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Inhibition of interferon-γ signaling by a mercurio-substituted dihydropsoralen in murine keratinocytes

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Abstract

Psoralens and ultraviolet light A (PUVA) are used in the treatment of a variety of epidermal proliferative and inflammatory disorders. These compounds are known to intercalate and photo crosslink DNA. Specific receptor proteins for psoralens have also been identified. We describe a novel activity of a thiol reactive derivative, iodomercurio-4′,5′-dihydrotrimethylpsoralen (iodomercurio-H_2TMP) in keratinocytes. Without UVA, this psoralen was found to be an effective inhibitor of interferon-γ (IFN-γ)-signaling as measured by induction of nitric oxide biosynthesis (IC_{50} = 0.8 μM). This activity was increased (IC_{50} = 0.1 μM) when the cells were depleted of intracellular glutathione (GSH) with buthionine sulfoximine. In keratinocytes, IFN-γ stimulates expression of inducible nitric oxide synthase (NOS2). Although iodomercurio-H_2TMP did not alter NOS2 enzymatic activity, it blocked IFN-γ-induced expression of NOS2 mRNA and protein, an effect that was enhanced in GSH-depleted cells. Iodomercurio-H_2TMP was found to readily inhibit IFN-γ signaling in transient transfection assays using NOS2 promoter/luciferase reporter constructs. NOS2 gene expression is known to require a variety of transcription factors including STAT-1, NF-κB and AP-1. Using mobility shift assays the psoralen, at concentrations that inhibit nitric oxide biosynthesis, had no effect on the DNA binding activity of STAT-1 or NF-κB. However, iodomercurio-H_2TMP was found to suppress AP-1. These data indicate that iodomercurio-H_2TMP acts at sulfhydryl-sensitive sites to inhibit NOS2. Moreover, this is dependent on early events in the IFN-γ signal transduction pathway. Inhibition of AP-1 suggests that the psoralen functions by interfering with an important transcription factor that regulates expression of NOS2 in keratinocytes.

Keywords: Nitric oxide; PUVA; Interferon-γ; Psoriasis; Skin

1. Introduction

The best characterized of the photosensitizing chemicals are the psoralens. Also known as furocoumarins, psoralen analogs such as 8-methoxypsoralen and 4,5′,8-trimethylpsoralen (TMP) are used clinically in the photochemotherapy of skin diseases such as psoriasis, mycosis fungoides, vitiligo and eczema [1–3]. Psoralens are known to intercalate into DNA in a dark reaction. After exposure to UVA light, they form mono- and bifunctional adducts with pyrimidine bases. This can result in cross-linking of the base paired strands of DNA [4]. These DNA modifications can be mutagenic and carcinogenic [5]. In previous studies, we identified a novel receptor for the psoralens that also appears to be involved in their biological activity [6,7]. This receptor is a 22,000 molecular weight protein that regulates several protein kinases important in cell growth including the epidermal growth factor (EGF) receptor

Abbreviations: AP-1, activator protein-1; BSO, buthionine [*S*,*R*] sulfoximine; COX-2, cyclooxygenase-2; GAS, gamma-activated sequence; GSH, glutathione; IFN-γ, interferon-γ; Iodomercurio-H_2TMP, 4,8-dimethyl-5′-iodo-methyl-4′,5′-dihydropsoralen; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NF-κB, nuclear factor kappa B; STAT-1, signal transducer and activator of transcription; TCA, trichloroacetic acid; TMP, 4,5′,8-trimethylpsoralen; UVA, ultraviolet light A (320–400 nm)

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tyrosine kinase and an EGF receptor serine kinase [8–10]. With the aim of developing psoralen analogs that retain biological activity, but are less toxic, we have synthesized a number of 4′,5′-dihydroderivatives of TMP which are unable to cross-link DNA [11]. One important class of these derivatives that are potent inhibitors of keratinocyte growth are mercurio-substituted 4′,5′-dihydropsoralens [12]. These compounds are particularly useful as models to localize reactive psoralens in cells and evaluate their mechanism of action in the skin.

Skin diseases such as psoriasis are characterized by excessive proliferation of keratinocytes and inflammation [13]. A characteristic feature of inflamed tissue is the production of nitric oxide, a free radical gas that can cause tissue injury. Many studies have demonstrated that reducing nitric oxide in tissues can suppress inflammation and a variety of nitric oxide antagonists have been developed [14,15]. Nitric oxide is formed in cells via the enzyme nitric oxide synthase using L-arginine as the substrate and is known to be important in normal skin physiology [16]. We previously reported that keratinocytes generate nitric oxide via an inducible form of nitric oxide synthase (NOS2) following treatment with the inflammatory cytokine interferon-γ (IFN-γ), and that this activity is regulated by EGF [17,18]. The present studies show a unique activity of one of the psoralen derivatives that we have synthesized, iodomercurio-H_2TMP (see Fig. 1 for structure). This compound was found to be a potent inhibitor of IFN-γ-induced nitric oxide production by keratinocytes. Unlike most inhibitors of nitric oxide which directly target NOS2 activity, we found that iodomercurio-H_2TMP interferes with the expression of the enzyme. This compound may be useful for understanding mechanisms regulating IFN-γ signaling and nitric oxide biosynthesis in skin-derived cells.

2. Materials and methods

2.1. Cells and reagents

PAM 212 mouse keratinocytes were obtained from Stuart Yuspa (NIH, Bethesda, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Iodomercurio-H_2TMP was synthesized as described by Whittemore [19]. [γ-^{32}P] ATP (111 TBq/mmol) and [α-^{32}P] CTP (111 TBq/mmol) were obtained from NEN Life Science Products (Boston, MA). Sulfosalicylic acid was obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions of the mercurio-psoralen were prepared in DMSO and stored at −20 °C. Final DMSO concentrations in the treatment groups were <0.1%. Mouse IFN-γ was kindly provided by Dr. Sidney Pestka (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ). Protein was quantified using the Detergent Compatible (Dc) Protein Assay (BioRad, Hercules, CA) using bovine serum albumin (BSA) as the standard.

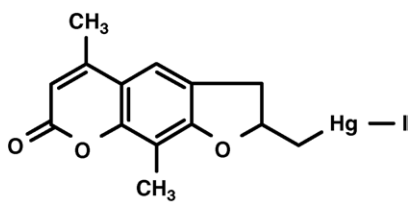

Fig. 1. Structure of iodomercurio-H_2TMP.

2.2. Cell viability assays

The effects of iodomercurio-H_2TMP on keratinocyte viability was assayed using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS, Promega, Madison, WI) or by trypan blue dye exclusion. Keratinocyte cell growth was evaluated as previously described [20,21]. Briefly, cells were plated at low density (5×10^3 cells/well) in six well tissue culture dishes and allowed to adhere overnight. The medium was then replaced with phenol red-free DMEM supplemented with increasing concentrations of the mercuriopsoralens. After 5 days, cells were removed from the plates and enumerated using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

2.3. GSH assays

Intracellular GSH was quantified using a modification of the method of Guaiquil et al. [22]. Briefly, 2.5×10^5 cells/well in six well dishes were treated for 30 min with increasing concentrations of the iodomercurio-H_2TMP, washed with PBS (3 × 2 mL), and then precipitated with 0.6 mL of ice cold 5% TCA. After 10 min, 150 μL of the TCA extract was mixed with 50 μL of 1 M $NaHPO_4$ buffer pH 7.5 containing 0.72 mM 5,5′-dithio-bis(2-nitrobenzoic acid) and 0.4% sulfosalicylic acid. Absorbance was measured 2 min later at 405 nm using a Perkin-Elmer HTS2000 microplate reader.

2.4. Western blotting

Cells were lysed in 10 mM phosphate buffer containing 1% SDS, 1 mM leupeptin, 1 mM pepstatin A, 0.1 mM aprotinin, 1 mM EDTA and 0.2 mM PMSF. Extracts containing 15 μg of protein were separated on 7.5% SDS polyacrylamide gels, and then transferred onto nitrocellulose membranes [23]. After blocking with 5% non-fat dried milk in TBS buffer (Tris-buffered saline) with 0.01% Tween 20 for 1 h, membranes were probed with primary antibody overnight at 4 °C followed by secondary antibody for 1 h at room temperature. Anti-phospho-STAT-1 and anti-STAT-1 antibodies (Transduction Labs, Lexington,

KY) were used at a dilution of 1:2000 and the anti-NOS2, anti-COX2, c-Jun, p50 and p65 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:4000. All antibodies were diluted in 2.5% milk in TBS/Tween buffer. Horse-radish-peroxidase-conjugated goat anti rabbit IgG (BioRad, Hercules, CA), diluted 1:20,000 in 1% milk in TBS, was used as the secondary antibody. Antigen–antibody complexes were visualized on the blots using enhanced chemiluminescence (ECL) reagents (NEN Life Science Products).

2.5. NOS2 and AP-1 luciferase reporter assays

The origin and use of the murine NOS2 promoter/luciferase reporter construct in keratinocytes has been reported previously by our laboratories [23]. Similar methods were used to assess the effects of the mercuriopsoralen on AP-1 using an AP-1 luciferase reporter construct (pAP-1-luc, Promega). With both the NOS2 and AP-1 reporter constructs, the cells were co-transfected with a *Renilla* luciferase plasmid (pRL-TK, Promega) as a control. Results are reported as the ratio of firefly to *Renilla* luciferase activity in the extract.

2.6. Gel shift assays

Briefly, cells were scraped from the culture dishes, centrifuged (400 × *g*, 10 min) and the pellets resuspended in 400 μL of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 10 μg/mL, pepstatin, 2 μg/mL aprotinin, 1 mM DTT, 2 μg/mL 1-chloro-3-tosylamido-7-amino-2-heptanone, and 10 mM sodium molybdate). After 15 min on ice, NP40 was added to a final concentration of 0.5%. The sample was then mixed rapidly, the nuclei sedimented (4000 × *g*, 1 min), and supernatants containing cytosolic proteins collected. The pellet was resuspended in 100 μL of nuclear extract buffer B (hypotonic buffer A supplemented with 20% glycerol and 0.4 M KCl) and shaken vigorously at 4 °C for 30 min. After centrifugation (13,000 × *g*, 15 min), nuclear extracts were collected and stored at −70 °C. For gel shift assays, 15 μg of nuclear extract protein was incubated with 1 ng of ^{32}P-labeled GAS probe, (sense, 5′-GATCGATTTCCCCGAAT-3′, antisense, 5′-CATGATTTCGGGGAATC-3′), ^{32}P-labeled AP-1 (sense, 5′-GATCCTTCGTGACTCAGCGGGATCCTTCGTGACTCAGCGG-3′, antisense, 5′-GATCCCGCTGAGTCACGAAGGATCCCGCTGAGTCACGAAG-3′) or ^{32}P-labeled NF-κB (sense, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, antisense, 5′-GCCTGGGAAAGTCCCCTCAACT-3′) at room temperature for 15 min in a reaction volume of 25 μl containing 30 μg BSA and 3 μg poly (dI/dC) in binding buffer (7.5% glycerol, 1 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 35 mM NaCl, and 7.5 mM HEPES, pH 8) [24]. The DNA:protein complex was separated on 4.5% native polyacrylamide gels. Binding specificity was determined by competition with excess (100×) unlabeled oligonucleotide. For supershift assays, the extracts were preincubated for 10 min with 1 μg of appropriate antibodies prior to their addition to the reaction mix. Radioactive bands were detected by autoradiography with Kodak MS film.

2.7. Assay for nitric oxide production and nitric oxide synthase assay

Nitric oxide production by IFN-γ stimulated keratinocytes was quantified by the accumulation of nitrite in the cell culture medium using the Greiss reaction with sodium nitrite as the standard as previously described [25]. Nitric oxide synthase activity was measured as the formation of citrulline from arginine as previously described [18] using a modification of the assay of Bredt and Schmidt [26].

2.8. RT-PCR

RNA was isolated from the keratinocytes using TriZol reagent (Gibco BRL, Rockville, MD) following the manufacturer's instructions. First strand cDNA synthesis utilized Superscript II (Gibco BRL) with 0.4 μg RNA in each reaction. PCR was then performed using 1 μL cDNA, NOS2 mouse upstream primer: 5′-GCTCATCCGGTACGCTGCTA-3′ and downstream primer: 5′-TCCAATCTCGGTGCCCATGTA-3′ (Ambion Gene-Specific Relative RT-PCR kit) and Taq DNA polymerase (GIBCO BRL). PCR amplification was performed according to the manufacturer's instructions using 18S as an internal standard. The PCR products were amplified using a 1 min hot start at 95 °C, and 25 cycles of 30 s at 95 °C, 30 s at 56 °C and 60 s at 74 °C and a final extension at 74 °C for 10 min. Products were separated by electrophoresis using 6 M urea polyacrylamide gels followed by autoradiography. Data were captured using the Eagle Eye II digital documentation system (Stratagene, La Jolla, CA).

All experiments were repeated at least twice with similar results. One representative experiment is shown.

3. Results

3.1. Inhibition of nitric oxide production by iodomercurio-H_2TMP

Initially, we examined the effects of iodomercurio-H_2TMP on nitric oxide biosynthesis in mouse keratinocytes. The psoralen was found to inhibit nitric oxide production in a concentration-dependent manner (Fig. 2, IC_{50} = 0.8 μM). These effects were not due to cytotoxicity as determined by MTS and trypan blue assays, or to growth inhibition (data not shown). Iodomercurio-H_2TMP also

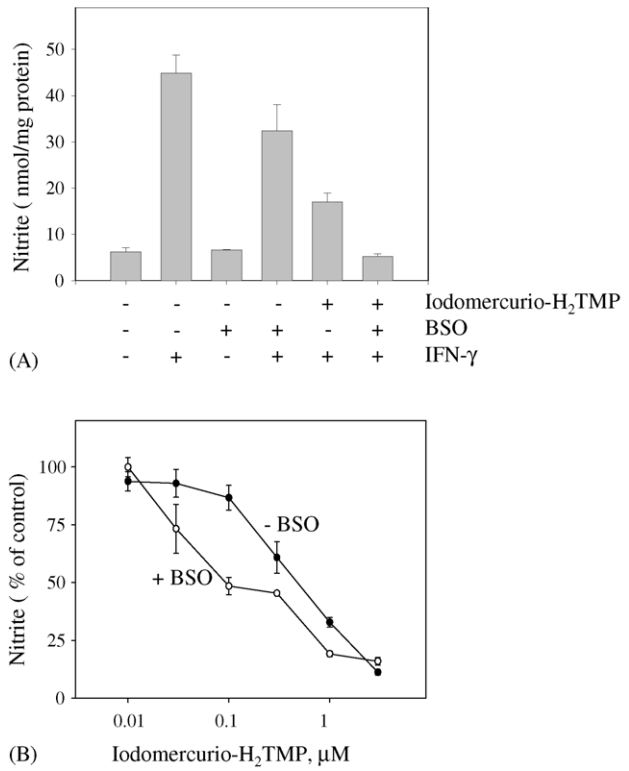

Fig. 2. Effects of iodomercurio-H_2TMP on nitric oxide biosynthesis in keratinocytes. (A) Cells were preincubated without and with BSO (10 μM) for 6 h and then with iodomercurio-H_2TMP (1 μM). After 30 min, IFN-γ (100 U/mL) was added. Nitrite was assayed in the cell culture medium 18 h later. (B) Cells were treated as in A except with increasing concentrations of the psoralen. Each point is the mean ± S.E. of triplicate samples. Experiments were repeated two times with similar results. One representative experiment is shown.

had no direct effect on the activity of NOS2. Thus, in enzyme assays, we found that the rate of citrulline formation from arginine in psoralen (3 μM) treated extracts was not significantly different from untreated extracts (5.1 ± 0.3 nmoles/(mg protein h) versus 4.6 ± 0.5 nmoles/(mg protein h), respectively). The psoralen did, however inhibit IFN-γ-induced NOS2 protein expression (Fig. 3, panel A). This was correlated with a decrease in steady state expression of NOS2 mRNA (Fig. 3, panel B). Inhibition of NOS2 was not due to a generalized suppression of gene expression in the cells as evidenced by the fact that iodomercurio-H_2TMP had no significant effect on the induction of STAT-1 or phospho-STAT-1 protein by IFN-γ (Fig. 3, panel C). COX-2, a protein constitutively expressed in the cells, was also unaltered by psoralen treatment (Fig. 3, panel D).

Organic mercurials are known to react with intracellular sulfhydryls [27]. We found that the activity of iodomercurio-H_2TMP was dependent on intracellular GSH. Thus, treatment of the cells with BSO, which reduced GSH by approximately 75% (0.24 ± 0.02 mmole GSH/10^6 control cells versus 0.06 ± 0.01 mmole GSH/10^6 in BSO-treated cells, mean ± S.E., $n = 3$), increased the sensitivity of the cells to the inhibitory effects of the psoralen on nitric oxide production (IC_{50} = 0.1 μM, Fig. 2), as well as expression of NOS2 mRNA and protein (Fig. 3, panel B). These data suggest that iodomercurio-H_2TMP acts to inhibit NOS2 expression at a sulfhydryl sensitive site. Concentrations of the psoralen that inhibited nitric oxide biosynthesis did not directly alter intracellular levels of GSH (0.22 ± 0.02 mmole GSH/10^6 treated control cells versus 0.25 ± 0.03 mmole GSH/10^6 cells treated with 3 μM iodomercurio-H_2TMP, mean ± S.E., $n = 3$), indicating that this was not responsible for the effects of the psoralens on NOS2. Also of interest was the finding of increased expression of NOS2 mRNA in GSH-depleted cells. This may be due to oxidative stress which has previously been reported to induce expression of nitric oxide synthase [28].

3.2. Effects of iodomercurio-H_2TMP on IFN-γ-induced signaling

Initially, we determined if iodomercurio-H_2TMP interfered with IFN-γ signaling in transient transfection assays using a wild-type NOS2 promoter/luciferase reporter construct. A *Renilla* luciferase construct was used as a control. In previous work, we reported that these constructs were highly active in the keratinocytes [23]. We found that the psoralen, at concentrations of 1 and 3 μM inhibited NOS2 promoter activity by 61 and 81%, respectively, but not control promoter activity (data not shown). These data suggest that iodomercurio-H_2TMP is able to interfere with IFN-γ-mediated signal transduction. Binding of IFN-γ to its receptor activates a receptor-associated kinase known as Jak [29]. This leads to tyrosine phosphorylation of the transcription factor STAT-1 which translocates to the nucleus and binds regulatory elements on the promoter regions of IFN-γ responsive genes [30]. In the keratinocytes, we found that IFN-γ readily induced phosphorylation of STAT-1 (Fig. 4, panel A). Iodomercurio-H_2TMP had no effect on this activity indicating that it did not inhibit early events in IFN-γ signaling.

In further studies, we analyzed the effects of iodomercurio-H_2TMP on STAT-1 binding to the IFN-γ-activated sequence (GAS), an important transcriptional regulatory element in the NOS2 promoter [30,31]. Using mobility shift assays, we found that IFN-γ readily induced GAS binding activity in the keratinocytes (Fig. 4, panel B). Supershift assays demonstrated that the STAT-1 was phosphorylated in the oligonucleotide binding complex. Treatment of the cells with iodomercurio-H_2TMP had no effect on STAT-1 DNA binding activity (Fig. 4, panel B). Since depletion of intracellular GSH increased the sensitivity of the cells to iodomercurio-H_2TMP, we next determined if this treatment altered IFN-γ-induced STAT-1-GAS binding activity. GSH depletion had no effect on this activity except at the highest concentration of iodomercurio-H_2TMP tested (3 μM). This concentration of psoralens was significantly greater than that required to inhibit nitric oxide

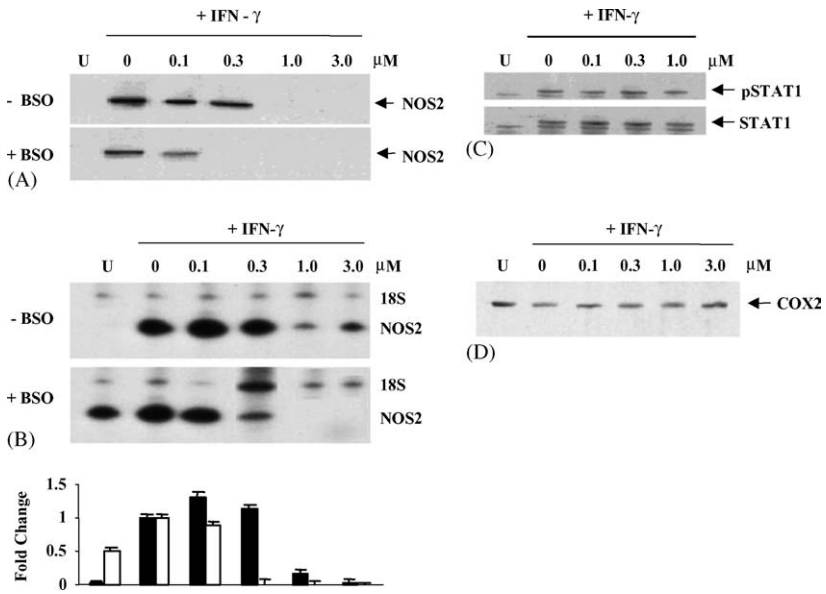

Fig. 3. Effects of iodomercurio-H_2TMP on NOS2 protein and mRNA expression in keratinocytes. (A) Effect of iodomercurio-H_2TMP on expression of NOS2 protein. Cells were treated with increasing concentrations of psoralens for 30 min followed by IFN-γ (100 U/mL, 18 h) and then analyzed for NOS2 expression by western blotting; U, untreated cells. (B) Effects of iodomercurio-H_2TMP on expression of NOS2 mRNA. Cells, treated as described above, were analyzed for NOS2 mRNA by RT-PCR. The fold change in NOS2 mRNA levels in BSO treated (open bars) and untreated (closed bars) cells was determined following normalization to 18S RNA. Graphed data in this panel are the mean ± S.E. ($n = 3$). (C) Effect of iodomercurio-H_2TMP on STAT-1 expression and phosphorylation. Cells were treated as above and analyzed for STAT-1 and phospho-STAT-1 (pSTAT1) by western blotting. (D) Effect of iodomercurio-H_2TMP on cyclooxygenase-2 (COX-2) expression. Cells were treated with increasing concentrations of the psoralen and then analyzed for COX-2 expression by western blotting. Each experiments was repeated two times with similar results. One representative experiment is shown.

production (Fig. 2). These data are consistent with the lack of inhibitory effects of the psoralen on nuclear localization and phosphorylation of STAT-1 (Fig. 4, panel A).

Activation of the transcription factor NF-κB has also been reported to be critical for expression of the NOS2 gene [31]. Analysis of keratinocytes by mobility shift assays showed that NF-κB was constitutively activated in these cells (Fig. 4C, left panel). LPS (1 μg/ml) did not further increase NF-κB activity (Fig. 4C, right panel). Supershift assays demonstrated that the complex consisted of p50 and p65 NF-κB subunits (Fig. 4C, right panel). As observed with STAT-1-GAS DNA binding activity, treatment of the cells with iodomercurio-H_2TMP had no significant effect on NF-κB DNA binding activity (Fig. 4C, left panel). NF-κB DNA binding was also unaffected by depletion of keratinocytes of GSH with BSO (Fig. 4C, left panel).

It is well known that the activity of the psoralens is enhanced by treatment with UVA light [3]. UVA light by itself was found to block nitric oxide production by the keratinocytes (data not shown) and thus, we were unable to compare the effects of the psoralen on nitric oxide production with and without UVA light treatment. However, despite its effects on nitric oxide production. UVA light treatment had no effect on STAT-1-GAS nuclear DNA binding activity in mobility shift assays, either in control or GSH-depleted cells (Fig. 4, panel B). Interestingly, UVA light was found to reduce NF-κB activity in both control and psoralen-treated cells (Fig. 4, panel C) and this may be an important mechanism underlying UVA light induced inhibition of nitric oxide production. A further decrease in NF-κB activity was observed at high concentrations of the psoralen and ultraviolet light.

Like NF-κB, AP-1 was also found to be constitutively activated in the keratinocytes (Fig. 5 and not shown). In mobility shift assays, AP-1 was supershifted with Jun antibodies, and inhibited with an unlabeled competitor probe. Unexpectedly, we found that iodomercurio-H_2TMP caused a dose-dependent inhibition of AP-1 binding activity (Fig. 5). To confirm these results, we examined AP-1 activity in the cells using an AP-1 luciferase reporter construct in transient transfections assays. We found that 0.3 and 1 μM concentrations of the psoralen inhibited AP-1 activity in the cells by 51 and 79%, respectively. In further studies, using gel shift assays, we found that the psoralen was significantly more effective in cells depleted of GSH with BSO. BSO alone had no affect on AP-1 binding activity. Taken together, these results indicate that AP-1 is a target for iodomercurio-H_2TMP and that this transcription factor is essential for IFN-γ-induced expression of keratinocyte NOS2.

4. Discussion

In the present studies, we characterized the activity of a novel psoralen derivative, iodomercurio-H_2TMP. This compound was found to be an effective inhibitor of nitric oxide biosynthesis in mouse keratinocytes. Unlike most psoralens, UVA light was not required for its biological

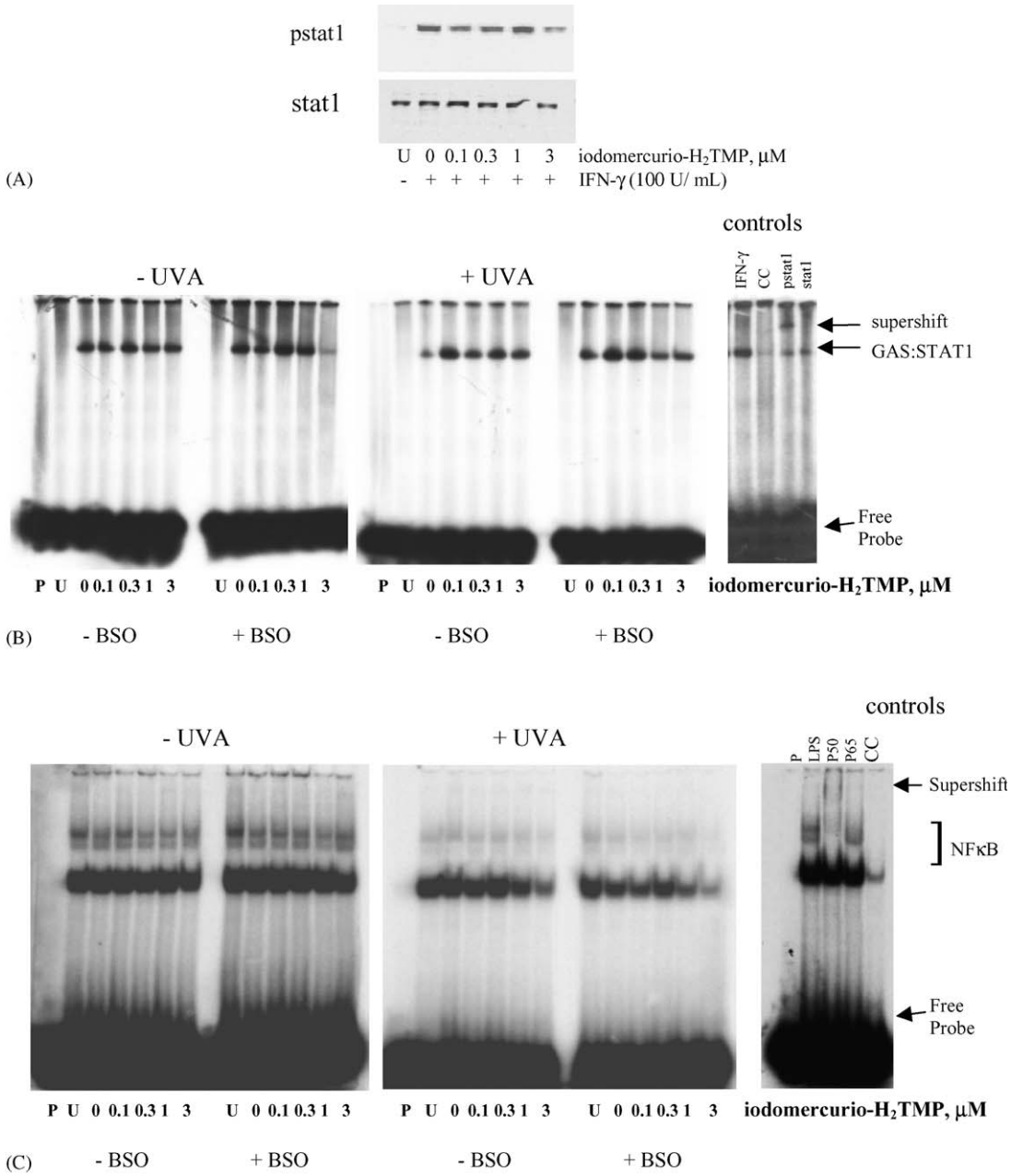

Fig. 4. STAT-1 activity and gel shift analysis of keratinocyte GAS and NF-κB binding proteins. Keratinocytes were incubated with increasing concentrations of iodomercurio-H_2TMP with and without UVA light. Some cultures were pretreated with BSO (10 μM, 6 h) prior to treatment with the psoralen. (A) Effect of iodomercurio-H_2TMP on STAT-1 and phospho-STAT-1 expression in nuclear extracts of PAM212 cells. Cells were treated with the psoralen in the absence and presence of IFN-γ (100 U/mL, 30 min). Nuclear extracts were then prepared and analyzed for STAT-1 and phospho-STAT-1 expression using western blotting; (B) effect of iodomercurio-H_2TMP on GAS:STAT1 binding. Following psoralen and/or UVA light treatment, cells were incubated with IFN-γ (100 U/mL, 30 min) and nuclear extracts prepared and analyzed in gel shift assays using a GAS sequence. In the control gel (right panel), a sample from cells treated with IFN-γ was analyzed in supershift assays using antibodies to either STAT-1 or phosphorylated STAT-1. Binding specificity was determined by incubation of the reaction mix with an excess (100×) of unlabelled competitor probe (CC). P, free probe; U, untreated cells (all other cells were treated with IFN-γ). (C) Effect of iodomercurio-H_2TMP on NF-κB binding. Nuclear extracts were analyzed using an NF-κB sequence. In the control gel (right panel), a sample from cells treated with LPS (1 μg/mL) was analyzed in supershift assays with antibodies to the p50 and p65 subunits of NF-κB. Binding specificity was determined by incubation of the sample with an excess (100×) of unlabeled competitor probe (CC). Each experiments was repeated two times with similar results. A representative experiment is shown.

activity which is most likely due to the mercurial substituent present in the compound. This is supported by our findings that an analog of iodomercurio-H_2TMP that lacks the mercury iodide (4,5′,8-trimethyl-4′,5′-dihydropsoralen) displayed no activity as an inhibitor of keratinocyte nitric oxide production (unpublished studies). Mercury has a high affinity for sulfur suggesting that cellular sulfhydryls are an important target for these compounds. In this

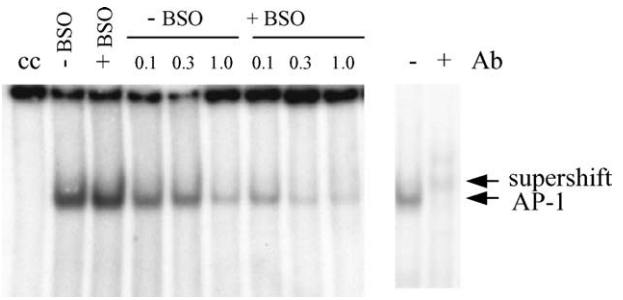

Fig. 5. Gel shift analysis of keratinocyte AP-1 binding. Keratinocytes were treated with iodomercurio-H_2TMP and/or BSO as described in the legend to Fig. 4. Nuclear extracts were prepared and analyzed for AP-1 using mobility shift assays as described in Section 2. Binding specificity was determined by incubation of the reaction mix with an excess (100×) of unlabelled competitor probe (cc). For supershifts, antibodies to Jun were added to the mobility shift assay. The experiment was repeated two times with similar results. A representative experiment is shown.

regard, we found that depleting the cells of GSH increased their sensitivity to iodomercurio-H_2TMP. It is well recognized that organic mercurials form complexes with thiol containing amino acids and proteins [27,32]. Similarly, when iodomercurio-H_2TMP was directly reacted with GSH or cysteine, thiol adducts were formed. However, these derivatives were inactive as inhibitors of nitric oxide biosynthesis indicating that the biological activity of iodomercurio-H_2TMP was dependent on the free mercury-iodide substituent on the psoralen (unpublished studies). Based on these findings, it appears that inhibition of nitric oxide production is not due to the formation of amino acid and peptide metabolites of iodomercurio-H_2TMP in keratinocytes.

Most inhibitors of nitric oxide biosynthesis target the enzyme, nitric oxide synthase [14,15]. In IFN-γ-treated keratinocytes, nitric oxide production is mediated by NOS2, the inducible form of the enzyme, and selective inhibitors of NOS2 have been described [14]. Unexpectedly, despite the fact that NOS2 contains thiol residues, iodomercurio-H_2TMP had no effect on its enzymatic activity. These data indicate that this psoralen is not an enzyme inhibitor and it does not appear to modify the enzyme. Moreover, if thiol adducts are formed, they do not denature the enzyme or interfere with cofactor binding and substrate utilization. Interestingly, iodomercurio-H_2TMP was found to reduce IFN-γ-induced expression of NOS2 protein. This was not due to a generalized inhibition of protein synthesis since the psoralen did not alter cellular STAT-1 expression or [^{3}H]-leu uptake into acid insoluble protein in the cells at concentrations causing inhibition of NOS2 expression (unpublished studies). Iodomercurio-H_2TMP did, however, cause a marked reduction in steady state expression of NOS2 mRNA. This is presumably due to inhibition of the synthesis of NOS2 mRNA which is thought to regulate expression of the protein [30]. However, it is also possible that iodomercurio-H_2TMP alters NOS2 mRNA turnover and this remains to be determined. The effects of iodomercurio-H_2TMP appeared to be specific for NOS2. Thus, as indicated above, the psoralen had no significant effect on induction of STAT-1 or constitutive expression of COX-2 at concentrations that inhibited nitric oxide production by the cells. Interestingly, the inhibitory effects of the psoralen on NOS2 mRNA and protein expression were augmented in cells depleted of GSH. This is consistent with our findings that nitric oxide production is reduced in GSH-depleted cells and supports the idea that this psoralen acts at a sulfhydryl-sensitive target.

A potential mechanism underlying the decrease in steady state NOS2 mRNA is that iodomercurio-H_2TMP interferes with IFN-γ-mediated signaling pathways leading to expression of the message. IFN-γ functions via a specific cell surface receptor consisting of a complex of at least two subunits [29]. Binding of IFN-γ causes oligomerization of the subunits initiating a signal that activates Jak1 and Jak2 receptor associated protein tyrosine kinases, phosphorylation of the IFN-γ receptor intracellular domain, followed by phosphorylation and activation of STAT-1, a latent transcriptional factor binding to GAS sequences in IFN-γ-sensitive genes including NOS2 [25,27]. We found that iodomercurio-H_2TMP had no major effect on STAT-1 phosphorylation or activation of GAS binding activity in keratinocytes, even after depletion of GSH, except at high concentrations. These data indicate that this psoralen does not interfere with IFN-γ receptor binding, oligomerization or phosphorylation. Similarly, the activity of NF-κB binding activity, another transcription factor important in the activation of NOS2 [30,31], was unaltered by treatment of the cells with the psoralen. Unlike GAS binding activity, NF-κB is constitutively expressed in the keratinocytes. Since NOS2 can be induced in the cells, it appears that NF-κB binding activity is not sufficient by itself to induce expression of the enzyme and additional transcription factors including GAS binding activity are required [30,31]. Alternatively, post-translational modifications, which are known to regulate NF-κB binding activity, may be involved in the process [33]. AP-1 was also constitutively expressed in the keratinocytes and this is likely to be important for activation of the NOS2 gene [34]. Inhibition of its activity in the psoralen-treated cells suggests that AP-1 is required for expression of NOS2. Further inhibition of AP-1 in GSH depleted cells suggests that the psoralen acts to inhibit the activity of the transcription factor on a sulfhydryl-sensitive site.

An important specific intracellular target for psoralens is a 22,000 molecular weight membrane and cytoplasmic receptor [6,7]. This receptor is known to be involved in the regulation of epidermal cell growth. Activation of the receptor by photoactivated psoralens down-regulates receptors for the epidermal growth factor (EGF) intrinsic tyrosine kinase and stimulates an EGF receptor serine kinase activity [8–10], processes that can be important in

suppressing the abnormal growth of cells in epidermal proliferative diseases such as psoriasis [35]. We have reported that EGF down-regulates nitric oxide production in keratinocytes and it is possible that the psoralen receptor is involved in this process [17]. We speculate that iodomercurio-H_2TMP may function by binding to and activating the psoralen receptor via sulfhydryl sensitive residues. However, at present, we cannot exclude the possibility that additional thiol containing proteins mediate the action of this compound. It should be noted that iodomercurio-H_2TMP has the potential to intercalate into DNA and thus, it may complex with thiol-sensitive DNA binding proteins. In this regard, the psoralens may also intercalate into the DNA probes used in the electrophoretic mobility shift assays although, if this was the case, then this activity had minimal biological effects.

There has been great interest in developing therapeutics that target the nitric oxide pathway [14]. Nitric oxide is produced in the skin and reduced proliferation in psoriatic lesions has been reported using the nitric oxide synthase inhibitor, N^G-monomethyl-arginine [36]. At the present time, it is uncertain if iodomercurio-H_2TMP will be useful therapeutically. Alkyl mercurials such as the mercury diuretics have been used clinically for many years [32]. However, concerns over toxicity of the heavy metal remain and this may be a problem with iodomercurio-H_2TMP. Further studies on the disposition of mercury derived from this compound are necessary before considering its use in the clinic. Nevertheless, the current studies suggest that iodomercurio-H_2TMP is useful as a model for elucidating pathways regulating nitric oxide production, as well as the action of the psoralens. It is important to note that although our studies demonstrated that iodomercurio-H_2TMP was effective in suppressing IFN-γ-induced NOS2 protein expression and nitric oxide production, we cannot exclude the possibility that this psoralen also inhibits expression of other IFN-γ-inducible genes. This could occur if the inhibitor was selective for the IFN-γ signaling pathway.

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