0026-895X/01/6005-972–980$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:972–980, 2001

Vol. 60, No. 5
982/930955
Printed in U.S.A.

Methimazole As an Antioxidant and Immunomodulator in Thyroid Cells: Mechanisms Involving Interferon-γ Signaling and H_2O_2 Scavenging

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Received March 22, 2001; accepted June 21, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The antithyroid drug, methimazole (MMI) is used to treat patients with Graves' hyperthyroidism. The major action of MMI is to inhibit synthesis of thyroid hormone in the thyroid gland. However, MMI also has antioxidant and immunomodulatory effects on thyrocytes and/or immune cells. This study identifies novel antioxidant and immunomodulatory effects of MMI involving the interferon-γ (IFN-γ) signaling pathway in thyroid cells. MMI inhibits transcription of the intercellular adhesion molecule-1 (ICAM-1) gene by modulating the function of transcription factor STAT1 (signal transducer and activator of transcription 1), which binds to the IFN-γ activated site of the ICAM-1 promoter. Furthermore, MMI rapidly eliminates H_2O_2 produced by IFN-γ treatment in thyroid cells and thus inhibits the H_2O_2-mediated phosphorylation of tyrosine 701 in STAT1. MMI also eliminates H_2O_2 in vitro. MMI facilitates electron transfer from NADPH to H_2O_2 using thioredoxin or glutathione, fulfilling a role similar to peroxiredoxin or glutathione peroxidase, respectively. MMI prevents the IFN-γ and H_2O_2-mediated reversible inactivation of phosphatases. These effects inhibit full activation of the IFN-γ-induced Janus kinase(JAK)/STAT signaling pathway in FRTL-5 thyroid cells. These results may in part explain the antioxidant and immunomodulatory effects of MMI in thyroid cells of Graves' disease patients.

The therapeutic effects of the antithyroid drug, methimazole (MMI) have been ascribed to its ability to decrease thyroid hormone production (Cooper, 1984). Antithyroid drugs (thionamides such as carbimazole and its active metabolite methimazole, and propyl thiouracil) are taken up by the thyroid gland as other anions similar to iodide (perchlorate, thiocyanate, and pertechnetate) (Cooper, 1984). Their target is the thyroid peroxidase. They block the iodination of tyrosine residues and the coupling of iodotyrosines into iodothyronines (Cooper, 1984). However, antithyroid drugs appear to interfere with the immunological abnormalities in Graves' hyperthyroidism: they cure 50% of patients provided they are maintained on drug therapy for at least 12 months, and they significantly decrease the titers of antithyroid antibodies in most patients (Weetman et al., 1984). Immunomodulatory effects of antithyroid drugs seem to be involved in reducing antigen expression (Singer et al., 1994; Volpe, 1994), and scavenging reactive free radicals (Imamura et al., 1986) generated from oxygen and/or iodide during peroxidation. Better knowledge of the antioxidant and immunomodulatory action of antithyroid drugs might help in understanding Graves' hyperthyroidism, especially the thyroid-immune dysfunction involved in its initiation or progression.

Abnormal MHC class I, class II, and intercellular adhesion molecule-1 (ICAM-1) gene expression in thyrocytes is associated with autoimmune thyroid diseases (Weetman and McGregor, 1994). The expression of these genes in thyrocytes may be related to secondary responses to cytokines such as IFN-γ, which are produced by lymphocytes infiltrating the thyroid (Weetman and McGregor, 1994). Abnormal expres-

This work was supported by HMP-98-M-2-0020, Ministry of Health and Welfare, and Biotech 2000 (98-N1-02-04-A-01), Molecular Medicine Research Group Program, National Research Laboratory Program, International Cooperative Research Program, Ministry of Science and Technology, and Research Fund from Korean Association of Internal Medicine, Korea.

H.K. and T.-H.L. contributed equally to this work.

ABBREVIATIONS: MHC, major histocompatibility complex; ICAM-1, intercellular adhesion molecule-1; MMI, methimazole; IFN, interferon; GAS, γ-activated sites; Gpx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; MAP, mitogen-activated protein; Prx, peroxiredoxin; JAK, janus kinase; STAT, signal transducer and activator of transcription; PIAS, protein inhibitors of activated STAT; SHP, SH2-containing phosphatases; SOCS, suppressor of cytokine signaling; TSH, thyroid-stimulating hormone; DCF, dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; PAGE, polyacrylamide gel electrophoresis; PTP, protein tyrosine phosphatase; TR, thioredoxin reductase; Trx, thioredoxin.

sion of MHC class I, class II, and ICAM-1 allow thyrocytes to become antigen-presenting cells and to be involved in amplification of the autoimmune reaction in the thyroid gland (Weetman and McGregor, 1994).

IFN-γ signaling initiates when IFN-γ binds to its receptor, which induces receptor dimerization (Ihle, 1995; Bach et al., 1997; Stark et al., 1998). After receptor dimerization, the receptor-associated Janus family tyrosine kinases, JAK1 and JAK2, transphosphorylate each other, which results in their activation (Bach et al., 1997; Stark et al., 1998). The cytoplasmic domains of the IFN-γ receptor are phosphorylated on tyrosine residues by the JAKs, enabling the recruitment of STAT1 (Darnell, 1997). The activated JAKs phosphorylate Y701 of STAT1, causing the phosphorylated STAT1 to dimerize by reciprocal SH2 phosphotyrosine interaction (Shuai et al., 1993) and enter the nucleus (Shuai et al., 1993), bind to IFN-γ-activated sites (GAS) promoter elements, and activate transcription of IFN-γ-responsive genes (Shuai et al., 1993; Darnell, 1997). STATs have been implicated in the activation of several genes important in inflammatory responses, including FcγRI (Decker et al., 1997), class II transactivator (Dong et al., 1999), interferon regulatory factor-1 (Rein et al., 1994), and ICAM-1 (Chung et al., 2000; Park et al., 2000a,b).

This study focuses on the mechanisms that contribute to the antioxidant and anti-inflammatory effects of MMI. Specifically, the experiments described here examine the ability of MMI to scavenge H_2O_2 as a peroxiredoxin (Prx) or glutathione (GSH) system and the ability of MMI to inhibit specific steps of IFN-γ signaling by inhibiting JAK/STAT activation in thyroid cells.

Experimental Procedures

Materials. Highly purified bovine TSH was purchased from Sigma Chemical Co. (St. Louis, MO). Rat recombinant IFN-γ was obtained from Invitrogen (Gaithersburg, MD). [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from DuPont Merck Pharmaceutical Co. (Wilmington, DE). The source of all other materials was the Sigma Chemical Co. unless otherwise noted.

Cell Culture. FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD) were a fresh subclone (F1) that had all the properties previously detailed (Kohn et al., 1986). Their doubling time with TSH was 36 ± 6 h; without TSH, they did not proliferate. After 6 days in medium with no TSH, addition of 1×10^{-10} M TSH stimulated thymidine incorporation into DNA by at least 10-fold. Cells were diploid and between their 5th and 20th passage. Cells were grown in 6H medium consisting of Coon's modified F12 supplemented with 5% calf serum, 1 mM nonessential amino acids, and a mixture of six hormones: bovine TSH (1×10^{-10} M), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Fresh medium was added to all cells every 2 or 3 days, and cells were passaged every 7 to 10 days. In individual experiments, cells were shifted to 5H medium with no TSH and 5% calf serum.

RNA Isolation and Northern Analysis. Total cellular RNA was isolated by standard procedures and Northern analysis was performed as described (Park et al., 2000). Final washes were carried out at 65°C in 1× SSPE (150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4). The rat ICAM-1 probe was the whole cDNA sequence from this gene, obtained by polymerase chain reaction using the published rat sequence (Park et al., 1999) and cloned in the *Eco*RI site of plasmid pUC19. The recombinant plasmid was sequenced to confirm accuracy and fidelity during construction. Northern blot analysis for PIAS-1 and PIAS-3 was carried out using total RNA, which was transferred to a membrane and hybridized with a probe from the pCMV5-PIAS1 (*Xho*I-*Bgl*II fragment) (Liu et al., 1998) and pCMV5-PIAS3 (*Xho*I-*Bgl*II fragment) (Chung et al., 1997). Hybridization probes for SOCS-1 and SOCS-3 were the purified inserts of the expression vectors pEF-SOCS-1 and pEF-SOCS-3, respectively (Starr et al., 1997). All probes were radiolabeled using a random priming protocol (Amersham Pharmacia Biotech, Arlington Heights, IL).

Construction of Promoter/Luciferase Chimeric Plasmids. Chimeric expression plasmids were constructed using fragments of the 5′-flanking region of the rat ICAM-1 gene as described previously (Park et al., 2000). Mutations were generated in promoter sequences by polymerase chain reaction using primers incorporating the mutated sequence. Amplified fragments were ligated into the pGL2-basic vector containing a luciferase reporter gene, and the correct DNA sequence was confirmed by DNA sequencing analysis. The 5′-deletion mutants included pCAM-175, pCAM-175 GAS mut, and pCAM-97, containing the indicated fragment of the ICAM-1 promoter starting from the numbered nucleotide at the 5′-end and extending to +1 base pairs, the start of protein translation. All plasmid preparations were purified twice by CsCl gradient centrifugation.

Transfection. Stably transfected FRTL-5 cells were constructed with pGL2-basic, pCAM-175, or pCAM-97. Near confluent FRTL-5 cells in 6H medium were cotransfected with 20 μg of plasmid DNA and 10 μg of pRcNeo. pRcNeo contains a portion of the human early cytomegalovirus promoter from pRc/CMV vector. After 2 days, 400 μg/ml of G418 (Invitrogen) was added to the medium, and after 3 weeks the G418-resistant colonies were pooled and used for experiments. To test the effect of cytokines and hormones, cells were grown to 70 to 80% confluence in 6H medium then maintained without TSH (5H medium) for 5 days, at which time they were exposed to various concentrations of the indicated agents (IFN-γ, MMI, H_2O_2) for 24 h and luciferase activity was measured.

Western Blot Analysis. Immunoblot analyses were performed using anti-PIAS-1, anti-PIAS-3, anti-SOCS-1, or anti-SOCS-3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The antibodies against MAP kinase p44/p42, STAT1 and STAT3, or phosphorylated forms of MAP kinase p44/p42, STAT1 (Y701, S727) or STAT3 (Y705, S727) were affinity purified rabbit polyclonal IgG (New England Biolabs, Beverly, MA and Upstate Biotechnology, Inc., Lake Placid, NY). The antibodies against JAK1 and JAK2 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA), and the rabbit polyclonal anti-JAK1 pYpY$^{1022/1023}$ and anti-JAK2 pYpY$^{1007/1008}$, which specifically recognizes dual phosphorylated forms of JAK1 and JAK2, were obtained from Bioscience International (Camarillo, CA). For the Western blot, adherent FRTL-5 cells were stimulated with various agents for the indicated period of time at 37°C. The treated cells were scraped, lysed by addition of SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 30% glycerol, 125 mM dithiothreitol, 0.03% (w/v) bromophenol blue] and separated by 10% SDS-PAGE along with biotinylated molecular weight standards. The proteins were transferred to a nitrocellulose membrane by electrotransfer for 2 h. After soaking the membrane in blocking buffer (1× Tris-buffered saline, 0.1% Tween-20 with blocking reagent 5% milk), the membrane was incubated with primary antibody overnight at 4°C. Blots were developed using horseradish peroxidase-linked anti-rabbit secondary antibody and chemiluminescent detection system (Phototope-HRP Western Blot Detection Kit, New England Biolab).

Assay of Intracellular H_2O_2 Generation. Intracellular H_2O_2 was measured in FRTL-5 cells with a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described previously (Kim et al., 2000). Briefly, phosphate-buffered saline-washed FRTL-5 cells were stimulated with IFN-γ (100 U/ml) with or without MMI (1 mM), rapidly washed once with Krebs-Ringer solution, and then incubated in Krebs-Ringer solution containing DCFH-DA (5 μg/ml). DCFH-DA is nonpolar and readily diffuses into cells where it is hydrolyzed to the nonfluorescent polar derivative DCFH and trapped within the

cells. In the presence of H_2O_2, DCFH is oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). DCF fluorescence was measured with a Zeiss Axiovert 135 inverted microscope equipped with a X20 Neoflur objective and Zeiss LSM410 confocal attachment. To avoid photo-oxidation of DCFH, fluorescent images were collected with a single rapid scan (4-line average; total scan time, 4.33 s) and identical parameters such as contrast and brightness for all samples. The cells were then examined by differential interference contrast microscopy. Five groups of 10 to 20 subconfluent cells or 20 to 30 confluent cells were randomly selected from the image for each sample. The fluorescence intensity per cell was measured to obtain a value for each group, and the average of the five group values was calculated.

In Vitro Assay for Antioxidant Activity of MMI. The antioxidant activity of MMI was analyzed as described (Kang et al., 1998) with slight modification. The reaction was started by the addition of H_2O_2 to a reaction mixture containing 20 mM phosphate buffer, pH 7.0, and 10 mM MMI in a total volume of 100 μl. At the indicated times, 0.88 ml of trichloroacetic acid solution (10% w/v) was added to the 20 μl of reaction mixture followed by the addition of 0.2 ml of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 0.1 ml of 2.5 N KSCN to develop complex. Absorbance of the complex was measured 480 nm. As a control experiment, 10 mM dithiothreitol was added to the reaction mixture instead of MMI. The peroxidation activity of MMI was measured using a thioredoxin or a glutathione-dependent system (Luthman and Holmgren, 1982; Tonissen et al., 1989; Kang et al., 1998). Thioredoxin-dependent peroxidase activity of MMI was measured in a reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.18 μM yeast thioredoxin reductase (TR), 8 μM yeast thioredoxin (Trx), 0.2 mM NADPH, 1 mM H_2O_2, and 10 mM MMI. The control assay mixture did not contain TR, Trx, or H_2O_2. Glutathione-dependent peroxidase activity of MMI was measured in reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 0.15 μM glutathione reductase (GR), 0.5 mM GSH, 0.2 mM NADPH, 1 mM H_2O_2, and 10 mM MMI in a total volume of 0.5 ml. NADPH oxidation was monitored by measuring the decrease in A_{340} at 30°C.

Protein Tyrosine Phosphatase (PTP) Assay. The protein phosphatase activity of the total cellular lysate was determined by measuring free PO_4 generated from the phosphopeptide RRA(pT)VA using the molybdate-malachite green-phosphate complex assay as described by the manufacturer (Promega, Madison, WI). Cell lysates were prepared in a low detergent lysis buffer (0.25% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The phosphatase assay was performed in a PP2A-specific reaction buffer (final concentration 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin) using 100 μM phosphopeptide substrate and 1 μg of protein isolated from either IFN-γ and/or MMI-pretreated FRTL-5 cell lysate. After a 15-min incubation at room temperature, molybdate dye was added, and free phosphate was measured by optical density at 600 nM. A standard curve was prepared using free phosphate. Phosphatase activity was defined as picomoles of free PO_4 per microgram of protein per minute. For dephosphorylation of STAT1, cells (2×10^6) were stimulated with IFN-γ for 1 h to phosphorylate the Y701 residue of STAT1 and rapidly lysed with 1 ml of buffer consisting of 20 nM Tris-HCl, pH 7.4, 10 mM EGTA, 1% Triton X-100, 1 mM (*p*-amidinophenyl)methanesulfonyl fluoride, 50 U/ml aprotinin, 20 μg/ml leupeptin, and 20 μg/ml pepstatin. The cell lysate was incubated at 37°C in the presence or absence of 1 mM H_2O_2 and then the reaction was terminated by addition of the phosphatase inhibitors, 1 mM Na_3VO_4, 20 mM NaF, and 60 mM 2-glycerophosphate. Phosphorylation of tyrosine residues of STAT1 was analyzed and quantified by Western blotting with phosphospecific antibodies.

Other Assays. Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA) and used recrystallized bovine serum albumin as the standard.

Statistical Significance. All experiments were repeated at least three times with different cells. Values are the mean ± S.E. of these experiments. Significance between experimental values was determined by two-way analysis of variance.

Results

MMI Inhibits IFN-γ and H_2O_2-Induced Expression of ICAM-1. Abnormal ICAM-1 gene expression has been observed in patients with autoimmune thyroid diseases and is involved in the binding of lymphocytes to thyrocytes. In this study, ICAM-1 expression is examined in thyroid cells challenged with H_2O_2 or IFN-γ in the presence or absence of MMI. Figure 1A shows that H_2O_2 (100 μM) increases the level of the ICAM-1 transcript approximately 3-fold (Fig. 1A, lane 2) and IFN-γ (100 U/ml) increases the transcript approximately 4-fold. However, pretreatment of the cells for 4 h with MMI (500 μM) inhibits induction of ICAM-1 RNA by H_2O_2 and IFN-γ (Fig. 1B, lanes 3 and 5, respectively). One possible explanation for this effect is that H_2O_2 and IFN-γ activate GAS-dependent transcription of ICAM-1 and that MMI interferes with this activation process. The following experiments test this possibility.

FRTL-5 cells were stably transfected with luciferase reporter constructs (Park et al., 2000) with different 5′-flanking regions of the rat ICAM-1 promoter (Fig. 1C): pCAM-175 has a single copy of palindromic GAS and an Sp1 sequence;

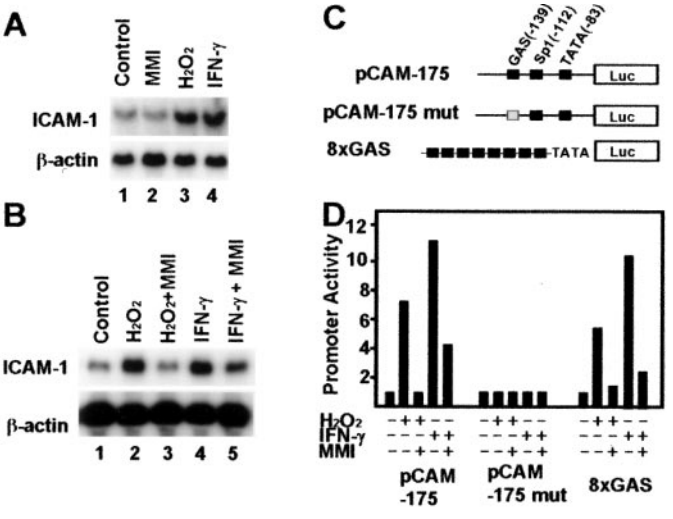

Fig. 1. Effects of MMI, H_2O_2, and IFN-γ on ICAM-1 gene expression in FRTL-5 cells. Panels A and B, FRTL-5 cells were grown to near confluence in complete 6H medium with 5% calf serum and maintained for 6 days with 3H medium that did not contain hydrocortisone, insulin, or TSH. RNA was isolated after 6 h of the indicated treatment and analyzed by Northern blot using probes for ICAM-1 and rat β-actin. The blots shown are representative of Northern analyses from one experiment. Each lane contained 20 μg of total RNA. Panel C, schematic representations of the 5′-region of the rat ICAM-1 promoter-luciferase constructs and 8xGAS-luciferase construct used in this study. This indicated that fragments of the ICAM-1 promoter region were cloned upstream of a promoterless firefly luciferase cDNA in the pGL2-basic vector (see *Experimental Procedures*). Panel D, FRTL-5 cells stably transfected with the indicated ICAM-1-luciferase chimeras were maintained in 5H 5% medium that did not contain TSH for 6 days before addition of H_2O_2 (100 μM), or MMI (500 μM), or both as noted. Luciferase assays were performed as described previously (see *Experimental Procedures*). The luciferase (Luc) activity from untreated cells was used as the control. All experiments were repeated at least three times. Data are normalized for transfection efficiency and are shown as the mean ± S.E.; significance ($p < 0.005$) was determined by two-way analysis of variance.

pCAM-175 GAS mut is a derivative of pCAM-175 in which the GAS sequence is mutated to a nonpalindromic form that does not bind STAT1 or STAT3; the 8× GAS-luciferase construct contains eight copies of the consensus GAS sequence TTCTCGGAA upstream of the minimal prolactin promoter (Horvai et al., 1997). FRTL-5 cells expressing these reporter constructs were treated with H_2O_2 or IFN-γ for 12 h, and the luciferase activity was measured. H_2O_2 increases the promoter activity of pCAM-175 7-fold and 8× GAS-luciferase 5-fold; IFN-γ increases the promoter activity of pCAM-175 11-fold and 8× GAS-luciferase 10-fold (Fig. 1D). In contrast, H_2O_2 and IFN-γ did not increase the promoter activity of pCAM-175 mut, which has a mutant core GAS sequence. Interestingly, pretreatment of the transfected cells with MMI for 4 h significantly reduces the ability of H_2O_2 and IFN-γ to induce expression of pCAM-175 and 8× GAS (Fig. 1D). These results suggest the following: 1) H_2O_2 and IFN-γ induce ICAM-1 expression in thyroid cells; 2) the antithyroid drug MMI blocks H_2O_2- and IFN-γ-dependent induction of ICAM-1; and 3) these effects involve transcriptional regulation mediated by the GAS element in the ICAM-1 promoter.

IFN-γ Rapidly Produces H_2O_2 and Stimulates Phosphorylation of Tyrosine Residues of STAT1 and STAT3 in FRTL-5 Cells. The results presented above suggest that H_2O_2 activates transcription factors which bind the palindromic GAS element in the ICAM-1 promoter and stimulate its expression. Therefore, intracellular concentration of H_2O_2 was monitored in cells with or without exposure to IFN-γ using the oxidation-sensitive fluorescent probe DCFH-DA and confocal microscopy (Fig. 2A). Minimal amounts of H_2O_2 were detected in untreated FRTL-5 cells. After exposure to IFN-γ (100 U/ml), DCF fluorescence increases to its maximal level within 15 min, and its level is maintained for 60 min (Fig. 2, A and B).

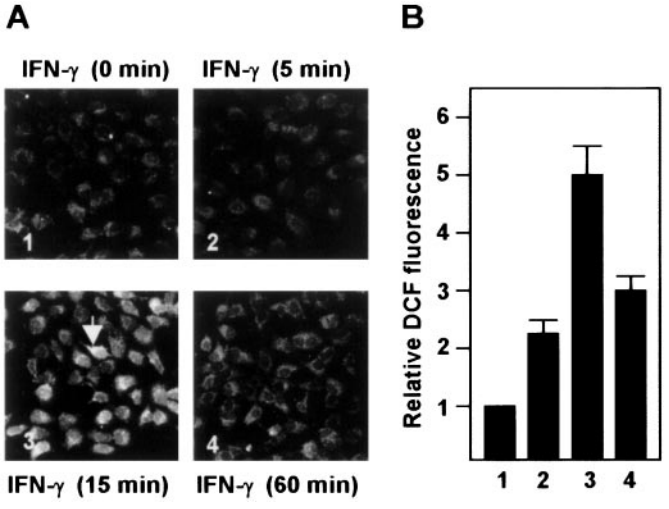

Fig. 2. Effect of IFN-γ on production of H_2O_2 in FRTL-5 cells. FRTL-5 cells were grown in 6H medium consisting of Coon's modified F12 supplemented with 5% calf serum, 1 mM nonessential amino acids, and a mixture of six hormones: bovine TSH (1 mU/ml), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). The cells were shifted to 5H medium with no TSH and 5% calf serum and cultured for an additional 7 days. DCF fluorescence was measured with a confocal microscope after incubation of the cells in the presence of IFN-γ for 0, 5, 15, or 60 min (panel A). Relative fluorescence intensity per cell was calculated as described under *Experimental Procedures*. Data shown are means ± S.E. of the values from five groups of 20 to 30 cells (panel B).

The ability of H_2O_2 to stimulate phosphorylation of tyrosine residues of JAK1, JAK2, STAT1, and STAT3 was also examined. Immunoblots were carried out with phosphospecific antibodies against JAK1, JAK2, STAT1, or STAT3 using extracts from cells treated with or without H_2O_2. Figure 3, A and B, shows that H_2O_2 stimulates the phosphorylation of Y1023, Y1024 in JAK1 and Y1007, Y1008 in JAK2, respectively, but the level of expression of JAK1 and JAK2 did not change significantly. Tyrosine-phosphorylated STAT1 was not detectable in FRTL-5 cells maintained in 5H 5% medium (Fig. 3C, lane 1). H_2O_2 stimulates the phosphorylation of Y701 in STAT1 in these cells, which leads to homodimer formation, nuclear translocation and DNA binding by STAT1. H_2O_2 (100 μM) induced significant phosphorylation of Y701 of STAT1 and Y705 of STAT3 within 5 min (Fig. 3, C and D), and the level of tyrosine-phosphorylated STAT1 and STAT3 returned to a basal level within 60 min. The level of expression of STAT1 and STAT3 in FRTL-5 cells did not change significantly after treatment with H_2O_2 (Fig. 3, C and D, lower). In cells treated with H_2O_2, there was no significant change in the amount or the serine/threonine phosphorylation state of MAP kinase 44/42 (Fig. 3, C and D).

MMI Enhances the Elimination of IFN-γ-Induced Intracellular H_2O_2 and Inhibits Exogenous H_2O_2-Induced Tyrosine Phosphorylation of STAT1 and STAT3. As shown above, IFN-γ generates intracellular H_2O_2 and exogenous H_2O_2 can cause phosphorylation of JAK1, JAK2, STAT1, and STAT3. However, in FRTL-5 cells pretreated with MMI followed by IFN-γ (100 U/ml), significantly less H_2O_2 (DCF fluorescence intensity) accumulates than in control cells without MMI (Fig. 4, A and B). Pretreatment of cells with MMI also prevents H_2O_2-induced phosphorylation of STAT1 and STAT3 (Fig. 5, A and B, lanes 3).

MMI Scavenges H_2O_2 in Vitro and Participates in Electron Transfer from Thioredoxin and Glutathione.

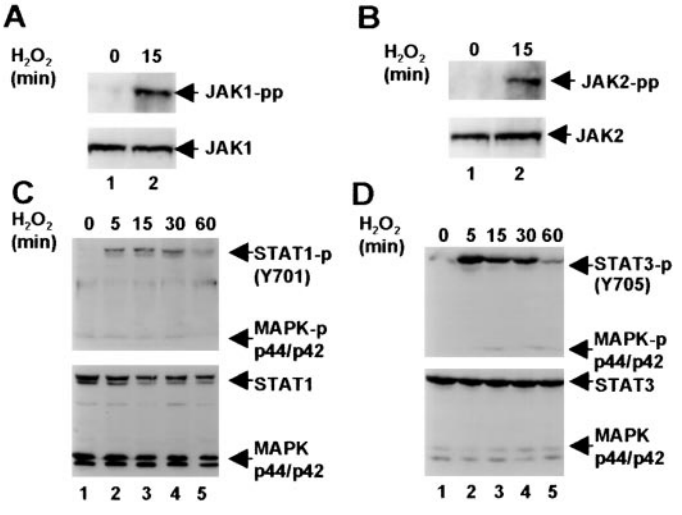

Fig. 3. H_2O_2 modulates the tyrosine phosphorylation of JAK1, JAK2, STAT1, and STAT3 in FRTL-5 cells. FRTL-5 cells were grown to near confluence in complete 6H medium with 5% calf serum, and cells were maintained for 6 days with 5H medium that did not contain TSH. Medium was replaced with fresh medium with H_2O_2 (100 μM). Total cell lysates were prepared and analyzed by SDS-PAGE. Phosphorylated forms of JAK1 (A), JAK2 (B), STAT1 (C), and STAT3 (D) were detected by phosphoprotein-specific antibodies. Phosphorylated forms of MAP kinase p44/42 were also detected by specific antibodies (D) (see *Experimental Procedures*).

An experiment was performed to directly monitor the interaction between MMI and H_2O_2 in vitro. The concentration of H_2O_2 was measured in the presence or absence of MMI and thioredoxin or glutathione. MMI alone causes a rapid and complete decrease in the concentration of H_2O_2 in vitro, while the concentration is stable in the control reaction (Fig. 6A). The concentration of H_2O_2 was reduced by approximately 50% by MMI in 5 min and reduced by approximately 99% in 100 min. These results suggest that MMI directly reduces H_2O_2 in vitro.

Prx and glutathione peroxidase (Gpx) efficiently reduce alkyl hydroperoxide (ROOH) (Fig. 6, D and E). The Trx-dependent system involves sequential oxidation/reduction steps with TR, Trx, and Prx (Fig. 6D). This reaction can be monitored by measuring the oxidation of NADPH. As shown in Fig. 6B, four components, MMI, TR, Trx, and H_2O_2, are required for this oxidation/reduction process to occur (i.e., NADPH oxidation does not occur in the absence of TR or Trx). If Trx or TR is replaced with MMI, Prx-mediated peroxidation does not occur. However, the reaction does proceed in the presence of MMI, TR, and Trx. This suggests a reaction pathway in which Trx reduces oxidized MMI, and reduced MMI reacts with ROOH to yield ROH and H_2O.

Glutathione-dependent reduction is another cellular system for scavenging ROOH that uses GR, GSH, and Gpx (Fig. 6E). As in the thioredoxin-dependent system, NADPH oxidation occurs in the presence of MMI, GR, and GSH but not in a control reaction lacking GR or GSH (Fig. 6C). When GSH or GR is replaced with MMI, Gpx-mediated peroxidation does not occur. These findings suggest that GSH reduces oxidized MMI and reduced MMI reacts with ROOH to yield ROH and H_2O.

Taken together, these results suggest that MMI has an intrinsic ability to scavenge H_2O_2 and that MMI may be involved in the peroxiredoxin or glutathione systems for eliminating cellular H_2O_2.

Kinetics of IFN-γ and MMI Effects on STAT1, STAT3, JAK1, and JAK2 Phosphorylation. The kinetics of STAT1 and STAT3 tyrosine phosphorylation were examined in the presence or absence of MMI in cells treated with IFN-γ (Fig. 7). STAT1 and STAT3 were detected by immunoblot analysis using antibodies recognizing all forms of the protein or recognizing phosphorylated forms of the protein. The concentration of STAT1 increases from a basal level in untreated cells to a maximal level after 48 h of exposure to IFN-γ (Fig. 7A, middle). A significant amount of the Y701 phosphotyrosine form of STAT1 is detected within 30 min after addition of IFN-γ and that level is maintained for 48 h (Fig. 7, A, upper and C). The STAT1/DNA complex was measured in these cells by electrophoretic mobility shift analysis with a GAS probe from the rat ICAM-1 promoter. The STAT1/DNA complex was detected and persisted for 72 h in cells treated with IFN-γ (data not shown). In cells simultaneously treated with IFN-γ and MMI, STAT1 expression is induced by IFN-γ and rapid phosphorylation of Y701 of STAT1 is observed (Fig. 7C, lane 2 versus lanes 3 and 4). However, in MMI-treated cells the extent of Y701 phosphorylation induced by IFN-γ is lower (Fig. 7C, upper lane 2 versus lanes 3 and 4).

STAT3 phosphorylation was also examined and the results were quite different from the results with STAT1 (Fig. 7, C and D). IFN-γ does not induce a high level of unphosphorylated STAT3, but it induces rapid and transient phosphorylation of Y705 of STAT3. Y705 phosphorylation of STAT3 is

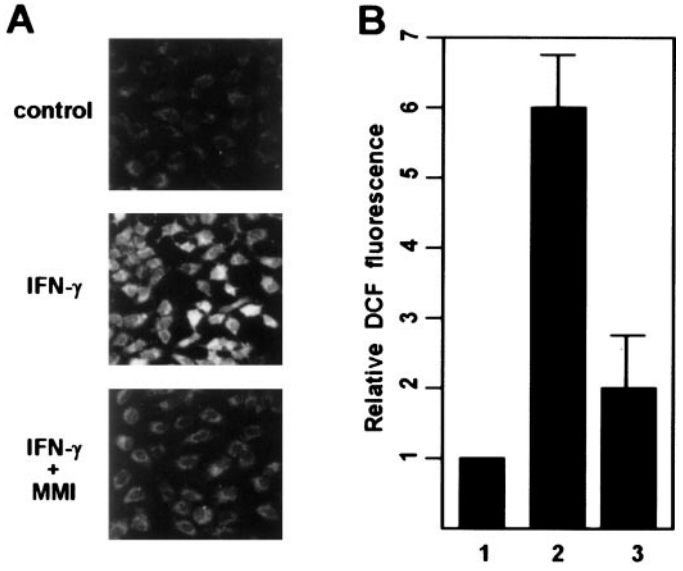

Fig. 4. Effect of MMI on IFN-γ-induced H_2O_2 in FRTL-5 cells. FRTL-5 cells were grown in 6H medium consisting of Coon's modified F12 supplemented with 5% calf serum, 1 mM nonessential amino acids, and a mixture of six hormones: bovine TSH (1 mU/ml), insulin (10 μg/ml), cortisol (0.4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). The cells were shifted to 5H medium with no TSH and 5% calf serum and cultured for an additional 7 days. DCF fluorescence was measured with a confocal microscope after incubation of the cells in the presence of IFN-γ and/or MMI (500 μM) for 15 min (A). Relative fluorescence intensity per cell was calculated as described under *Experimental Procedures*. Data shown are means ± S.E. of the values from five groups of 20 to 30 cells (B).

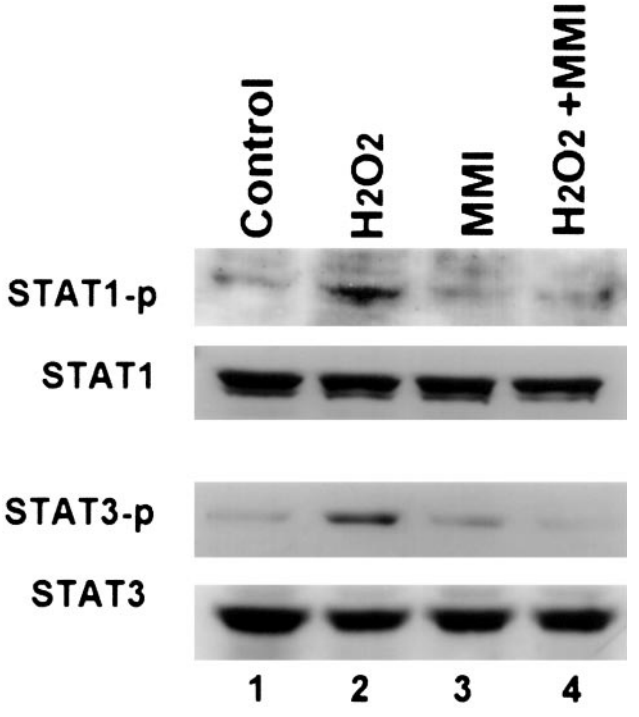

Fig. 5. Effects of H_2O_2-mediated tyrosine phosphorylation of STAT1 and STAT3. Experimental protocol was the same as in the legend to Fig. 3, except cells were cultured in the presence of H_2O_2 (100 μM), MMI (1 mM), or both. Total cell lysates were prepared and analyzed by SDS-PAGE. Phosphorylated forms of STAT1 and STAT3 were detected by specific antibodies (see *Experimental Procedures*).

maximal approximately 30 min after treatment with IFN-γ (Fig. 7C) and declines to near basal level by 12 h after IFN-γ treatment (Fig. 7B). In MMI-treated cells, the extent of Y705 phosphorylation induced by IFN-γ is lower (Fig. 7C, lane 2 versus lanes 3 and 4).

In a parallel experiment, the effects of MMI on tyrosine phosphorylation of JAK1 and JAK2 were determined. In MMI-treated cells, IFN-γ induced a lower level of $pYpY^{1022/1023}$-phosphorylated JAK1 and $pYpY^{1007/1008}$-phosphorylated JAK2.

Effects of MMI on PIAS-1, PIAS-3, SOCS-1, and SOCS-3 Expression and on PTPs Activity in FRTL-5 Cells. In earlier studies, several mechanisms were proposed for down-regulation of STAT signaling (Starr and Hilton, 1999; Tamir et al., 2000). There is now evidence for the involvement of at least three families of proteins in inhibiting IFN-γ-mediated-JAK/STAT signaling. These three families are the suppressors of cytokine signaling (SOCS) (Starr et al., 1997), protein inhibitors of activated STATs (PIAS) (Chung et al., 1997), and the SH2-containing phosphatases (SHP) (You et al., 1999; Tamir et al., 2000). The SOCS and PIAS proteins were detected by immunoblot using extracts from cells treated with MMI (Fig. 8). PIAS-1, PIAS-3, and SOCS-3 are not induced in cells treated with MMI for 12 h. The level of SOCS-1 increases gradually in cells exposed to MMI and is maintained at an elevated level for 12 h. These findings suggest that MMI up-regulates SOCS-1, which specifically inhibits JAKs that are activated by IFN-γ. However, MMI induced SOCS-1 after 2 h. This finding suggests that MMI inhibits rapid induction of STAT1 and STAT3 phosphorylation in response to IFN-γ, and this may not be mediated by induction of SOCS-1. MMI induction of SOCS-1 may be a plausible explanation for inhibition of IFN-γ-mediated prolonged phosphorylation of STAT1 Y701 (data not shown).

The PTPs SHP-1 and SHP-2 are involved in the negative regulation of hormone and cytokine-mediated JAK/STAT activation (Jiao et al., 1996; You et al., 1999; Tamir et al., 2000). All

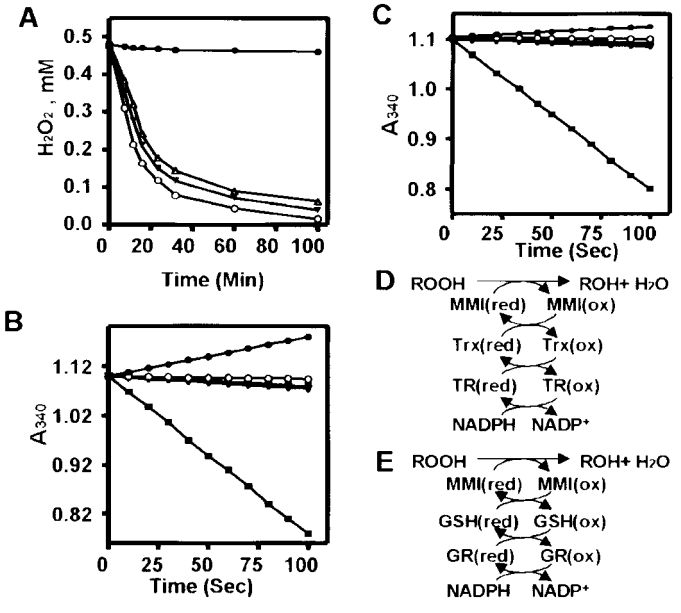

Fig. 6. In vitro H_2O_2-scavenging and thioredoxin- and glutaredoxin-dependent peroxidation activity of MMI. Panel A, H_2O_2 scavenging was started by the addition of 0.1 mM H_2O_2 to 50 mM phosphate buffer, pH 7.0, in a total volume of 100 μl containing a final concentration of 10 mM MMI and incubated at 30°C. At appropriate time points, 880 μl of 10% (w/v) trichloroacetic acid solution was added to stop the reaction, followed by the addition of 200 μl of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 100 μl of 2.5 N KSCN to develop the complex producing a purple color. The concentration of H_2O_2 was determined by measuring a decrease in absorbance at 480 nm. Assay mixture contained GSH (▼), MMI (▽), or GSH and MMI (○). The control reaction did not contain glutathione or MMI (●). Panel B, thioredoxin-dependent peroxidation activity of MMI. NADPH oxidation was monitored as the decrease in A_{340} in an 0.5-ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 5 μg of TR, 10 μg of Trx1, 0.2 mM NADPH, 1 mM H_2O_2, and 10 mM MMI (■) in a total volume of 0.5 ml. The control assays did not contain TR (●), Trx (○), MMI (▽), or H_2O_2 (▼). Panel C, oxidation of NADPH by GSH and GR in the presence of MMI and H_2O_2. The incubation mixture contained 50 mM Hepes-NaOH (pH 7.0), 5 μg of GR, 0.1 mM GSH, 0.2 mM NADPH, 1 mM H_2O_2, and 10 mM MMI (■) in a total volume of 0.5 ml. The control assays did not contain GR (○), GSH (●), MMI (▽), or H_2O_2 (▼).

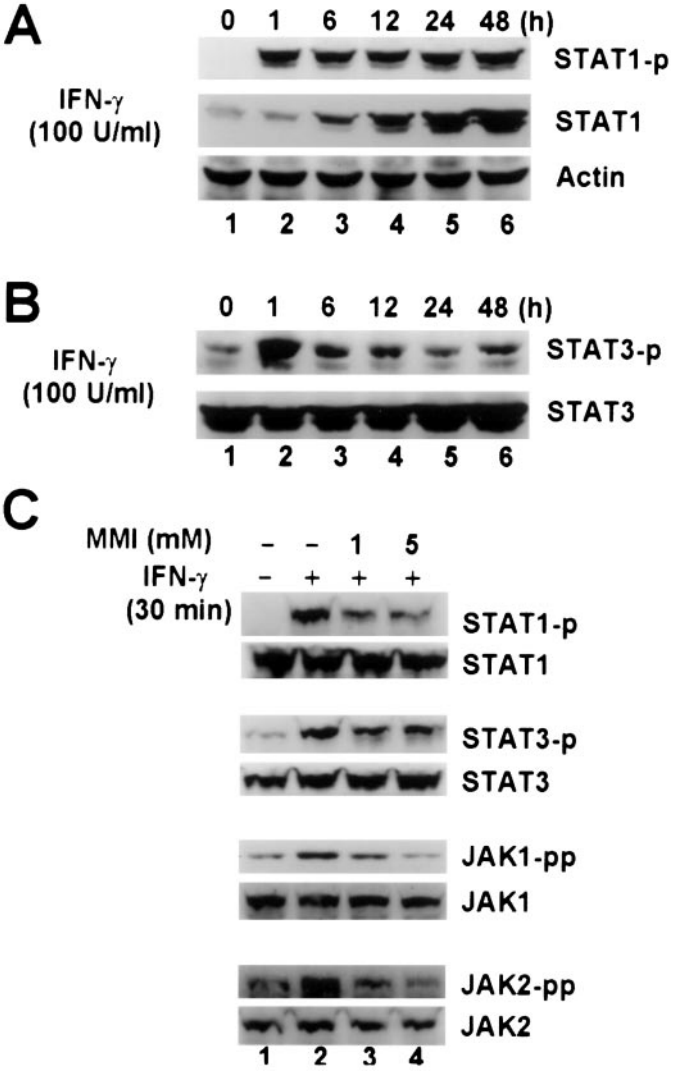

Fig. 7. MMI modulates the IFN-γ-induced tyrosine phosphorylation of STAT1, STAT3, JAK1, and JAK2. Panel A, FRTL-5 cells were grown to near confluence in complete 6H medium with 5% calf serum and maintained for 6 days with 5H medium lacking TSH. The medium was replaced with fresh medium containing IFN-γ (100 U/ml). Total cell lysates were prepared after treatment and analyzed by SDS-PAGE. Phosphorylated forms of STAT1 (Y701) and STAT3 (Y705) were detected using phosphospecific antibodies. Panel B, FRTL-5 cells were grown to near confluence in complete 6H medium with 5% calf serum and maintained for 6 days with 5H medium that did not contain TSH. The medium was replaced with fresh medium containing IFN-γ (100 U/ml) and/or MMI (1 or 5 mM). Total cell lysates were prepared after 30 min of treatment and analyzed by SDS-PAGE. Phosphorylated forms of STAT1 (Y701), STAT3 (Y705), JAK1, and JAK2 were detected by phosphospecific antibodies.

PTPs contain an essential cysteine residue which has a low pK_a value in the signature active site motif, HCXXGXXR(S/T). Intracellular H_2O_2, generated after growth factor stimulation, can interact with this cysteine residue in the PTPs, form Cys-SOH, and reversibly inactivate the phosphatase. This reversible inactivation of PTPs by intracellular H_2O_2 may allow sufficient increase in tyrosine phosphorylation (Lee et al., 1998). The level of Y701 phosphorylated STAT1 in whole cell lysates from FRTL-5 cells treated with IFN-γ for 1 h spontaneously decreases until 3 h in vitro (Fig. 9B, upper). However, addition of exogenous H_2O_2 to the IFN-γ-treated cell lysate prevents dephosphorylation of Y701-phosphorylated STAT1; this suggests that exogenous H_2O_2 inactivates the dephosphorylation process in vitro (Fig. 9A, lower).

The phosphatase activity in protein lysates obtained from FRTL-5 cells treated with H_2O_2 and/or MMI was determined by measuring free phosphate liberated from phosphopeptide substrates, END(pY)INASL and DADE(pY)LIPQQG, which serve as substrates for many protein tyrosine phosphatases (Fig. 9B). Treatment of FRTL-5 cells with H_2O_2 inhibits PTPs by 50%, but treatment with H_2O_2 and MMI reverses this effect (Fig. 9B). PTP activity was measured in lysates of FRTL-5 thyroid cells treated with IFN-γ (Fig. 9C). PTP activities decreased in response to IFN-γ, with maximal suppression 15 min after IFN-γ treatment (Fig. 9C). This corresponds to the point in time when a maximal amount of H_2O_2 is generated in response to IFN-γ (Fig. 2). In cells treated with MMI, PTP activities did not decrease after IFN-γ treatment (Fig. 9C). These findings support the idea that MMI prevents H_2O_2-mediated reversible inactivation of protein tyrosine phosphatases in response to IFN-γ. Thus, MMI_2 may be responsible for diminished JAK/STAT phosphorylation in response to IFN-γ.

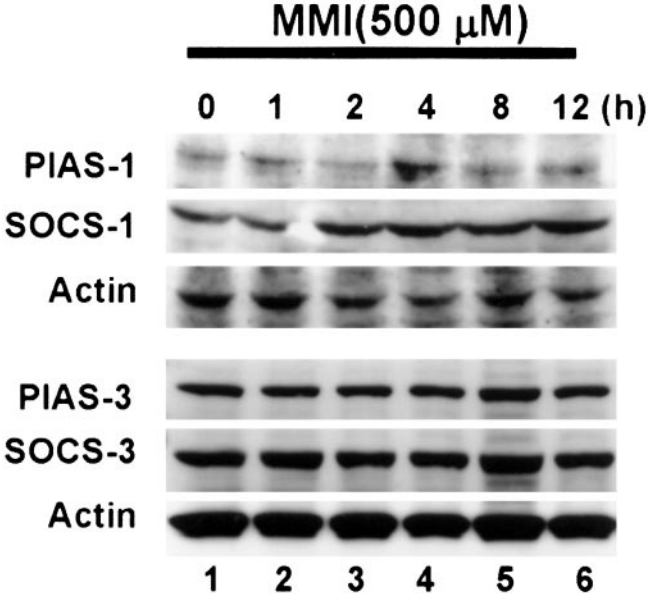

Fig. 8. Effects of MMI on PIAS-1, PIAS-3, SOCS-1, and SOCS-3 in FRTL-5 cells. FRTL-5 cells were grown to near confluence in complete 6H medium with 5% calf serum and maintained for 6 days with 5H medium that did not contain TSH. The medium was replaced with fresh medium containing MMI (500 μM). Total cell lysates were prepared 30 min after treatment and analyzed by SDS-PAGE and Western blot with appropriate antibodies.

Discussion

This study focuses on the effect of MMI on the action of IFN-γ in thyrocytes. The results show that IFN-γ produces a significant amount of H_2O_2 in thyroid cells and exogenous H_2O_2 induces tyrosine phosphorylation of STAT1 and STAT3. Interestingly, MMI accelerates elimination of H_2O_2 produced in response to IFN-γ and also inhibits the tyrosine phosphorylation of STAT1 and STAT3 by exogenous H_2O_2. In addition, we show that MMI eliminates H_2O_2 by a one-electron reduction from TR and GSH. By eliminating H_2O_2, MMI prevents physiological reversible inactivation of phosphatases in response to IFN-γ signaling. The net effect is that MMI inhibits full activation of the JAK/STAT signaling pathway in FRTL-5 thyroid cells.

A scheme depicting the proposed effect of MMI on IFN-γ-mediated signaling is shown in Fig. 10. This scheme is based on the following observations: 1) H_2O_2 produced by growth factors inhibits protein tyrosine phosphatases through reversible oxidation of an active site cysteine (Lee et al., 1998),

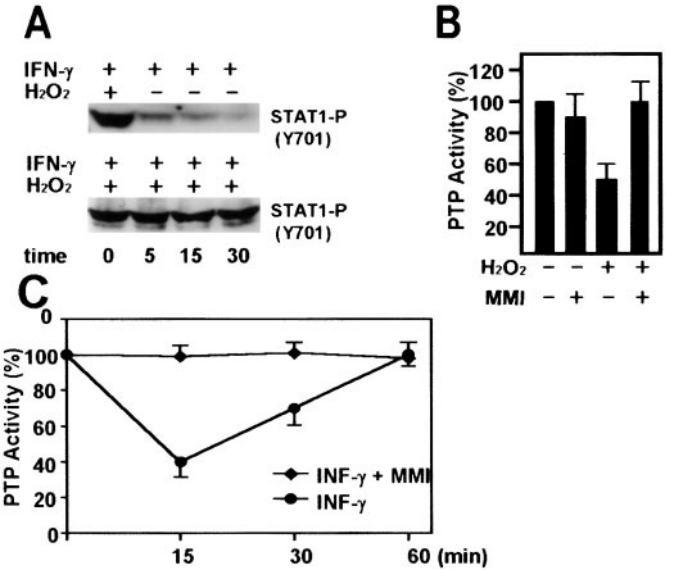

Fig. 9. Panel A, effect of H_2O_2 on dephosphorylation of STAT1. FRTL-5 cells were grown to near confluence in complete 6H medium with 5% calf serum and maintained for 6 days with 5H medium that did not contain TSH. The medium was replaced with fresh medium containing IFN-γ (100 U/ml) for 1 h to phosphorylate the Y701 residue of STAT1. Then, they were rapidly lysed with 1 ml of buffer (see *Experimental Procedures*). The resulting cell lysate mixture was incubated at 37°C in the presence or absence of 1 mM H_2O_2, and then the reaction was terminated by the addition of the phosphatase inhibitors, 1 mM Na_3VO_4, 20 mM NaF, and 60 mM 2-glycerophosphate. The phosphorylation level of tyrosine residues of STAT1 was analyzed by Western blotting using phosphospecific antibodies. Panels B and C, effect of H_2O_2, MMI, and IFN-γ on protein tyrosine phosphatase activity. The protein phosphatase activity of total cellular lysate was determined by measuring free PO_4 generated from the phosphopeptide RRA(pT)VA using the molybdate-malachite green-phosphate complex assay as described by the manufacturer (Promega). Cell lysates were prepared in a low detergent lysis buffer [0.25% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin]. The phosphatase assay was performed in a PP2A-specific reaction buffer [final concentration 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin[using 100 μM phosphopeptide substrate and 1 μg of protein from FRTL-5 cell lysate. After a 15-min incubation at room temperature, molybdate dye was added, and free phosphate was measured by optical density at 600 nM. A standard curve was prepared using free phosphate. Phosphatase activity was defined as picomoles of free PO_4 per microgram of protein per minute.

and this active site cysteine oxidation is related to functional inactivation of SHP-1 (Cunnick et al., 1998); and 2) SHP-1 is directly associated with JAK2 and related to dephosphorylation of the kinase in growth hormone and cytokine signaling (Lee et al., 1998). In summary, MMI eliminates H_2O_2 produced by IFN-γ through its reducing activity, which is coupled to oxidation/reduction of Trx and/or GSH. H_2O_2 may potentiate IFN-γ-mediated JAK/STAT activation by concurrent inhibition of PTPs, but MMI inhibition of H_2O_2-induced PTP inactivation results in a reduced level of JAK/STAT activation in IFN-γ-treated cells. This model explains previous evidence that the antioxidant and immunomodulatory roles of MMI in thyroid cells are mainly accomplished by H_2O_2-scavenging in thyroid cells. MMI (1-methyl 2-mercaptoimidazole) has an SH group in its basic imidazole ring structure and the SH group serves as a drug oxidation site. The oxidation/reduction of MMI through electron exchange with TR and/or GSH may occur mainly in the SH group of MMI.

MMI has immunomodulatory effects in the thyroid and the immune system. Despite some results to the contrary, numerous in vitro and in vivo studies have shown that antithyroid drugs have an immunomodulatory effect (Leclere, 1987). However, the pharmacological relevance of the immunomodulatory effects have been questioned because most studies, including this study, are carried out using drug concentrations in the range of 10^{-4} to 10^{-5} M. Nevertheless, MMI is concentrated in the thyroid, and uptake of this drug is greatly increased in patients with Graves' disease. Studies of patients with Graves' disease demonstrate intrathyroid MMI concentrations of 500 to 2,000 ng/g (about 5×10^{-5} M) (Jansson et al., 1983).

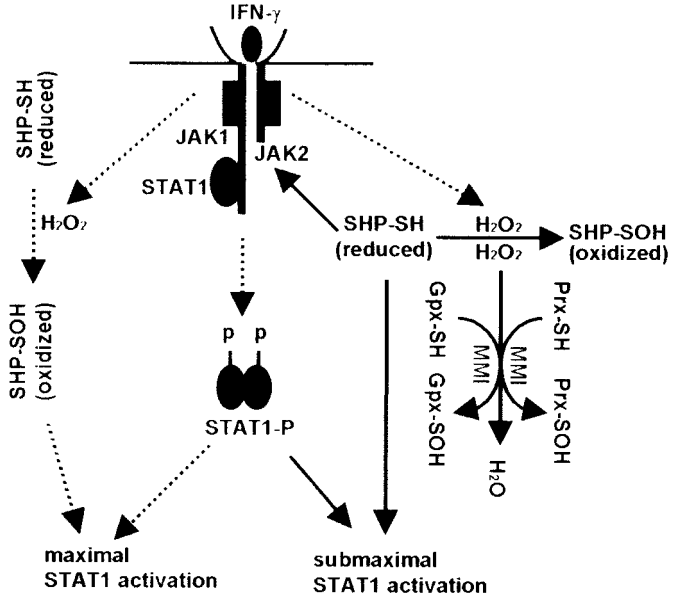

Fig. 10. Proposed scheme of the inhibitory effects of MMI on IFN-γ signaling in thyroid cells. In the absence of MMI (dotted pathway), IFN-γ induces sequential activation of JAK/STAT1 by tyrosine phosphorylation. H_2O_2 generated by IFN-γ concurrently inactivates PTPs, especially SHP, by cysteine oxidation. Reversible PTP inactivation allows full activation of JAK/STAT1 in response to IFN-γ in thyroid cells. However in the presence of MMI (solid pathway), MMI reduces the level of H_2O_2 by using electron transfer from Trx and/or Gpx, and prevents reversible PTP inactivation. Because MMI prevents reversible inactivation of PTPs, the JAK/STAT1 signaling pathway is submaximally activated.

Recent evidence suggests that certain forms of reactive oxygen species such as H_2O_2 may play a role in signal transduction and may regulate specific transcription factors. These transcription factors may include NF-κB (Li and Karin, 1999), AP1 (Li and Karin, 1999), and STATs (Carballo et al., 1999), which regulate immune response genes such as MHC class I and ICAM-1 in the thyroid gland. In the classical cytokine signaling pathway, the phosphorylation of tyrosine residues in STAT1 and STAT3 is mediated by nonreceptor tyrosine kinases such as JAK1, JAK2, JAK3, and Tyk2 (Ihle, 1995; Bach et al., 1997; Darnell, 1997; Stark et al., 1998). Activated STAT1 and STAT3 regulate many cellular processes including development (Takeda et al., 1997) apoptosis (Bromberg and Darnell, 2000; Wang et al., 2000), and transcription (Decker et al., 1997). Activated STATs can lead to abnormal expression of class II transactivators, MHC class I, class II, and ICAM-1 genes, which contributes to the pathogenesis of autoimmune thyroid diseases. This study shows that IFN-γ increases the level of activated STAT1 for a prolonged period of up to 72 h (Fig. 7A). The consequences of this prolonged activation of STAT1 is not known. Several mechanisms may be involved in the prolonged activation of STAT1: for example, 1) overexpression of the IFN-γ receptor; 2) defects in internalization of the IFN-γ receptor; and 3) altered function of inhibitors that negatively regulate the IFN-γ receptor. This study provides evidence that MMI increases the level of SOCS-1 in FRTL-5 thyroid cells. SOCS-1 negatively regulates JAKs through direct binding. Thus, this is a possible mechanism by which MMI could inhibit IFN-γ-induced prolonged activation of STAT1 (data not shown).

Thyroid cells utilize several cellular defense systems against oxidative damage including antioxidant proteins, superoxide dismutase, catalase, and glutathione. However, the exact mechanisms regulating intracellular H_2O_2 are not known. It has been shown that Prx I and II are physiologically involved in regulating the level of H_2O_2 in FRTL-5 thyroid cells (Kim et al., 2000). MMI induces Prx I RNA and protein in FRTL-5 thyroid cells; therefore, this may have been involved in its ability to eliminate H_2O_2 produced in response to IFN-γ.

In summary, this study provides the first evidence that MMI accelerates H_2O_2 scavenging in vivo in cells exposed to IFN-γ. The biochemical mechanism of the MMI-mediated reduction of H_2O_2 involves electron transfer using Trx or GSH. In addition, MMI inhibits the activation of JAK/STAT1 signaling that is triggered by IFN-γ. These molecular mechanisms may be related to the therapeutic effects of MMI in patients with autoimmune thyroid disease.

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