Cyclosporin A Exacerbates Skin Irritation Induced by Tributyltin by Increasing Nuclear Factor κB Activation

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In searching for pharmacologic agents able to reduce xenobiotic-induced skin irritation, we found that cyclosporine A exacerbates the skin irritation induced by tributyltin. We previously demonstrated the involvement of interleukin-1α and tumor necrosis factor α in tributyltin-induced skin irritation. Here, we show that cyclosporine A (28 mg per kg), at a dose that results in systemic immunosuppression, potentiates tributyltin-induced skin irritation through increased tumor necrosis factor α production, associated with increased tributyltin-induced activation of transcription factor nuclear factor κB in cyclosporine-A-treated mice. On the other hand, under the same experimental conditions, cyclosporine A prevented the elicitation phase of oxazolone-induced contact allergy, but was ineffective in preventing benzalkonium-chloride-induced skin irritation. Using a murine keratinocyte cell line (HEL30) we demonstrated, also *in vitro*, that the cyclosporine A potentiates tributyltin-induced nuclear factor κB activation and cytokine production, this being preceded by an increase in cellular oxidative activity, essential for nuclear factor κB activation, that is time and dose (0.1–10 μM) dependent. This effect was not exclusive to tributyltin but could be extended to other mitochondrial poisons such as sodium arsenate. It has been reported that cyclosporine A binds to cyclophilins. An 18-mer antisense phosphorothioate oligodeoxynucleotide was used to target mitochondrial cyclophilin D mRNA. After 24 h exposure to the oligonucleotide, the amount of cyclophilin D in the cells was decreased by 54% as judged by Western blot analysis. Cyclophilin D suppression prevented cyclosporine A potentiation of tributyltin-induced cellular oxidative activity, indicating the key role of the binding of cyclosporine A to mitochondrial cyclophilin D in mediating this effect. ***Key words: organitans/cyclophilin/mitochondria/keratinocytes. J Invest Dermatol 117:1627–1634, 2001***

Depending on the country, dermatoses comprise 20%–70% of all occupational diseases. Irritation of the skin is important, and it is commonly thought to account for approximately 60%–80% of the clinically recognized cases of human contact dermatitis, most of the remainder being allergic contact dermatitis (Wahlberg, 1996). Occupational and accidental exposure to tributyltin (TBT), a biocidal agent used mainly in wood preservation and marine antifouling paints, results in skin and eye irritation, and severe dermatitis has been reported after direct TBT contact with the skin (reviewed by World Health Organization, 1990).

The biochemical mechanisms involved in skin irritation are complex. Skin inflammatory reactions are under the control of a network of cytokines and lipid mediators (Boss and Kapsenberg, 1993; Corsini and Galli, 1998). We have previously demonstrated the involvement of interleukin-1α (IL-1α) (Corsini *et al*, 1996b) and tumor necrosis factor α (TNF-α) (Corsini *et al*, 1997) in TBT-induced skin irritation in the mouse. Both cytokines if injected into the skin independently trigger cutaneous inflammation (Kupper, 1990). Furthermore, both IL-1 and TNF-α are functionally relevant to a variety of inflammatory skin diseases, both in rodents and in humans (Wakefield *et al*, 1991; Groves *et al*, 1995; 1996; Gniadecki, 1998). Inducible expression of cytokines is controlled by the activity of transcription factors (Muegge and Durum, 1990). In particular, nuclear factor κB (NF-κB) and activator protein-1/c-*jun* (Rhoades *et al*, 1992) have been implicated in TNF-α and IL-1α expression. We have shown earlier that TBT is able to induce, both *in vitro* and *in vivo,* a dose-dependent NF-κB activation, which is followed by cytokine production (Corsini *et al*, 1996a; 1997). Furthermore, TBT skin edema and TNF-α production were significantly reduced by topical treatment with dexamethasone and pentamidine, two anti-inflammatory agents, with the dexamethasone decreasing TBT-induced NF-κB activation (Corsini *et al*, 1997). Triorganotins affect mitochondrial activity, inhibiting ATP synthesis, disturbing the proton gradient, and generating reactive oxygen species (ROS), and induce mitochondrial swelling (Snoeij *et al*, 1987; Marinovich *et al*, 1990; Corsini *et al*, 1996a). ROS are involved in the activation of transcription factors, such as NF-κB and AP-1, and in the production of cytokines (Baeuerle and Henkel, 1994). We were able to demonstrate that TBT-induced NF-κB activation and cytokine production are dependent on mitochondria (Corsini *et al*, 1996a; 1998), depletion of which by prolonged treatment with ethidium bromide resulted in a dramatic reduction of TBT-induced NF-κB activation and IL-1α produc-

Manuscript received December 29, 2000; revised July 30, 2001; accepted for publication August 4, 2001.

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Abbreviations: cremophor, cremophor:ethanol 10:1; CyA, cyclosporine A; DCFH, dichlorodihydrofluorescein diacetate di(acetomethyl ester); EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; TBT, tributyltin.

tion. This indicates that mitochondria serve as mediators of the effect of TBT on ROS generation.

In searching for other pharmacologic agents able to modulate TBT-induced skin irritation, we found that cyclosporine A (CyA), a potent immunosuppressant, exacerbates skin irritation induced by TBT rather than preventing it. CyA is a lipophilic, cyclic fungal undecapeptide that is extensively used as immunosuppressive agent in the prevention of allograft rejection and in a variety of autoimmune diseases (Kahan, 1989). CyA is also highly effective in the treatment of psoriasis, atopic dermatitis, and many other inflammatory dermatoses (Ellis *et al*, 1991; Berth-Jones, 1996; Ruzicka, 1996). CyA was chosen because *in vitro* it inhibits mitochondrial permeability transition-pore formation and subsequent mitochondrial swelling by displacing bound cyclophilin D and decreasing the Ca^{2+} sensitivity of the pore (Griffiths and Halestrap, 1991; Savage *et al*, 1991; Broekemeier *et al*, 1992). Because of this protective effect on mitochondria and the importance of mitochondria in TBT toxicity, we examined the effect of CyA on TBT-induced skin irritation.

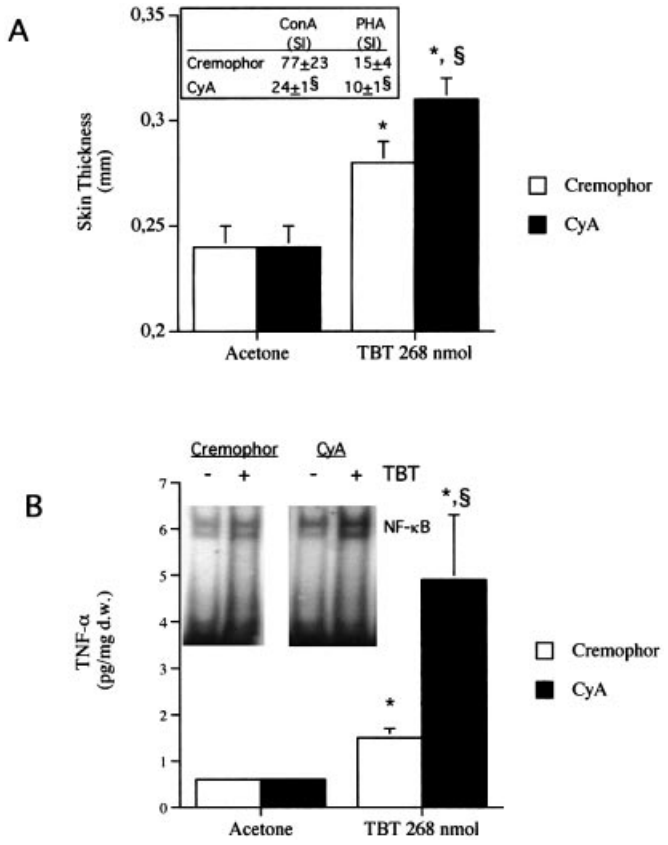


Figure 1. CyA exacerbates TBT-induced skin irritation. Mice were treated by intraperitoneal injection with CyA (28 mg per kg) or cremophor:ethanol (cremophor) as vehicle control 24 and 3 h before topical application of TBT (268 nmol) or acetone as vehicle control. Skin thickness (*A*) and TNF-α content (*B*) were measured 2 h after TBT application. (*A*) *Inset* shows the lymphoproliferative response to ConA and PHA stimulation following CyA or vehicle treatment. Mean ± SD ($n = 3$). Student's t test, *$p < 0.05$ *versus* vehicle control; §$p < 0.05$ *versus* animals treated with TBT and cremophor. (*B*) *Inset* shows the NF-κB activation 5 min after TBT application. Equal amounts (5 μg) of nuclear extract were analyzed by EMSA, with a ^{32}P-labeled DNA probe detecting the binding activity of NF-κB.

MATERIALS AND METHODS

Chemicals Tributyltin chloride was obtained from Aldrich (Steinhein, Germany), cyclosporine A was kindly provided by Novartis (Basel, Switzerland), cremophor, benzalkonium chloride, 4-ethoxy-methylene-2-phenyloxazol-5-one (oxazolone), and sodium arsenate from Sigma (St. Louis, MO), and dichlorodihydrofluorescein diacetate di(acetomethyl ester) (DCFH) from Molecular Probes (Eugene, OR). All reagents were purchased at the highest purity available. For the *in vivo* studies CyA was dissolved in cremophor:ethanol (10:1; cremophor) and TBT in acetone, whereas for the *in vitro* studies CyA and TBT were both dissolved in dimethylsulfoxide (DMSO).

Animals Female BALB/C mice 9–12-wk-old (Charles River, Calco, Italy) were used throughout these studies. Mice were randomized into treatment groups, housed three to four per cage over wood-chip bedding, and allowed food and water *ad libitum*. Before treatment mice were quarantined for 2 wk and were acclimatized to a 12 h light–dark cycle.

Animal exposure Animals ($n = 3$–4) were injected intraperitoneally with 200 μl of CyA (11.4 or 28 mg per kg) or cremophor:ethanol (10:1) as vehicle control 24 and 3 h before TBT or acetone treatment. This schedule of treatment was chosen to guarantee a systemic distribution and effect of CyA. Mice received different amounts (134–268 nmol) of TBT dissolved in 10 μl of acetone on both sides of both ears or an equal volume of acetone alone. The doses were chosen from data in the literature (Middleton, 1982). Ears were treated on both sides to maximize induction of cytokine production. At various times after treatment, mice were sacrificed, the increase in ear thickness was measured with a micrometer, and the ears were removed. One ear was used to determine the dry weights, measured after heating at 105°C for 24 h. The other ear was used either for histology or for the TNF-α content of whole ear, determined in a skin homogenate prepared as previously described (Corsini *et al*, 1997). For histology, ears were fixed in phosphate-buffered saline (PBS, pH 7.4) with 10% formalin, dehydrated, and embedded in paraffin. Hematoxylin–eosin-stained sections were analyzed under light microscopy.

To assess the effect of CyA on benzalkonium-chloride-induced skin irritation, mice were treated with CyA (28 mg per kg) as above described, and then 10 μl of benzalkonium chloride or acetone was applied on both sides of the ears. Animals were killed 24 h later and ear skin thickness was measured.

To assess the effect of CyA on oxazolone-induced contact allergy, mice were shaved on their dorsal surface and the following day sensitized by topical application of 100 μl of 2% oxazolone dissolved in 4:1 acetone:olive oil mixture (AOO). Four days after sensitization, mice were treated with CyA (28 mg per kg) as above described. On day 5 after CyA mice were challenged with 10 μl of 0.5% oxazolone by topical

Table I. CyA exacerbates TBT-induced TNF-α production *in vivo*[a]

Time (h)	Cremophor/acetone (pg per mg dry weight)	CyA/acetone (pg per mg dry weight)	Cremophor/TBT (pg per mg dry weight)	CyA/TBT (pg per mg dry weight)
1	0.5 ± 0.1	0.6 ± 0.1	1.9 ± 0.1**	4.8 ± 1.5**,§
2	0.7 ± 0.1	0.7 ± 0.1	2.5 ± 0.9*	20.9 ± 5.3**,§§
4	0.9 ± 0.1	0.8 ± 0.2	1.8 ± 0.4*	4.0 ± 0.5**,§§
24	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2

[a]Three female BALB/C mice per group were injected intraperitoneally with cremophor or CyA 11.4 mg per kg body weight 18 and 3 h before topical application of acetone or TBT 134 nmol. The animals were sacrificed at different times thereafter. Total ear TNF-α (per mg dry weight) was measured as cytotoxicity against sensitive L929 cells (Rosenthal *et al*, 1995). Results are expressed as mean ± SD. Statistical analysis by Dunnett's test: *$p < 0.05$ and **$p < 0.01$ *versus* the relative control groups; §$p < 0.05$ and §§$p < 0.01$ *versus* cremophor/TBT group.

application to both sides of the right ear. AOO was applied to the left ear as vehicle control. Immunologic responses to oxazolone were assessed 24 h after challenge by measuring the increase in skin thickness.

Assay for TNF-α TNF-α content was assayed by determining the cytotoxicity of TNF-α against sensitive L929 cells, as previously described (Rosenthal and Corsini, 1995). The results are expressed in pg per mg dry tissue. TNF-α concentration was calculated against a standard curve with known amounts of recombinant murine TNF-α (R&D Systems).

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA) Nuclear extracts were prepared from chopped ear skin as previously described (Corsini *et al*, 1997). The supernate containing nuclear proteins was stored at –80°C until used in the EMSA. EMSA was performed as follows. Binding reaction mixtures (20 μl) containing 5 μg protein of nuclear extract, 0.5 μg poly(dI-dC).poly(dI-dC) (Sigma), and 10,000 cpm ^{32}P-labeled probe in binding buffer [10 mM HEPES pH 7.9, 50 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, 10% (vol/vol) glycerol, 1% Ficoll, and 0.2 μg per ml albumin] were incubated for 30 min at room temperature before separation in a 7% acrylamide gel in 1 × Tris borate EDTA followed by autoradiography. A double-stranded oligonucleotide containing the binding site for NF-κB (5′-GTCTCGCAATTCCCCTCTCTCAG-3′) was labeled with α-^{32}P-dATP (Amersham, Buckinghamshire, U.K.) using T4 polynucleotide kinase (Amersham). We have previously characterized the specificity of the probe used (Corsini *et al*, 1996a).

Lymphoproliferative assays Spleens were removed and single-cell suspensions were prepared in culture medium. Aliquots containing 2×10^6 cells were added to 96-well flat bottom microtiter plates.

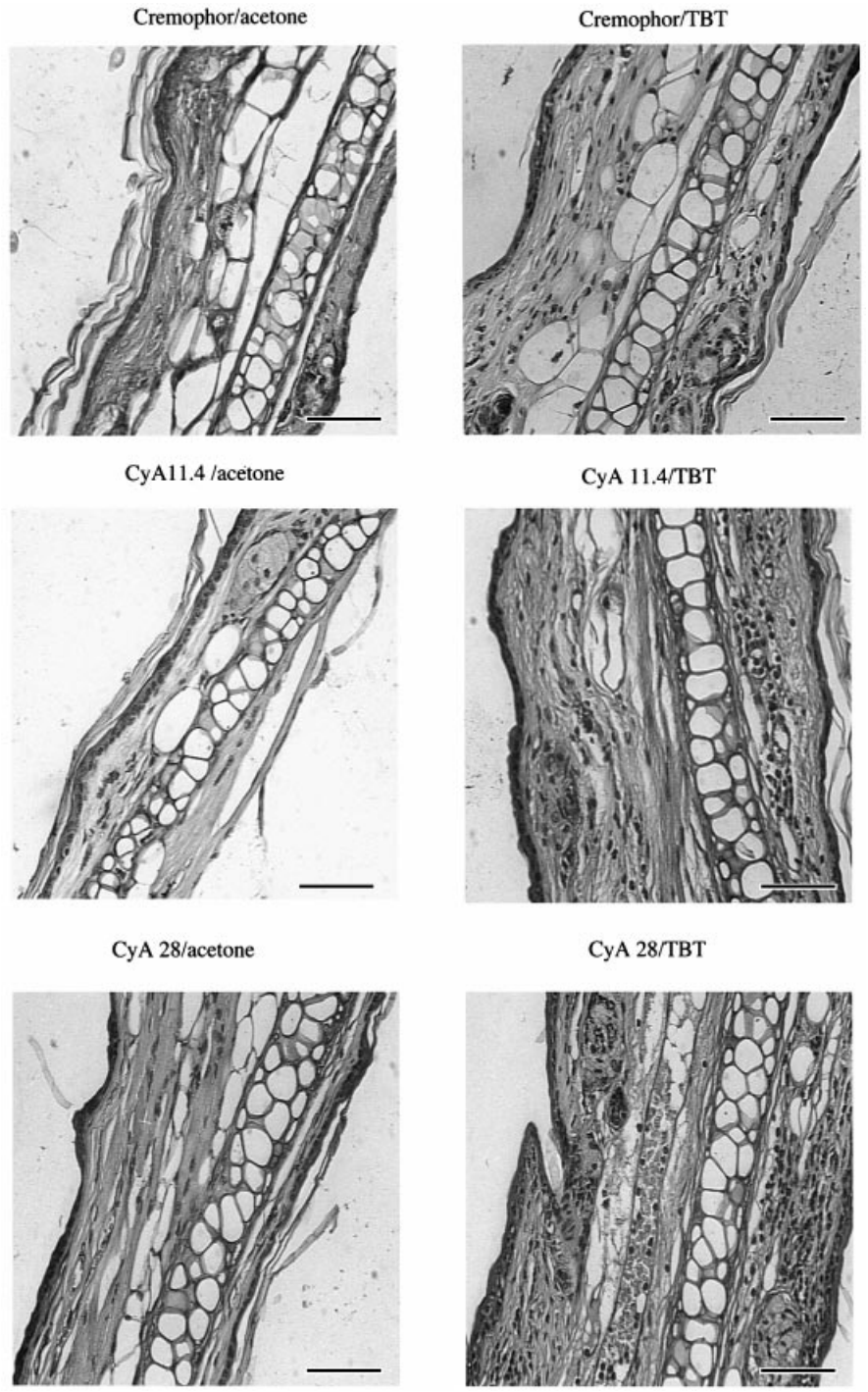
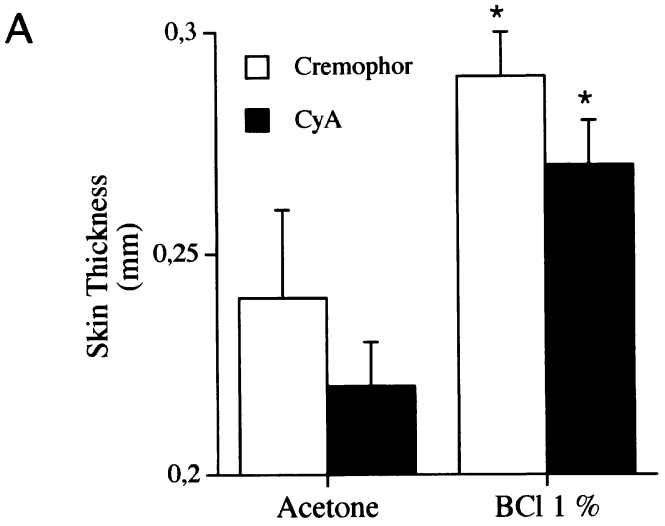


Figure 2. Hematoxylin and eosin staining of ear following CyA and TBT treatment. Mice were treated by intraperitoneal injection with CyA (11.4 and 28 mg per kg) or cremophor:ethanol (cremophor) as vehicle control 24 and 3 h before topical application of TBT (268 nmol) or acetone as vehicle control for 2 h. *Scale bar*: 50 μm.



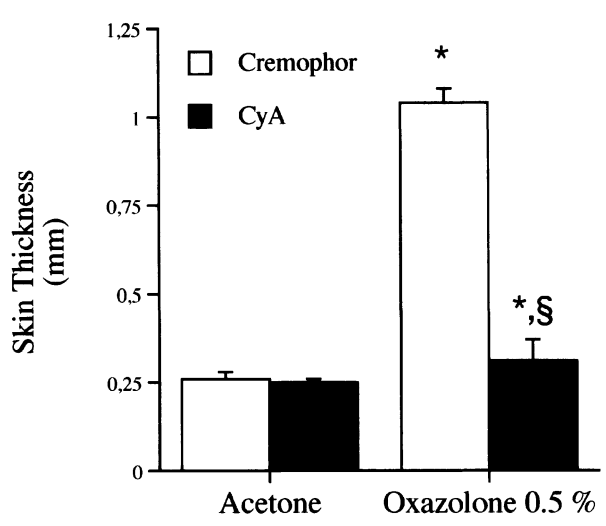


Figure 3. CyA acts via a mechanism that appears to involve immunomodulation rather than mere nonspecific anti-inflammatory effects. Mice were treated by intraperitoneal injection with CyA (28 mg per kg) or cremophor:ethanol (cremophor) as vehicle control 24 and 3 h before topical application of benzalkonium chloride (1%) or challenged with oxazolone (0.5%) or vehicle control. Skin thickness was measured 24 h after application. Mean ± SD ($n = 4$). (*A*) Effect of CyA on benzalkonium-chloride-induced skin irritation; (*B*) effect of CyA on oxazolone-induced contact allergy. Student's *t* test, *p < 0.05 *versus* vehicle control; §p < 0.05 *versus* animals treated with oxazolone and cremophor.

Lymphocytes were stimulated with either 2 μg per ml concanavalin A (ConA; Boehringer Mannheim, Germany) or 1.2% of phytohemagglutinin (PHA; Gibco, Piasley, U.K.). The blastogenic response was determined by the ability of cells to incorporate ^{3}H-thymidine into DNA as described previously in detail (Smialowicz, 1995). Results are expressed as the stimulation index SI, calculated as follows:

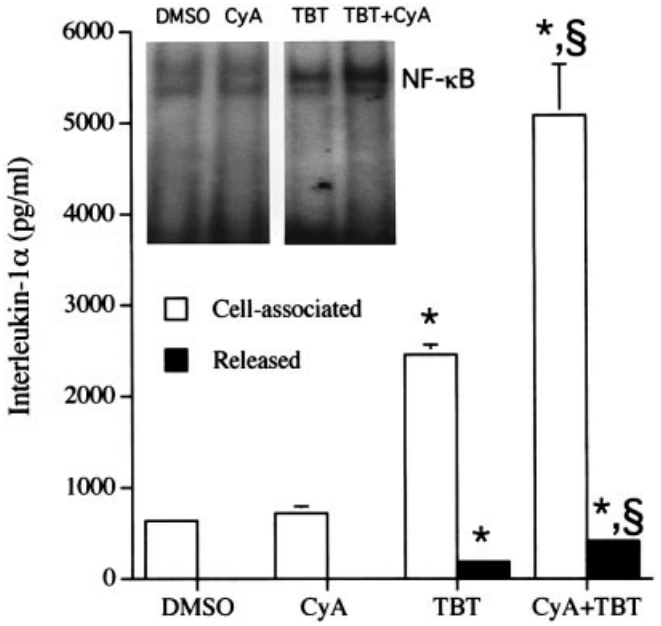


Figure 4. Action of CyA and TBT on a murine keratinocyte cell line. Cells were treated for 1 h with CyA (10 μM) or DMSO as vehicle control and then TBT (2.5 μM) was added for 30 min for NF-κB activation or for 24 h for IL-1α production, both released and cell-associated. Mean ± SD ($n = 3$). Student's *t* test, with *p < 0.05 *versus* vehicle control, §p < 0.05 *versus* cells treated with TBT. *Inset*, NF-κB activation. *Lane 1*, cells + DMSO; *lane 2*, cells + CyA; *lane 3*, cells + TBT; *lane 4*, cells + CyA + TBT. Equal amounts (5 μg) of nuclear extract were analyzed by EMSA, with a ^{32}P-labeled DNA probe detecting the binding activity of NF-κB.

$$SI = (cpm)_{stimulated}/(cpm)_{unstimulated}$$

Cell culture and treatment Murine keratinocyte cell line HEL30 (kindly supplied by Dr. N.E. Fusenig, Cancer Research Center, Heidelberg, Germany) was cultured as previously described (Corsini *et al*, 1994). Cells were mantained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin (100 U per ml), and streptomycin (100 μg per ml) (medium) at 37°C in a 5% CO_2 humidified incubator.

For the measurement of oxidative activity (see below) cells were grown to confluence on sterile round glass slips (Knittel Glaser, Germany) in a 24-well plate (Corning, NY). For the determination of IL-1α content, confluent cells were treated for 1 h at 37°C with 0.3 ml of medium containing CyA or DMSO as vehicle control and then TBT (2.5 μM) dissolved in DMSO was added. The DMSO concentration in the culture medium never exceeded 0.1%. After 24 h of incubation monolayers were washed once with PBS and lyzed in 0.3 ml PBS containing Triton X-100 (0.5%), and IL-1α was determined by enzyme-linked immunosorbent assay (ELISA) (see below). To assess the effect of CyA on TBT- or As-induced NF-κB activation, confluent cells in 60 mm Petri dishes were incubated at 37°C for 1 h with CyA (10 μM), and then TBT (2.5 μM) or As (50 μM) was added for 30 min and nuclear cell extracts were prepared as previously described (Corsini *et al*, 1996a).

To reduce cyclophilin D expression an 18-mer antisense oligonucleotide (Primm, Milan, Italy) corresponding to nucleotides 561–578 in the rat cyclophilin D sequence (Woodfield *et al*, 1997) was utilized: 5′-TATTTTCTTCACAACATC-3′ (antisense). As control, the sense complement oligonucleotide was used. Both oligonucleotides contained phosphorothioate linkages to limit degradation. Confluent cells were treated for 24 h with 3 μM phosphorothioate oligonucleotide in culture medium (Doyle *et al*, 1999) and then cyclophilin D immunoreactivity and cellular oxidative activity were evaluated as described. The treatment was not cytotoxic for the cells as assessed by lactated dehydrogenase leakage (data not shown).

Western blot analysis For the immunoreactivity of cyclophilin D, monolayers in a 60 mm Petri dish were washed once with PBS and

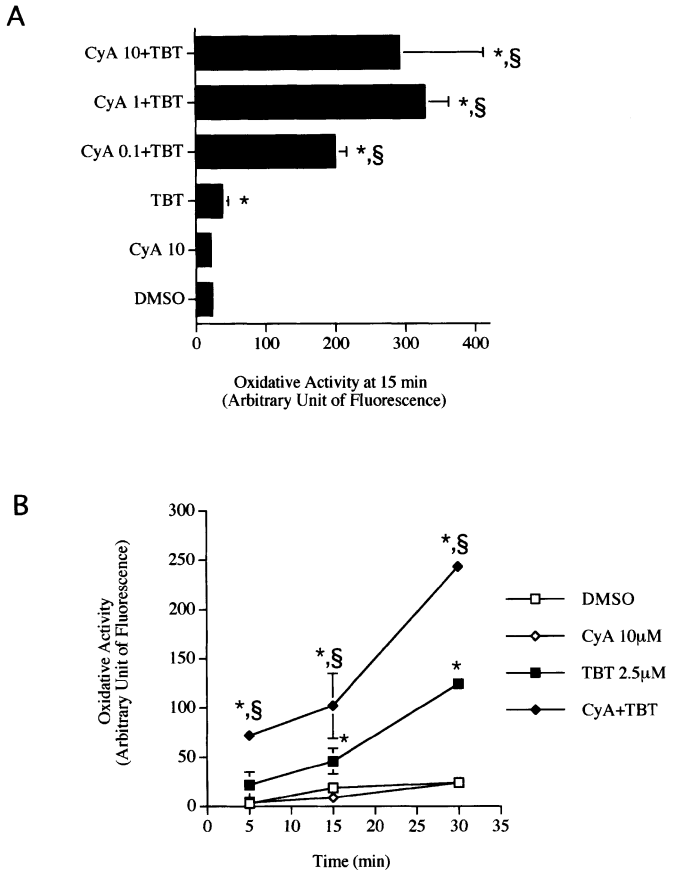


Figure 5. CyA potentiates TBT-induced cellular oxidative activity in live cells, as measured by DCFH oxidation. Confluent HEL30 cells were loaded for 1 h with 10 μM DCFH and treated with different concentrations of CyA (0.1–10 μM) and then TBT (2.5 μM) or DMSO as vehicle control was added for different times. (*A*) Effects 15 min after TBT treatment; (*B*) time course of TBT-induced oxidative activity. Mean ± SD ($n = 3$). Dunnett's *t* test, *p < 0.05 *versus* vehicle control; §p < 0.05 *versus* cells treated with TBT.

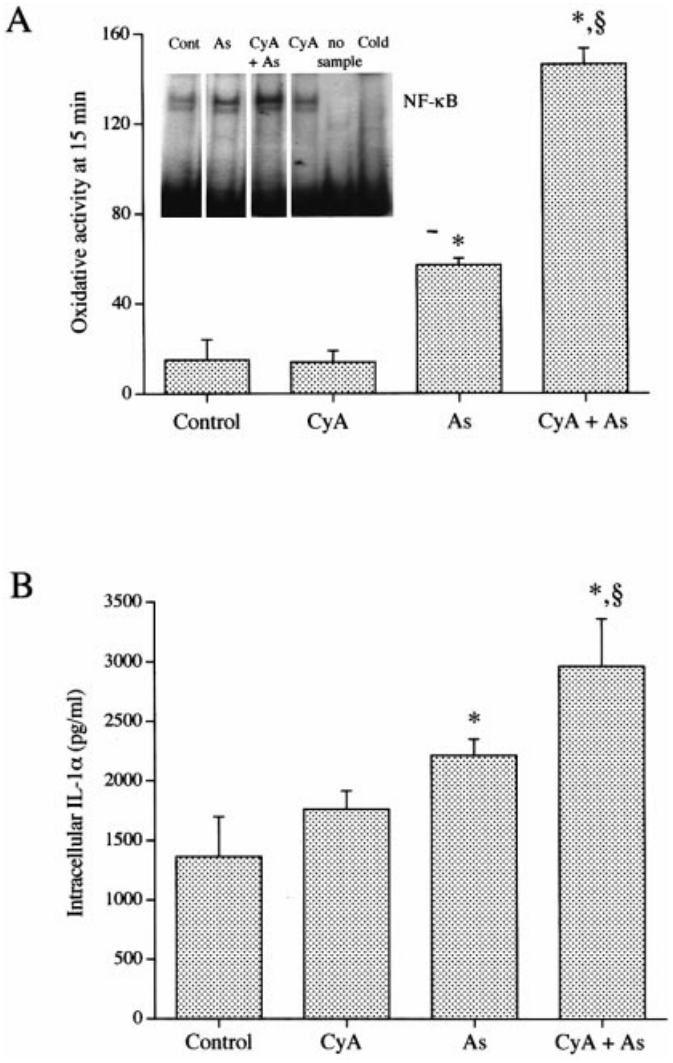


Figure 6. CyA potentiates sodium-arsenate-induced oxidative activity, NF-κB activation, and IL-1α production in HEL30 cells. (*A*) Oxidative activity and NF-κB activation following CyA (1 μM) and sodium arsenate (50 μM). For cellular oxidative activity, confluent HEL30 cells were loaded for 1 h with 10 μM DCFH and treated with CyA (1 μM), and then As (50 μM) was added for 15 min. For NF-κB activation, confluent cells were treated for 1 h with CyA (1 μM) or DMSO as vehicle control and then As (50 μM) was added for 30 min. Equal amounts (5 μg) of nuclear extract were analyzed by EMSA, with a ^{32}P-labeled DNA probe detecting the binding activity of NF-κB. In *lane 5* no nuclear extract was added (no sample), whereas in *lane 6* to the control nuclear extract a 100-fold excess of cold probe was used to demonstrate NF-κB complex specificity (cold). (*B*) Cell-associated IL-1α. Confluent cells were treated for 1 h with CyA (1 μM) or DMSO as vehicle control and then As (50 μM) was added for 24 h for IL-1α. Mean ± SD ($n = 3$). Dunnett's *t* test, *p < 0.05 *versus* vehicle control; §p < 0.05 *versus* cells treated with As.

scraped in PBS; after centrifugation, the pellet was lyzed in 100 μl of homogenation buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5% Triton X-100, 50 μM phenylmethylsulfonyl fluoride, 2 μg per ml aprotinin, 1 μg per ml pepstatin, and 1 μg per ml leupeptin) and denatured in 100 μl of Laemmli sample buffer (Laemmli, 1970) for 5 min at 100°C. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad, Richmond, CA). Ten micrograms of protein were then electrophoresed into a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were transferred to PVDF membrane (Amersham, Little Chalfont, U.K.) in 20 mM Tris, 150 mM glycine, 20% methanol, 0.03% SDS. The cyclophilin D was visualized using a cyclophilin D antiserum diluted at 1:500 as the primary antibody and developed using enhanced chemiluminescence according to the manufacturer's instruction (Amersham). Molecular weight references were obtained by running one lane with prestained standard (Amersham). The image of the immunoblotting was acquired with a Nikon CCD video camera module. The optical density of the bands was calculated and analyzed by means of the Image 1.47 program for digital image processing (Wayne Rasband, Research Service Branch, NIMH, NIH, Bethesda, MD).

Oxidative activity in living cells Confluent cells were washed once with Hank's balanced salt solution without phenol red (HBSS) containing 2% bovine serum albumin and were loaded with DCFH 10 μM (stained) or DMSO (unstained) for 1 h at 37°C in the presence or absence of different concentrations of CyA (0.01–10 μM). Then cells were washed once with HBSS and oxidative activity was assessed as follows. Glass coverslips in quartz cuvettes containing 2 ml of HBSS were treated with TBT (2.5 μM), sodium arsenate (50 μM), or DMSO as vehicle control. ROS production was measured as DCFH oxidation by the intensity of the emission of 525 nm fluorescence excited by 503 nm (Perkin Elmer LS 50B), at time 0 and 5, 15, and 30 min following treatment both in stained and unstained cells. Results are expressed as the change in fluorescence (in arbitrary units, AU) calculated as follows:

$$AU = (I_{stained} - I_{unstained})_{tx} - (I_{stained} - I_{unstained})_{t0}$$

where I represents the intensity of fluorescence.

ELISA IL-1α content was measured by a specific sandwich ELISA as previously described (Corsini *et al*, 1996b). The limit of sensitivity for this assay is 15 pg per ml. Results are expressed as pg per ml. Preliminary

Table II. CyA prevents TBT-induced changes in mitochondrial membrane potential[a]

Treatment	Median channel fluorescence
Control DMSO	73.1 ± 8.6
CyA 1 μM	78.7 ± 4.4
TBT 2.5 μM	52.2 ± 4.8*
CyA + TBT	69.8 ± 2.3§§

[a]HEL30 cells were treated for 1 h at 37°C in the presence or absence of CyA (1 μM). TBT (2.5 μM) or DMSO as vehicle control was then added. After 30 min at 37°C, culture medium was removed and cells were loaded with rhodamine 123 10 μg per ml in medium for 30 min at 37°C. Cells were then analyzed as described in the *Materials and Methods*. Mean ± SD ($n = 3$). Statistical analysis by Dunnett's test: *$p < 0.05$ *versus* control; §§$p < 0.01$ *versus* TBT.

studies showed that the maximum TBT or CyA concentrations tested did not interfere with the ELISA.

Mitochondrial potential Rhodamine 123 (Sigma) was used to detect changes in mitochondrial membrane potential following TBT and CyA treatment in HEL30 cells (Ronot *et al*, 1986). Confluent cells in 24-well plates were treated for 1 h at 37°C in the presence or absence of CyA (1 μM). Then cells were treated with TBT (2.5 μM) or DMSO as vehicle control. After 30 min at 37°C, culture medium was removed and cells were loaded with rhodamine 123 10 μg per ml in medium for 30 min at 37°C. Cells were then rinsed, trypsinized, suspended in PBS, and analyzed on a FACScalibur flow cytometer (Becton Dickinson Italia, Milan, Italy). Forward angle light scatter and integral green fluorescence were measured for 10,000 events. The median channel fluorescence for each histogram was determined by integral analysis with the CellQuest software interfaced to the flow cytometer and was used to compare the effects of CyA on TBT-induced changes in the mitochondrial potential.

Statistical analysis All experiments were performed at least twice; representative results are shown. Statistical significance was determined by Student's *t* test or Dunnett's multiple comparison test as indicated, after ANOVA.

RESULTS

CyA exacerbates TBT-induced skin irritation **Figure 1(*A*)** shows that CyA at a dose (28 mg per kg) that results in system immunosuppression (*inset*), actually exacerbates TBT-induced skin irritation, as measured by increased skin thickness 2 h after TBT treatment. This could be explained by an increase, at the same time point, of TBT-induced TNF-α production (**Fig 1*B***). The increase in TNF-α was associated with increased NF-κB activation (*inset*), which is essential for TNF-α neosynthesis.

A similar effect has also been observed at lower CyA (11.4 mg per kg) and TBT (134 nmol) doses. As shown in **Table I**, TNF-α production by CyA + TBT follows the same kinetics as cremophor + TBT, but the effect is much more striking with CyA, which also produced a significantly greater increase in skin thickness at 24 h than did cremophor (0.35 ± 0.01 *vs* 0.31 ± 0.01 mm, $p < 0.05$; 0.27 ± 0.02 mm was the skin thickness in both acetone mice groups). The potentiation of TBT-induced skin irritation by CyA was confirmed by histopathologic examination of ear skin, which revealed a dose-related increase in neutrophil infiltration following CyA plus TBT treatment (**Fig 2**).

CyA prevents oxazolone-induced contact allergy but is ineffective in benzalkonium-chloride-induced skin irritation Under the same experimental conditions, CyA (28 mg per kg) was ineffective in preventing benzalkonium-chloride-induced skin irritation (**Fig 3*A***), but effectively prevented the elicitation phase of oxazolone-induced contact allergy (**Fig 3*B***). These data are consistent with the idea that CyA acts via a mechanism that appears to involve immunomodulation rather than mere nonspecific anti-inflammatory effects (Anderson, 1985), and suggest that CyA-induced exacerbation of skin irritation is specific for TBT.

Role of ROS and mitochondria in CyA-induced cytokine production To better characterize and understand the effect of CyA in TBT-induced skin irritation, we used a murine keratinocyte cell line (HEL30) that has been reported to contain cellular binding sites for CyA (Gschwendt *et al*, 1987). In this cell line, similarly CyA potentiated TBT-induced NF-κB activation (**Fig 4**, *inset*) as well as TBT-induced IL-1α production (**Fig 4**). Under these experimental conditions, CyA alone did not affect ROS production, NF-κB activation, or cytokine production.

We have previously shown that mitochondria-derived ROS play a fundamental role in TBT-induced NF-κB activation and cytokine production (Corsini *et al*, 1996a, 1997, 1998). Here we demonstrated that CyA-induced NF-κB activation following TBT treatment is preceded by a dose (0.1–10 μM) and time dependent increased cellular oxidative activity (**Fig 5*A***, ***B***), which activates NF-κB. This effect was shown by mitochondrial poisons other than TBT such as sodium arsenate. Also in this case, we have previously shown that mitochondria play a key role in ROS production, NF-κB activation, and cytokine production (Corsini *et al*, 1999). As shown in **Fig 6**, CyA potentiates arsenic-induced NF-κB activation (**Fig 6*A***, *inset*), cellular oxidative activity (**Fig 6*A***), and IL-1α production (**Fig 6*B***).

We have previously shown in HEL30 cells that TBT treatment is associated with rapid mitochondrial swelling (Corsini *et al*, 1996a). It is known that *in vitro* CyA is able to inhibit mitochondrial permeability transition-pore formation and subsequent mitochondrial swelling (Griffiths *et al*, 1991; Savage *et al*, 1991; Broekemeier *et al*, 1992). Here, we demonstrated (**Table II**) that TBT 2.5 μM reduced the median fluorescence to 70% of that observed for control cells and CyA completely prevented TBT-induced changes in mitochondrial membrane potential, preserving mitochondrial integrity.

To further characterize the role of mitochondria, an 18-mer antisense phosphorothioate oligodeoxynucleotide was designed to target a translated region of the mitochondrial target of CyA cyclophilin D mRNA. After 24 h exposure to the oligonucleotide, the amount of cyclophilin D in the cells was decreased by 54% ± 14% (mean ± SD of three independent experiments) as judged by densitometric analysis of Western blots (**Fig 7*A***). Cyclophilin D suppression prevented CyA potentiation of TBT-induced cellular oxidative activity (**Fig 7*B***), indicating the key role of the binding of CyA to mitochondrial cyclophilin D in mediating this effect. Control sense oligonucleotide did not affect cyclophilin D expression or cellular oxidative activity (**Fig 7**).

DISCUSSION

We found that CyA exacerbates TBT-induced skin irritation. At a dose that results in system immunosuppression, CyA potentiated TBT-induced skin irritation, as assessed by increased skin thickness and histopathologic evaluation, but effectively prevented oxazolone-induced contact allergy. This exacerbation could be explained by increased TNF-α production in the mice treated with CyA followed by TBT, which was associated with increased NF-κB. Then, in a murine keratinocyte cell line *in vitro* CyA potentiated TBT-induced NF-κB activation and cytokine production, this being preceded by a dose- and time-dependent increased cellular oxidative activity. This effect was also found for sodium arsenate, a mitochondrial poison that, like TBT, stimulates mitochondria to generate ROS, NF-κB, and IL-1α and induces mitochondrial swelling (Corsini *et al*, 1999). A key role of the binding of CyA to mitochondrial cyclophilin D in mediating cellular oxidative activity by TBT was demonstrated.

The concentrations used in our *in vitro* studies (0.1–10 μM) are similar to those achieved following exposure *in vivo*. In particular, it has been demonstrated that skin biopsies obtained from psoriatic patients treated for 7 d with a high dose of CyA (14 mg per kg per d) had intracellular concentrations (based on tissue wet weight) of 2.8 μM (Ellis *et al*, 1991); with lower doses of CyA (3–7.5 mg per

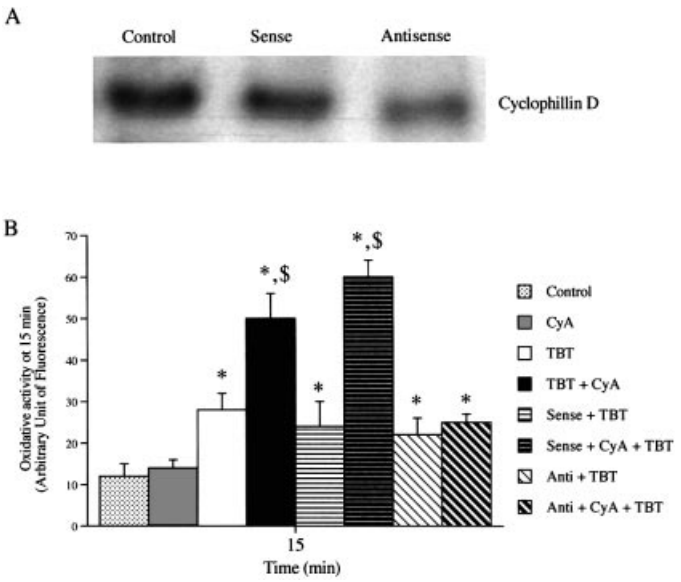


Figure 7. Antisense oligonucleotide suppression of cyclophilin D prevents CyA potentiation of TBT-induced ROS production. (*A*) Representative Western blot analysis of cyclophilin D immunoreactivity in cell homogenates of HEL30 cells treated with medium alone (control), antisense oligonucleotide (3 μM), or sense oligonuleotide (3 μM). Protein was loaded at 20 μg. (*B*) Oxidative activity. For cellular oxidative activity, confluent HEL30 cells were treated for 24 h with medium alone, antisense oligonucleotide (3 μM), or sense oligonucleotide (3 μM), then loaded for 1 h with 10 μM DCFH and treated with CyA (1 μM), and then TBT (2.5 μM) was added for 15 min. Mean ± SD ($n = 3$). Dunnett's t test, *$p < 0.05$ *versus* relative control; §$p < 0.05$ *versus* cells treated with TBT alone.

kg per d), intracellular epidermal CyA concentrations were around 1.0 μM (Cooper *et al*, 1992).

In this investigation we used TBT, a biocidal agent able to induce skin and eye irritation in humans and rodents, because we have previously characterized its molecular mechanism of action, showing *in vivo* that both IL-1α and TNF-α play an important role in TBT-induced skin irritation (Corsini *et al*, 1996b, 1997), and that the production of these pro-inflammatory cytokines by TBT is preceded by NF-κB activation. *In vitro*, we have shown the pivotal role of mitochondria as the source of second messenger molecules important for TBT-induced ROS generation, NF-κB activation, and cytokine production (Corsini *et al*, 1996a, 1998).

CyA is a potent immunosuppressive agent, highly effective in the treatment of several skin diseases, e.g., psoriasis and severe refractory atopic dermatitis (Ellis *et al*, 1991; Berth-Jones, 1996). Besides its effect on T cells, *in vitro* CyA is able to inhibit mitochondrial permeability transition-pore formation and subsequent mitochondrial swelling by displacing bound cyclophilin D and decreasing the Ca^{2+} sensitivity of the pore (Savage *et al*, 1991; Griffiths *et al*, 1991; Broekemeier *et al*, 1992). This protective effect on mitochondria allows complete recovery of mitochondrial membrane potential, and has been shown to prevent cell death in several experimental models (Crompton *et al*, 1992; Ankarcrona *et al*, 1996; Bernardi, 1996). Mitochondrial swelling is a common feature after exposure to organotins (Snoeij *et al*, 1987), and we have shown in HEL30 cells that TBT treatment is associated with rapid mitochondrial swelling (Corsini *et al*, 1996a). This, together with the protective role of CyA on mitochondria, led us to investigate the potential anti-inflammatory effect of CyA on TBT-induced skin irritation. Surprisingly, we found that CyA actually exacerbates TBT-induced skin irritation.

We demonstrated that in its protective role CyA leads to preservation of mitochondrial integrity, as assessed by rhodamine 132 uptake, so that when combined with mitochondrial poisons CyA increases the generation of ROS and pro-inflammatory cytokine production, resulting in an exacerbation of skin reactions. Indeed, by reducing the expression of cyclophilin D, the mitochondrial target of CyA, by antisense oligonucleotide treatment, the potentiation of TBT-induced cellular oxidative activity by CyA was completely prevented. In contrast, the antisense oligonucleotide for cyclophilin A, the cytosolic isoform of rotamase that also binds CyA, was ineffective (data not shown), further supporting the key role of the binding of CyA to mitochondrial cyclophilin D in mediating TBT-induced oxidative stress.

Taken together, these data may contribute to explain the variable response to treatment of human skin diseases with CyA, with some patients showing a paradoxically increased reactivity and some showing no change (e.g., Ellis *et al*, 1991; Chawla *et al*, 1996). We speculate in the nonresponsive patients a concomitant environmental exposure to mitochondrial poisons or in the case of the use of CyA in atopic dermatitis exposure to allergens somehow affecting mitochondria. In this regard, it is important to remember that the skin toxicants arsenic and organotins are also common water contaminants. Nevertheless, even if our *in vitro* data did not show any effect of CyA alone on the parameters considered, we cannot exclude in patients undergoing CyA treatment for skin diseases a different mitochondrial sensitivity to CyA itself, that may result in ROS generation and pro-inflammatory cytokine neosynthesis.

This work has been partially supported by UNIPRO.

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