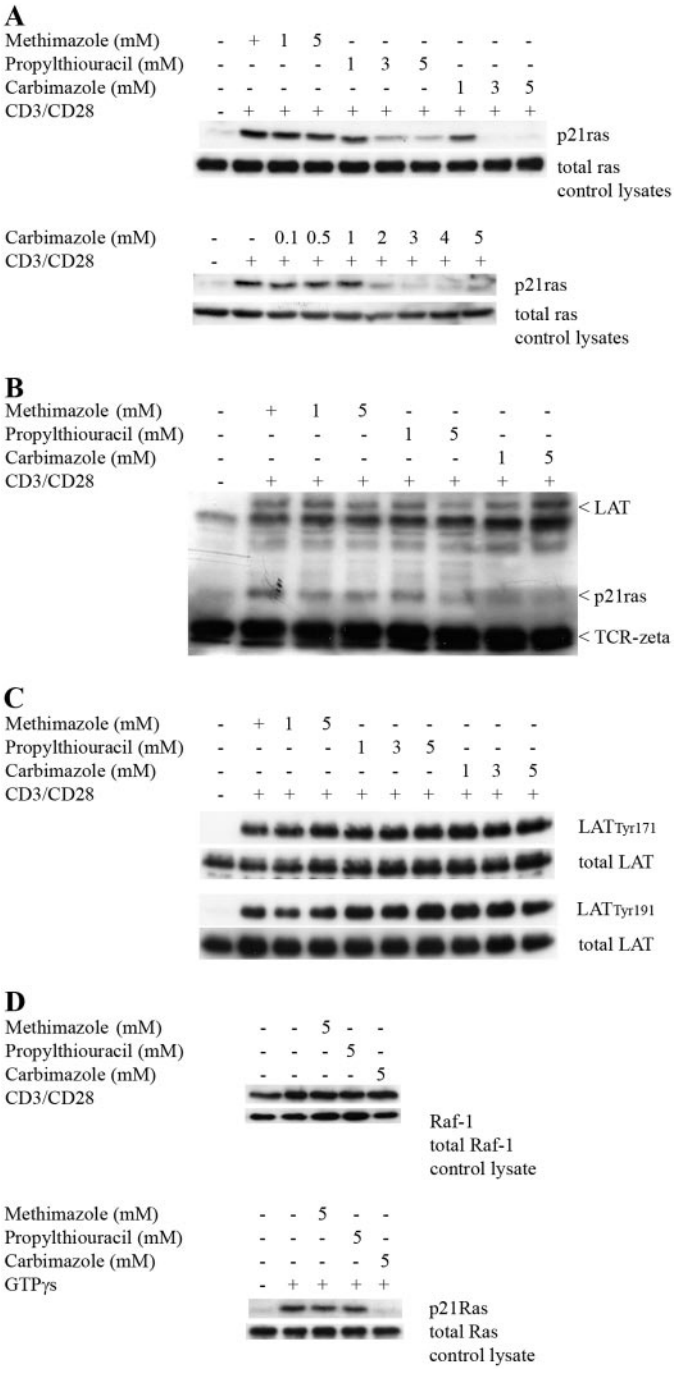

Fig. 7. Propylthiouracil and carbimazole inhibit ras activation. Western blot experiments are shown. Peripheral human T lymphocytes (2×10^7) were pretreated with thionamides for 2 h and stimulated with 0.5 CD3/CD28 T cell Expander Dynabeads per cell for 10 min. On the other hand, p21ras was activated by 100 μM GTPγS at 30°C for 30 min. within cellular lysates. A, effect of thionamides on Ras activity was determined by a Raf-1 pull-down assay. Active Ras was pulled down from whole-cell lysates with Raf-1 RBD fusion proteins. Precipitated proteins (top) show the active fraction of the GTPase, whereas the lower panel shows the total amount of p21ras in cell lysates (control lysates). B, protein phosphorylation was detected by an anti-phosphotyrosine (4G10) horseradish peroxidase-conjugated antibody using whole-cell lysates. Then, tyrosine phosphorylation of LAT was analyzed by protein-specific antibodies (C). Equal loading was demonstrated by detection of total LAT, independent of the phosphorylation status. In D, Raf-1 protein was pulled down by H-ras(V12) glutathione Sepharose beads from cellular lysates (top). Finally, p21Ras was pulled down by Raf-1 RBD fusion proteins using GTPγS-activated cell lysates (bottom). Representative blots of three independent experiments are shown.

Carbimazole, a sulfur-containing imidazole derivate, represses calcineurin-dependent dephosphorylation of NFAT, its nuclear translocation, and autophosphorylation of CamKII. These events require functional calmodulin (Rao et al., 1997; Hudmon and Schulman, 2002), a ubiquitous Ca^{2+}-mediating signal-transducing molecule, which suggests that inhibition by carbimazole is mediated by a disabled calmodulin. Indeed, the use of ionomycin that forms transmembrane pores and leads to direct cytoplasmic calcium entry excludes calcium flux-regulating mechanisms in carbimazole-mediated NFAT suppression.

Carbimazole also inhibits p21Ras. In contrast to the pyri-

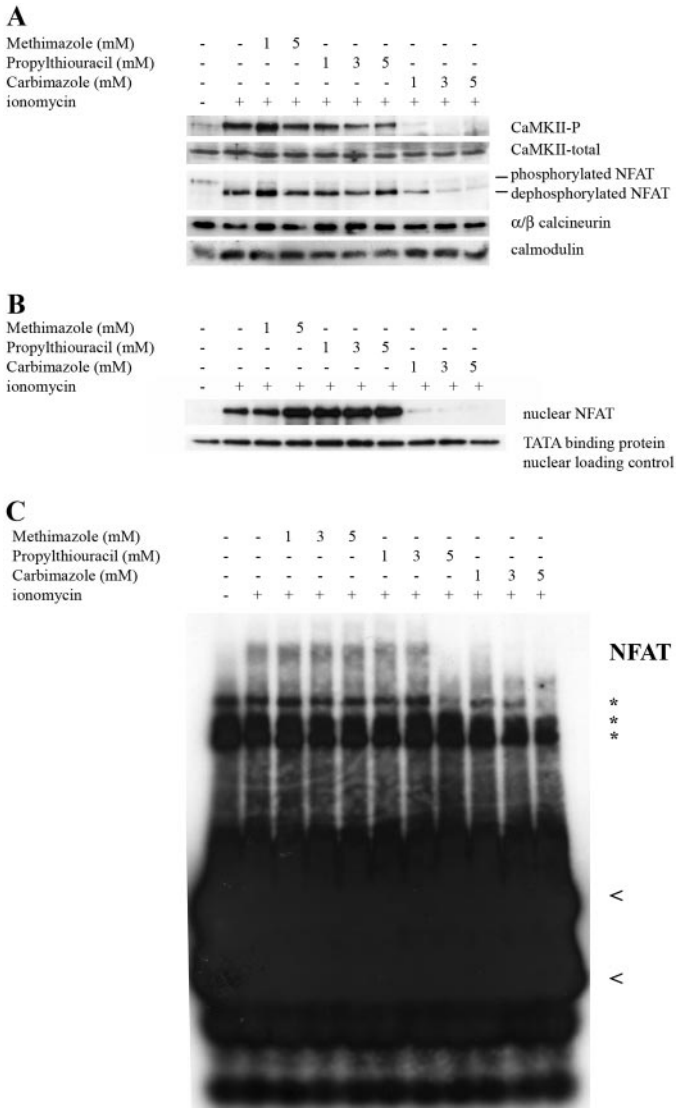

Fig. 8. Carbimazole inhibits CaMKII autophosphorylation, NFAT dephosphorylation, and interaction of NFAT with its DNA consensus sequence. A, Western blots are shown. Cells were pretreated with 1 to 5 mM thionamides for 2 h and stimulated with 1 μg/ml ionomycin for the last 10 min. Total lysates were prepared and analyzed for CaMKII autophosphorylation or NFAT dephosphorylation by specific antibodies. Detection of total CaMKII, α/β calcineurin, or calmodulin demonstrates equal protein amounts in each extract. B, nuclear protein extracts of T-lymphocytes were analyzed to detect nuclear NFAT and the presence of the TATA binding protein TBP18 as a nuclear loading control. C, a representative electrophoretic mobility shift assay is shown. Nuclear extracts from peripheral human T lymphocytes were used. Cells were pretreated with 0 to 5 mM thionamides for 2 h and 1 μg/ml ionomycin for the last 1 h of the experiment. Top blot, NFAT-DNA complex and nonspecific binding activity of the probe (*). Bottom blot, nonbound oligonucleotide (<).

midine-containing thioureylenes, inhibition by carbimazole is direct because p21Ras activity was also blocked after GTPγS stimulation of cellular lysates. This experimental setting excludes both the proximal activation cascade and active enzymatic post-translational modifications as possible drug targets. In fact, we demonstrate unchanged protein tyrosine phosphorylation of the p21Ras-activating cascade. Furthermore, the Ras-associated GTPase activating proteins that directly regulate the GTP/GDP exchange of Ras (Wittinghofer et al., 1997) are not affected by carbimazole, as determined by GTPase activating protein assays in vitro (data not shown).

Bioreactive sulfur is central for the inhibitory potential of carbimazole, propylthiouracil, and the thiobarbiturates, as demonstrated by comparison with methimazole or the oxyanalogs of the barbiturates. Thiobarbiturates inhibit immunorelevant transcription factors, but their oxyanalogs show only marginal effects on their activation (Loop et al., 2002; Humar et al., 2004b). Likewise, carbimazole and methimazole demonstrate opposite effects depending on the biochemical activity of the associated sulfur group. Methimazole, because of its stable aromatic structure, displays no immunoinhibitory function. In contrast, carbimazole is not an aromatic compound but includes redox-reactive sulfur. Carbohydrate side chains (ethylcarbamate) and components of the heterocycle (thiourea, malonic acid, imidazole) have no immunoregulatory function and do not significantly alter NFAT/AP-1 reporter gene expression (M. Humar and H. Dohrmann, unpublished observations).

Several publications confirm that molecular targets of the thioureylene-mediated immunosuppression are susceptible to interactions with reactive sulfur (King, 1986; Tan et al., 1996; Heo et al., 2005). The structural analysis of calmodulin suggests that cysteines participate in the formation of an EF-hand motif and thus regulate the Ca^{2+}-induced conformational transition of calmodulin. Their inactivation leads to a decrease in affinity for Ca^{2+} and a loss of ability to activate target enzymes, phosphodiesterases, and calcineurin (King, 1986). In addition, it has been described that sulfhydryl reagents inactivate calcineurin, and at least one cysteine residue in the catalytic subunit of calcineurin seems to be important for establishing the activated form of calcineurin (Tan et al., 1996). On the other hand, Ras proteins possess an NKCD motif that forms interactions with the guanine nucleotide base and contains a redox-active cysteine. Cysteine 118 in conjunction with phenylalanine 28 mediates the redox-dependent GDP dissociation (Heo et al., 2005). The sulfur side chain of carbimazole might directly interact with these redox-active cysteines and thus block the activity of the calcineurin/calmodulin complex or p21Ras.

After administration of phorbol ester (PMA) and ionomycin, MAP kinase phosphorylation or c-Jun NH_2-terminal kinase activity is largely unchanged in the presence of propylthiouracil, whereas AP-1 is still repressed. This observation suggests that AP-1 might be regulated by a dominant additional but unknown mechanism independent of PKC, calcium, and the induction of MAP kinases. Observations using thiopental, a barbiturate that also contains a pyrimidine nucleus, support this hypothesis. Similar to propylthiouracil, thiopental inhibits AP-1 without affecting its activation of the MAP kinase cascade upon PKC and calcium mobilization (Humar et al., 2004a). Further experiments demonstrated a reduced c-Fos mRNA expression upon PMA/ionomycin activation and thiopental treatment, whereas transcriptional activation of c-Jun was normal (M. Humar, unpublished data).

Deregulation of essential switching points in cellular signaling often leads to disease (Molkentin, 2000; Wilkins and Molkentin, 2004; Friday and Adjei, 2005). Profound alterations in intracellular calcium handling have been shown to be the leading cause of cardiac hypertrophy and progressive heart failure and involve activation of calmodulin, calcineurin, and NFAT-dependent gene transcription (Molkentin, 2000; Wilkins and Molkentin, 2004). It is noteworthy that hyperthyroid patients with active myocardial damage or cardiomyopathy described a complete remission upon antithyroid treatment with thionamides (Martí et al., 1995; Hardiman et al., 1997; Khandwala, 2004). These observations may now be explained by the thionamide-mediated inhibition of calcineurin, as described by us. Indeed, the use of pharmacological calmodulin/calcineurin-inhibitors has led to beneficial effects (Wilkins and Molkentin, 2004). New pharmacological agents, designed on the structural basis of mononuclear thioureylenes, might enlarge the pool of these agents.

Likewise, small G proteins are constitutively activated in many tumors (Friday and Adjei, 2005). Clinical studies inhibiting the tumor-promoting farnesylation and geranylgeranylation of Ras are promising (Sebti and Hamilton, 2000; Friday and Adjei, 2005). Active thioureylenes might block p21Ras by interfering with cysteine thiols within the protein that are relevant for farnesylation and geranylgeranylation. In addition, carbimazole-like pharmaceuticals might directly inhibit p21Ras function and thus block its transforming activity.

The maximal concentration of thionamides used in our study was 50- to 500-fold higher than the amount a patient may obtain daily (Werner et al., 1989). However, it must be taken into account that patients obtain long-term antithyroid treatment and 10-fold higher doses of thionamides at the onset of therapy (Passath et al., 1987). For related agents, such treatment has been associated with drug accumulation in immunologically relevant tissue (Turcant et al., 1985). It is now known that the turnover of thionamides is slow in tissue compared with a short plasma half-life (Jansson et al., 1983). Comparing ex vivo and in vivo enzyme activities is challenging, but clinical observations such as thionamide-mediated immunosuppression or remission of cardiac disease support our results because they both depend on the examined pathways (Martí et al., 1995; Hardiman et al., 1997; Volpé, 2001; Bandyopadhyay et al., 2002).

For a valid evaluation of heterocyclic thioureylenes in calmodulin/calcineurin or Ras-associated diseases, more case observations and clinical studies are necessary. We suggest the analysis of hyperthyroid patients with secondary symptoms associated with calcium deregulation, malignant Ras transformation, or inappropriate MAPK signaling and ongoing therapy with thionamides. Upon treatment, differences in the clinical manifestation of secondary disease-related symptoms might be related to our observations. In addition, animal models designed for the experimental treatment of heart disease or tumor therapy might prove beneficial effect of heterocyclic thioureylenes.

In summary, our observations uncover immunomodula-

tory thioureylene-mediated molecular mechanisms. Because these compounds affect ubiquitously expressed proteins that are involved in numerous aspects of cellular responses, extensive consequences of treatment might be expected. On the other hand, further characterization and modification of heterocyclic thio-derivates might lead to new therapeutic applications.

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References

Bandyopadhyay U, Biswas K, and Banerjee RK (2002) Extrathyroidal actions of antithyroid thionamides. *Toxicol Lett* **128:**117–127.
Cantrell D (1996) T cell antigen receptor signal transduction pathways. *Annu Rev Immunol* **14:**259–274.
Castellanos MC, Lopez-Giral S, Lopez-Cabrera M, and de Landazuri MO (2002) Multiple Cis-acting elements regulate the expression of the early T cell activation antigen CD69. *Eur J Immunol* **32:**3108–3117.
Castellanos MC, Munoz C, Montoya MC, Lara-Pezzi E, Lopez-Cabrera M, and de Landazuri MO (1997) Expression of the leukocyte early activation antigen CD69 is regulated by the transcription factor AP-1. *J Immunol* **159:**5463–5473.
Correa-Sales C, Tosta CE, and Rizzo LV (1997) The effects of anesthesia with thiopental on T lymphocyte responses to antigen and mitogens in vivo and in vitro. *Int J Immunopharmacol* **19:**117–128.
Farwell AP and Braverman LE (1996) Thyroid and anti-thyroid drugs, in *Goodman and Gilman's the Pharmacological Basis of Therapeutics* (Hardman JG and Limbird LE eds), 9th ed, pp 1383–1409, McGraw-Hill, New York.
Foletta VC (1996) Transcription factor AP-1, and the role of Fra-2. *Immunol Cell Biol* **74:**121–133.
Friday BB and Adjei AA (2005) K-Ras as a target for cancer therapy. *Biochim Biophys Acta* **1756:**127–144.
Hardiman O, Molloy F, Brett F, and Farrell M (1997) Inflammatory myopathy in thyrotoxicosis. *Neurology* **48:**339–341.
Heo J, Prutzman KC, Mocanu V, and Campbell SL (2005) Mechanism of free radical nitric oxide-mediated ras guanine nucleotide dissociation. *J Mol Biol* **346:**1423–1440.
Hollowell JG, Staehling NW, Flanders WD, Hannon WH, Gunter EW, Spencer CA, and Braverman LE (2002) Serum TSH, T_4, and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). *J Clin Endocrinol Metab* **87:**489–499.
Hudmon A and Schulman H (2002) Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J* **364:**593–611.
Humar M, Andriopoulos N, Pischke SE, Loop T, Schmidt R, Hoetzel A, Roesslein M, Pahl HL, Geiger KK, and Pannen BH (2004a) Inhibition of activator protein 1 by barbiturates is mediated by differential effects on mitogen-activated protein kinases and the small G proteins Ras and Rac-1. *J Pharmacol Exp Ther* **311:**1232–1240.
Humar M, Pischke SE, Loop T, Hoetzel A, Schmidt R, Klaas C, Pahl HL, Geiger KK, and Pannen BH (2004b) Barbiturates directly inhibit the calmodulin/calcineurin complex: a novel mechanism of inhibition of nuclear factor of activated T cells. *Mol Pharmacol* **65:**350–361.
Jansson R, Dahlberg PA, Johansson H, and Lindström B (1983) Intrathyroidal concentrations of methimazole in patients with Graves's disease. *J Clin Endocrinol Metab* **57:**129–132.
Khandwala HM (2004) A case of congestive heart failure due to reversible dilated cardiomyopathy caused by hyperthyroidism. *South Med J* **97:**1001–1003.
King MM (1986) Modification of the calmodulin-stimulated phosphatase, calcineurin, by sulfhydryl reagents. *J Biol Chem* **261:**4081–4084.
Loop T, Liu Z, Humar M, Hoetzel A, Benzing A, Pahl HL, Geiger KK, and BH J P (2002) Thiopental inhibits the activation of nuclear factor kappaB. *Anesthesiology* **96:**1202–1213.
Macián F, Lopez-Rodriguez C, and Rao A (2001) Partners in transcription: NFAT and AP-1. *Oncogene* **20:**2476–2489.
Martí V, Ballester M, Obrador D, Moya C, Carrio I, and Pons-Llado G (1995) Active myocardial damage in hyperthyroidism. A concurrent mechanism of heart failure reversed by treatment. *Eur Heart J* **16:**1014–1016.
Molkentin JD (2000) Calcineurin and beyond: cardiac hypertrophic signaling. *Circ Res* **87:**731–738.
Nelson BH (2002) Interleukin-2 signaling and the maintenance of self-tolerance. *Curr Dir Autoimmun* **5:**92–112.
Nishina K, Akamatsu H, Mikawa K, Shiga M, Maekawa N, Obara H, and Niwa Y (1998) The inhibitory effects of thiopental, midazolam, and ketamine on human neutrophil functions. *Anesth Analg* **86:**159–165.
Orgiazzi J and Millot L (1994) Theoretical aspects of the treatment with antithyroid drugs. *Ann Endocrinol (Paris)* **55:**1–5.
Passath A, Leb G, and Warnkross H (1987) Pharmacology and dosage of thyrostatic drugs. *Acta Med Austriaca* **14:**72–74.
Pastor MI, Woodrow M, and Cantrell D (1995) Regulation and function of P21ras in T lymphocytes. *Cancer Surv* **22:**75–83.
Pearce SH (2004) Spontaneous reporting of adverse reactions to carbimazole and propylthiouracil in the UK. *Clin Endocrinol (Oxf)* **61:**589–594.
Rao A, Luo C, and Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* **15:**707–747.
Rincón M, Flavell RA, and Davis RA (2000) The JNK and P38 MAP kinase signaling pathways in T cell-mediated immune responses. *Free Radic Biol Med* **28:**1328–1337.
Salman H, Bergman M, Bessler H, Alexandrova S, Beilin B, and Djaldetti M (1998) Effect of sodium thiopentone anesthesia on the phagocytic activity of rat peritoneal macrophages. *Life Sci* **63:**2221–2226.
Sebti SM and Hamilton AD (2000) Farnesyltransferase and geranylgeranyltransferase I inhibitors and cancer therapy: lessons from mechanism and bench-to-bedside translational studies. *Oncogene* **19:**6584–6593.
Tan RY, Mabuchi Y, and Grabarek Z (1996) Blocking the Ca^{2+}-induced conformational transitions in calmodulin with disulfide bonds. *J Biol Chem* **271:**7479–7483.
Turcant A, Delhumeau A, Premel-Cabic A, Granry JC, Cotineau C, Six P, and Allain P (1985) Thiopental pharmacokinetics under conditions of long-term infusion. *Anesthesiology* **63:**50–54.
Volpé R (2001) The immunomodulatory effects of anti-thyroid drugs are mediated via actions on thyroid cells, affecting thyrocyte-immunocyte signalling: a review. *Curr Pharm Des* **7:**451–460.
Wange RL (2000) LAT, the linker for activation of T cells: a bridge between T cell-specific and general signaling pathways. *Sci STKE* **2000:**RE1.
Werner MC, Romaldini JH, Bromberg N, Werner RS, and Farah CS (1989) Adverse effects related to thionamide drugs and their dose regimen. *Am J Med Sci* **297:**216–219.
Whitmarsh AJ, and Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* **74:**589–607.
Wilkins BJ and Molkentin JD (2004) Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. *Biochem Biophys Res Commun* **322:**1178–1191.
Wittinghofer A, Scheffzek K, and Ahmadian MR (1997) The interaction of Ras with GTPase-activating proteins. *FEBS Lett* **410:**63–67.

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