Role of Mitochondria in Tributyltin-Induced Interleukin-1α Production in Murine Keratinocytes

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Tributyltin (TBT) salts are well known skin irritants in rodents and humans. TBT induced both the intracellular production of interleukin-1α (IL-1α) and its release into culture medium in a murine keratinocyte cell line (HEL30). Here, we report that mitochondria are important for TBT-induced IL-1α production.

Confluent cells were treated with increasing concentrations of TBT (0–2.5 μM) or dimethylsulfoxide as vehicle control. At different times thereafter (0–24 h), nuclear extracts were analyzed for nuclear factor-κB (NF-κB) binding activity by electrophoretic mobility shift assay, and the released and cell-associated IL-1α was measured by enzyme-linked immunosorbent assay. TBT induced a direct and concentration-related activation of NF-κB, which peaked at 2 h and was blocked by pyrrolidinedithiocarbamate, a potent NF-κB inhibitor, and rotenone, an inhibitor of the electron entry from complex I to ubiquinone. Rotenone also induced a concentration-related inhibition of IL-1α synthesis induced by TBT, but rotenone did not completely abrogate TBT-induced IL-1α production, which suggests that other transcription factors may be involved in IL-1α production.

Prolonged treatment with ethidium bromide, an inhibitor of mitochondrial DNA and RNA synthesis, was used to partially deplete cells of functional mitochondria. After 5 d of treatment, mitochondrial conversion of tetrazolium bromide to formazan was reduced by 50%, and IL-1α release was decreased by 65%, whereas no induction of intracellular IL-1α was observed. This effect was not due to inhibition of protein synthesis, because identical incorporation of [^{3}H]leucine into protein in control and ethidium bromide–treated cells was identical. This impairment of mitochondrial metabolism inhibited NF-κB activation by TBT. These findings indicate that mitochondria may be the source of second messenger molecules important for TBT-induced IL-1α production. ***Key words: NF-κB/transcription factors/organotins/reactive oxygen species. J Invest Dermatol 107:720–725, 1996***

The biocidal agent tributyltin (TBT) is used mainly in wood preservation, marine anti-fouling paints, disinfection of circulating industrial cooling waters, and slime control in paper mills. The annual world production of organotin compounds has grown rapidly from 500 tons in 1950 to more than 50,000 tons in 1986 (Snoeij *et al*, 1987). Exposure of workers occurs principally during the manufacture and formulation of TBT compounds, in application and removal of TBT paints, and from the use of TBT in wood preservatives. Exposure of the general public may come from contaminated food, particularly fish and shell-fish, and from domestic application of wood preservatives. Occupational exposure of workers to TBT can result in skin and eye irritation, and severe dermatitis has been reported after direct contact with the skin (reviewed by World Health Organization, 1990).

Skin irritation involves resident epidermal cells, fibroblasts of dermis, and endothelial cells as well as invading leukocytes interacting with each other under the control of a network of cytokines and lipid mediators (Boss and Kapsenberg, 1993). Because of their anatomic location, a role for cytokines, especially interleukin-1α (IL-1α), produced by keratinocytes has been proposed (Kupper, 1990). The hypothesis is that when the epidermis is damaged, active IL-1α is released (Nickoloff *et al*, 1990). This release stimulates further production of IL-1α together with synthesis of other cytokines such as interleukin-6 and interleukin-8 and of intercellular adhesion molecules, thus amplifying the response (Kupper, 1989).

Many other factors increase IL-1α production, including lipopolysaccharide, various toxins, cytokines, ultraviolet radiation, and phorbol esters, but the molecular mechanisms responsible for xenobiotic induction of IL-1α production are not understood. In mouse keratinocytes IL-1α–induced IL-1α gene expression is regulated by tyrosine kinase in combination with serine-threonine kinase, leading to activation of mitogen-activated protein kinase and later converging into activation of transcription factor activator protein-1 (AP-1) (Lee *et al*, 1994).

We have previously shown (Corsini *et al*, 1996) that TBT-induced IL-1α production is partially inhibited by addition for 24 h of a neutralizing antibody against IL-1α, which suggests an autocrine effect of IL-1α on its own production. Because the inhibition is only partial, however, other mechanisms may be involved in TBT-induced IL-1α production. Indeed, we also showed (Corsini *et al*, 1996) that TBT-induced intracellular production of IL-1α can

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Abbreviations: AP-1, activator protein-1; EBr, ethidium bromide; EMSA, electrophoretic mobility shift assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NF-κB, nuclear factor-κB; PDTC, pyrrolidinedithiocarbamate; TBT, tributyltin chloride.

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be modulated by treatment with antioxidants, which suggests a role for oxidative species in the mechanism of action of TBT in IL-1α induction. An early toxic action of TBT in rat skin is to inhibit oxidative metabolic pathways (Middleton, 1982); triorganotins, in general, bind to a component of the adenosine triphosphate (ATP) synthase complex, disturb the proton gradient, and inhibit mitochondrial ATP synthesis (Snoeij *et al*, 1987; Marinovich *et al*, 1990). We hypothesize that this diverts electrons from the respiratory chain into the formation of reactive oxygen species known to be involved in activation of transcription factors and production of cytokines (Ilnicka *et al*, 1993; Lee and Ilnicka, 1993; Baeuerle and Henkel, 1994).

It has also been reported that TBT^+ (a dissociation product of the parent TBTX compound) and O_2^- can combine chemically at room temperature in protein-free medium to produce the [μ-(superoxo)bis(tributylstannyl(I)] radical (Rivera *et al*, 1992); this may also be relevant in an integral cell system, indicating another possible mechanism, that of activating of TBT-induced transcription factors. Inducible expression of IL-1α is controlled by regulation of the activity of transcription factors, mainly nuclear factor-κB (NF-κB) and AP-1 (Muegge and Durum, 1990; Fenton, 1992). Here, we focused our attention on TBT-induced NF-κB activation, because NF-κB is a primary transcription factor and its activation occurs without new protein synthesis.

In the resting cell, NF-κB resides in the cytoplasm as a latent form complexed to the inhibitory protein IκB (Baeuerle and Baltimore, 1988). Following cell stimulation, NF-κB is rapidly activated by its release from IκB, which allows its migration into the nucleus and subsequent binding to DNA. Activation of NF-κB is generally controlled by reactive oxygen species and the cellular redox state and can be modulated by antioxidants (Schreck *et al*, 1991, 1992). NF-κB controls the inducible expression of various genes that are involved in immune responses and inflammatory and cellular defense mechanisms. Target genes include cytokines and their receptors, acute-phase proteins, and several viral enhancers. Because of the pivotal role of IL-1α in immune and inflammatory reactions of the skin, the ability of TBT to induce IL-1α production both *in vivo* and *in vitro* (Corsini *et al*, 1996), and considering that one of the intracellular targets of organotins is mitochondria [reviewed in Snoeij *et al*, 1987), we investigated the role of the latter in TBT-induced IL-1α production.

Here, we report that TBT promptly induced activated transcription factor NF-κB prior to IL-1α production. The depletion of functional mitochondria by long-term treatment with ethidium bromide resulted in a dramatic reduction of TBT-induced NF-κB activation and IL-1α production, which suggests that mitochondria serve as mediators of TBT effects and gene-regulatory signaling pathways.

MATERIALS AND METHODS

Chemicals Tributyltin chloride (TBT) was obtained from Aldrich (Steinhein, Germany); pyrrolidinedithiocarbamate (PDTC), butylated hydroxyanisole, cycloheximide, ethidium bromide (EBr), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and rotenone were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were purchased at the highest purity available.

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 maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Sigma), penicillin (100 U per ml), and streptomycin (100 μg per ml) (medium) at 37°C in a 5% CO_2 humidified incubator.

Confluent cells plated in a 24-well plate (Corning, Inc., Corning, NY) were treated with 0.3 ml of medium containing different concentrations of TBT dissolved in dimethylsulfoxide (DMSO) or with DMSO alone as vehicle control. DMSO concentration in the culture media never exceeded 0.1%. After 24 h of incubation, the culture medium was recovered and centrifuged for enzyme-linked immunosorbent assay (see below) determination of IL-1α release. The monolayers were washed once with PBS and lysed in 0.3 ml of PBS containing Triton X100 (0.5%), and the intracellular IL-1α was determined.

Mitochondrial Function Assay To assess the role of mitochondria in TBT-induced IL-1α production, cells were treated for 1 h with different concentrations of rotenone, an inhibitor of complex-I, and then made 1 μM in TBT; 24 h later, IL-1α production was measured. In addition, to confirm the role of mitochondria as TBT intracellular signal transducers, we depleted cells from functional mitochondria; 10^5 cells per ml were seeded and treated for 5 d with ethidium bromide (200 ng per ml) in medium supplemented with glucose (4.5 mg per ml) and uridine (5 μg per ml), because respiration-deficient cells have been reported to become pyrimidine auxotrophs (King and Attardi, 1989). At this time, cells were confluent and the medium was made 1 μM in TBT or DMSO (0.05% final concentration, vol/vol) was added for 30 min or 24 h and NF-κB activation or IL-1α production was assessed.

To evaluate mitochondrial function, MTT conversion to formazan was carried out as follows: 10^4 cells were seeded in a 96-well plate and treated for 5 d with or without EBr (200 ng per ml), after which TBT (1 μM) was added for 20 h; finally, 10 μl of MTT solution in PBS (5 mg per ml) were added to each well, and MTT conversion to formazan was evaluated spectrophotometrically 4 h later as previously described (Gerlier and Thomaset, 1986). Results are expressed as per cent of control.

Protein Synthesis To assess that the reduction in TBT-induced IL-1α production in EBr-treated cells was not due to inhibition of protein synthesis, [^{3}H]leucine incorporation into protein was evaluated as follows: after 5 d of treatment with or without EBr (200 ng per ml), 1 μCi of [^{3}H]leucine (Amersham, Buckinghamshire, UK) was added to each well in the presence or absence of TBT (1 μM). [^{3}H]Leucine incorporation into protein was assessed 24 h later (Corsini *et al*, 1992). Results are expressed in counts per min/μg of protein. Protein content of cell lysate was measured spectrophotometrically according to Bradford (1976) using a commercially available kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Enzyme-Linked Immunosorbent Assay Costar 3690 plates (Costar, Cambridge, MA) were coated overnight at 4°C with 0.1 ml of monoclonal anti-murine IL-1α antibody (Genzyme, Cambridge, MA) diluted to 1 μg per ml in PBS. PBS containing Tween 20 (0.05%) was used to wash the plates. The washed plates were blocked with 0.1 ml of PBS containing bovine albumin (0.5%) and Tween 20 (0.05%) for 30 min at room temperature. Samples (0.1 ml) at different dilutions or recombinant murine IL-1α (Genzyme) were added for 1 h at room temperature. After three washes, 0.1 ml of rabbit anti-murine IL-1α antibody (Genzyme, 1:800 diluted) was added to each well for 1 h and then 0.1 ml of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) 1:1000 diluted for 1 h at room temperature. *p*-Nitrophenylphosphate (Sigma) was used as substrate, and the absorbance was measured at 405 nm. The limit of sensitivity for this assay was 15 pg per ml. Results are expressed in picograms per ml for extracellular IL-1α and picograms per mg of protein for intracellular IL-1α (to normalize the recovery of cell-associated IL-1α). Preliminary studies showed that the maximum TBT or rotenone concentrations tested did not interfere with the enzyme-linked immunosorbent assay.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA) Nuclear extracts were prepared essentially as described by Schreiber *et al* (1989). Confluent cells in 60-mm petri dishes were washed once with cold PBS after which 2 ml of a hypotonic lysis buffer were added to each dish (buffer A: 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ethylenediamine tetraacetic acid, 0.1 mM phenylmethylsulfonyl fluoride). Cells were incubated on ice for 15 min, after which 125 μl of a 10% Nonidet P-40 solution were added, and cells were scraped, mixed for 15 s, and then centrifuged for 30 s at 12,000 rpm. The pelleted nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% NP-40, centrifuged, and then suspended in 50 μl of buffer C [50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% (vol/vol) glycerol], mixed for 20 min, and centrifuged for 5 min at 12,000 rpm. The supernatant containing nuclear proteins was harvested, protein concentration was determined, and the supernatant was stored at −80°C until used in EMSA. EMSA was performed as follows. Binding reaction mixtures (20 μl) containing 5 μg of protein from nuclear extract, 0.5 μg polydeoxyinosinic-deoxycytidylic acid · polydeoxyinosinic-deoxycytidylic acid (Sigma), 10,000 cpm ^{32}P-labeled probe in binding buffer [10 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 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(hydroxymethyl)aminomethane-borate-ethylenediamine tetraacetic acid followed by autoradiography. A double-stranded oligonucleotide containing the binding site for NF-κB (5′-

Table I. TBT induces IL-1α Production in a Concentration-Dependent Fashion[a]

Concentration (μM)	Entracellular IL-1α (pg/ml)	Intracellular IL-1α (10^3 pg/mg)
0	n.d.	2.18 ± 0.34
0.1	n.d.	3.44 ± 0.46[a]
0.6	65 ± 14	5.38 ± 0.50[a]
1.0	371 ± 28	10.21 ± 1.92[b]
2.5	623 ± 110	7.52 ± 2.25[b]

[a] HEL30 cells were treated with different concentrations of TBT (0.1–2.5 μM) or DMSO as vehicle control (0 μM). After 24 h of treatment the release of IL-1α (extracellular IL-1α) and the cell-associated IL-1α (intracellular IL-1α) were measured by enzyme-linked immunosorbent assay. Mean ± SD of three to four determinations. Statistical analysis by Dunnett's test, [a] $p < 0.05$ and [b] $p < 0.01$ *versus* control (0 μM). n.d., not detectable, below limit of detection.

GTCTCGCAATTCCCCTCTCTCAG-3′) was labeled with [α-^{32}P]dATP (Amersham) using T4 polynucleotide kinase (Amersham).

Electron Microscopy HEL30 cells were treated with TBT (10 μM) or DMSO as vehicle control. After 5 min the cells were fixed in 3.3% glutaraldehyde in 25 μM phosphate buffer for 1 h at 4°C, then treated with 1% osmium tetroxide in phosphate buffer for 1 h at 4°C and dehydrated and embebbed in Araldite epoxy resin (Sigma).

Statistical Analysis All experiments were performed at least twice; representative results are shown. Results are expressed in mean ± SD. Statistical significance was determined by Student's t test or Dunnett's multiple comparison test, as indicated, after analysis of variance.

RESULTS

TBT Induces NF-κB Activation and IL-1α Production A murine keratinocyte cell line HEL30, known to produce IL-1α similarly to murine primary keratinocytes, was examined for its response to TBT. **Table I** shows that TBT induced a concentration-related increase in both intracellular and extracellular IL-1α. Intracellular IL-1α increased from 0.1 to 1 μM, declining thereafter because of cytotoxicity, as indicated by lactate dehydrogenase leakage (data not shown). TBT-induced IL-1α production is not due to endotoxin contamination, as shown by adding polymixin B: the percentage increase of intracellular IL-1α was 357 ± 32 (mean ± SD) and 331 ± 35 for TBT (1 μM) and TBT plus 15 μg polymixin B per ml, respectively.

To assess the role of mitochondria in TBT-induced IL-1α production, we first treated confluent keratinocytes with non-cytotoxic concentrations of rotenone, an inhibitor of the entry of electrons from complex I to ubiquinone. Rotenone significantly reduced both IL-1α release and the intracellular increase induced by 1 μM TBT **(Fig 1)**. Interestingly, no change in constitutive IL-1α levels was observed **(Fig 1*B*)**, which suggests that other mechanisms are probably involved in the maintenance of the constitutive IL-1α level; only the inducible expression of IL-1α is modulated by rotenone.

We next assessed the ability of TBT to induce NF-κB activation. TBT was able to induce a concentration-related activation of NF-κB **(Fig 2*A*)**, that peaked at 2 h and declined thereafter **(Fig 2*B*)**. The decline can be explained by the ability of NF-κB to control the expression of IκB by means of an inducible autoregulatory pathway: it has been reported that the reexpression of IκB is associated with a downregulation of NF-κB DNA-binding activity (Sun *et al*, 1993). p65 has been reported to be a potent inducer of IκB (Sun *et al*, 1993). Preliminary supershift analysis of our nuclear extract indicated p65 as one of the proteins present in the NF-κB dimer, which may explain the decline observed in NF-κB activation. Prior treatment with 10 μM cycloheximide, a protein synthesis inhibitor, did not prevent TBT-induced NF-κB **(Fig 2*A*)**, which indicates that TBT directly activates NF-κB. NF-κB activation preceded IL-1α production, which began 4 h after treatment (Corsini *et al*, 1996). TBT-induced NF-κB activation could be

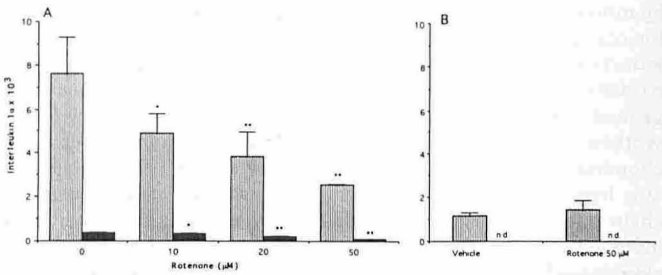

Figure 1. Concentration-dependent reduction of TBT-induced IL-1α production by rotenone. Confluent cells were treated for 1 h with different concentrations of rotenone (0–50 μM) and then TBT (1 μM) or DMSO vehicle control was added for 24 h. **Figure 1*A*** shows the concentration- dependent inhibition by rotenone of TBT-induced IL-1α release and intracellular IL-1α increase, whereas **Fig 1*B*** illustrates the lack of effect of rotenone on IL-1α synthesis in control cells. Values are mean ± SD of three to four determinations. Statistical analysis by Dunnett's multiple comparison test, with $^{*}p < 0.05$ and $^{**}p < 0.01$ *versus* TBT-treated cells (without prior rotenone treatment, 0 μM). n.d., not detectable, below limit of detection. □, intracellular IL-1α; ■, extracellular IL-1α.

modulated by PDTC, a potent antioxidant inhibitor of NF-κB activation, and by butylated hydroxyanisole **(Fig 3)**.

Figure 4 shows the effect of 20 μM rotenone on both TBT-induced NF-κB activation (*inset*) and intracellular IL-1α increase (*bar chart*). As expected, rotenone prevented NF-κB activation induced by TBT, confirming the role of mitochondrial ubiquinone in the generation of reactive oxygen species. Under these experimental conditions, intracellular IL-1α production was only partially inhibited **(Fig 4,** *bar chart*). Taken together these data indicate that inhibition of the mitochondrial formation of reactive oxygen species specifically modulates the activation of NF-κB and IL-1α neosynthesis.

Depletion of Functional Mitochondria Results in a Reduction of TBT Effects Organotin compounds are generally described as powerful metabolic inhibitors because of their ability to interfere with mitochondria (reviewed by Snoeij *et al*, 1987). Indeed, treatment of HEL30 cells with a toxic concentration of TBT (10 μM) resulted in very rapid (5 min) and dramatic morphologic changes in mitochondria: the organelles become round in shape and swollen **(Fig 5)**, confirming mitochondria as an important intracellular target of TBT. Lower concentrations of TBT were not tested.

In order to substantiate further the involvement of mitochondria in both TBT-induced NF-κB activation and IL-1α production, we treated cells for 5 d with ethidium bromide (200 ng per ml), a specific inhibitor of mitochondrial DNA and RNA synthesis (Knight, 1969; Wiseman *et al*, 1978). This procedure has been used to deplete cells of a functioning mitochondrial electron transport system (Nass, 1972; Wiseman *et al*, 1978; Shultze-Osthoff *et al*, 1993). Measurement of the conversion of MTT to formazan, to assess mitochondrial function, revealed that 5 d of treatment with EBr reduced MTT conversion by 50%, whereas no inhibition of protein synthesis was detected **(Table II)**. Addition of TBT (1 μM) in the control group resulted in a slight reduction of MTT conversion, associated with a significant increase in protein synthesis, which is in agreement with TBT-induced IL-1α neosynthesis. On the contrary, the addition of TBT in the EBr-treated group stimulated protein synthesis to a lesser extent, without affecting the effect of EBr on MTT conversion. **Table III** shows IL-1α release induced by TBT was 65% lower ($p < 0.01$) in EBr-treated cells, whereas intracellular IL-1α was at control (EBr 200 ng per ml) levels. This confirms the important role of mitochondria in TBT-induced IL-1α production. It is interesting to note **(Table III)** that EBr treatment increased the intracellular IL-1α level. The respiratory-deficient cells were also examined for the activation of NF-κB

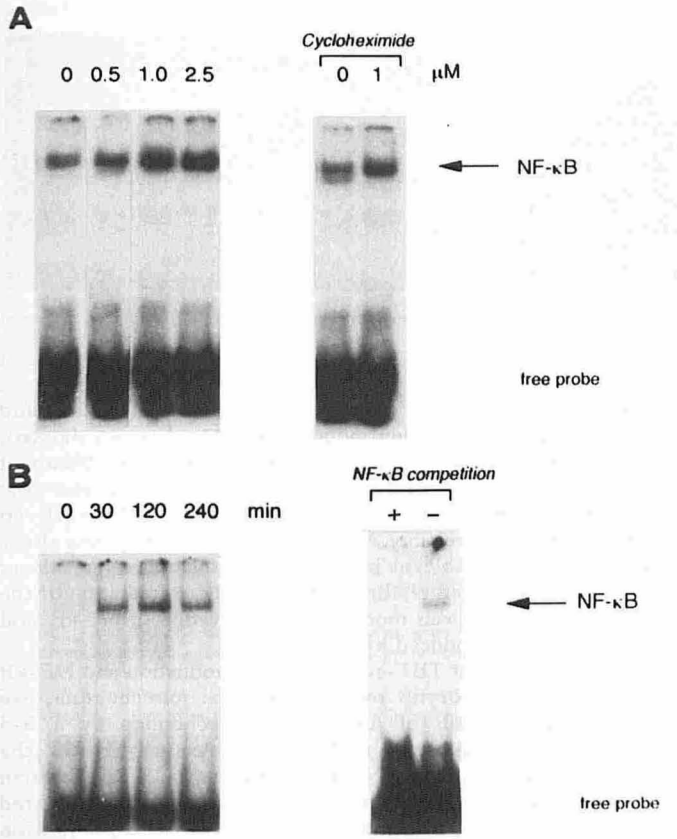

Figure 2. Concentration dependence and time course of TBT-induced NF-κB activation. Confluent cells were incubated in (*A*) for 30 min with different concentrations of TBT (0–2.5 μM) or, in (*B*) for different times (0–4 h) with 1 μM TBT. (*A*) also shows the effect of cycloheximide (10 μM) on TBT-induced NF-κB activation (0, control; 1 μM, TBT), and (*B*) shows the effect of NF-κB competition when a 100-fold excess of cold probe (+) was used to demonstrate NF-κB complex specificity. Cells were treated for 1 h with cycloheximide, and TBT (1 μM) was then added for 30 min. Equal amounts (5 μg) of nuclear extracts were analyzed by EMSA, with a ^{32}P-labeled DNA probe detecting the binding activity of NF-κB.

in response to TBT. In cells treated with TBT for 30 min, TBT induced NF-κB activation in control cells, whereas EBr-treated cells responded only weakly **(Fig 5)**, confirming that depletion of the mitochondrial electron transport system largely abrogates TBT-induced NF-κB activation.

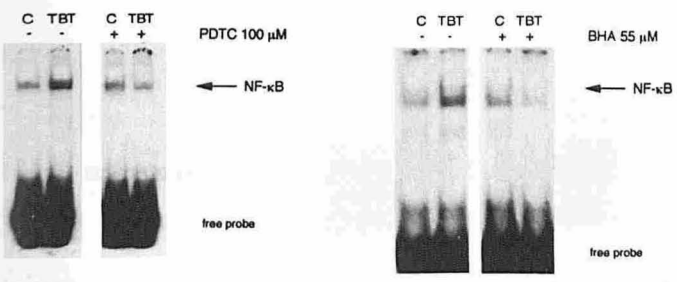

Figure 3. Antioxidants inhibit TBT-induced NF-κB activation. Confluent cells were treated for 1 h with PDTC (+, 100 μM), butylated hydroxyanisole (+, 55 μM), or DMSO vehicle control (−), and 1 μM TBT was then added for 30 min. Equal amounts (5 μg) of nuclear extracts were analyzed by EMSA, with a ^{32}P-labeled DNA probe detecting the binding activity of NF-κB.

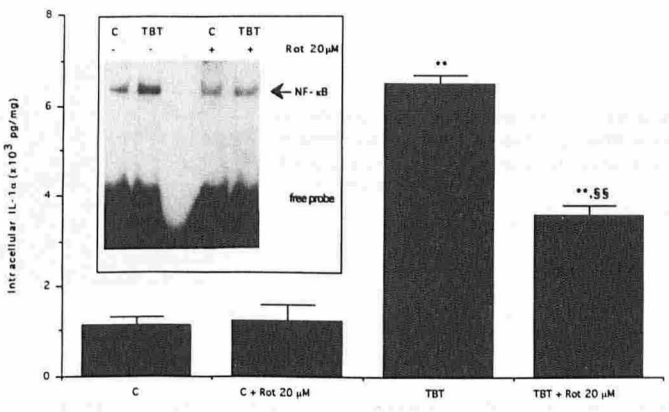

Figure 4. Rotenone inhibits TBT-induced NF-κB activation *(inset)* and intracellular IL-1α content *(bar chart).* Confluent cells were incubated for 1 h with rotenone (+, Rot 20 μM) or vehicle control (−) and then 1 μM TBT or DMSO vehicle control was added for 30 min (NF-κB activation) or for 24 h (intracellular IL-1α). In the *inset* (−) indicates without rotenone and (+) indicates with rotenone. Mean ± SD of three to four determinations; Student's t test, $^{**}p < 0.01$ *versus* relevant control and §§$p < 0.01$ *versus* TBT-treated cells.

DISCUSSION

The concentrations used in these studies were in the range reached *in vivo*. When TBT was applied (Middleton, 1982) to rat dorsal skin as a single cutaneous dose of 67 nmol per cm^2, steady-state levels of 0.44 nmol/mg dry weight were attained in the viable epidermis in 1 h. If skin contains 70% water and 20% protein, this is equivalent to 1.3 nmol/mg protein. The concentrations used for our *in vitro* studies (0.1–1 μM) are equivalent to 0.28–2.8 nmol/mg cellular protein. TBT was indeed a potent inducer of IL-1α *in vitro*. The lower intracellular IL-1α content at higher concentrations of TBT is due to TBT's cytotoxicty, which results in leakage rather than *de novo* synthesis of IL-1α. Others have shown (Snoeij *et al*, 1986; Marinovich *et al*, 1990) in other cell types that triorganotin compounds at concentrations higher than those disturbing mitochondrial respiration (>1 μM) severely damage cell membranes.

Our experiments document a requirement for mitochondria in TBT-induced IL-1α production. Effects of triorganotin salts on mitochondria have been studied extensively (reviewed in Snoeij *et al*, 1987), and three different types of interaction have been distinguished. In medium containing halides, the triorganotin salts cause an exchange of halide for hydroxyl ions across the mitochondrial membrane, resulting in a disturbance of the existing proton gradient; the triorganotins also bind to a component of the ATP synthase complex, inhibiting ATP production; and gross mitochondrial swelling was noted following incubation with trialkyltin compounds (see also **Fig 5**). In plant mitochondria, dibutylchloromethyltin chloride has been shown to interact with the function-

Table II. Mitochondrial Depletion Results in a Reduction of MTT Conversion[a]

Treatment	MTT Conversion (% of control)	Protein Synthesis (cpm/μg protein)
Control	100 ± 3	272 ± 72
EBr (200 ng/ml)	48 ± 3[b]	288 ± 49
TBT, 1 μM	80 ± 2[b]	1590 ± 106[b]
EBr + TBT	56 ± 1[b, c]	572 ± 61[b, c]

[a] HEL30 cells were treated with EBr for 5 d and then TBT (1 μM) or DMSO as vehicle control was added. After 24 h of treatment MTT conversion (OD value in control was 1.416 ± 0.025) and [^{3}H]leucine incorporation into proteins were assessed. Mean ± SD of six to eight determinations. Student's t test, [b] $p < 0.01$ *versus* control and [c] $p < 0.01$ *versus* TBT 1 μM.

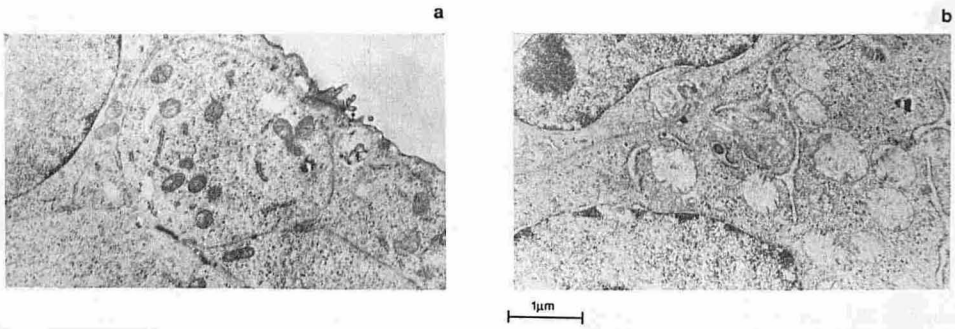

Figure 5. Electron microscopy shows swelling of mitochondria in HEL30 cells after treatment with TBT (10 μM). *(a)* Control cells. *(b)* Cells 5 min after treatment with TBT.

ally distinct pool of ubiquinone associated with oxidation of succinate and L-malate, thus inhibiting mitochondrial electron transport (Moore *et al*, 1980). Early investigations with isolated mitochondria showed that ubiquinone is the major source of reactive oxygen species derived from the mitochondrial chain (Cadenas *et al*, 1977) in which electrons are transferred from ubisemiquinone to molecular oxygen, resulting in the formation of superoxide anions. We speculate that TBT may induce increased radical production at the ubiquinone site of the respiratory chain.

Inhibition of electron entry to the ubiquinone pool of the respiratory chain by the complex I inhibitor rotenone indeed resulted **(Fig 1)** in a significant and concentration-related reduction of TBT-induced IL-1α release and intracellular IL-1α increase. This does not exclude the possibility that TBT may also affect mitochondrial calcium homeostasis, which also controls the mitochondrial respiratory chain (Richter and Frei, 1988), but it is not clear whether changes in calcium metabolism precede or succeed the oxidative stress.

Inducible expression of IL-1α is regulated via transcription factors, mainly NF-κB and AP-1 (Muegge *et al*, 1990; Fenton, 1992). NF-κB activation is generally controlled by reactive oxygen species and the cellular redox state and can be inhibited by antioxidants—especially PDTC (Schreck *et al*, 1992)—or iron chelators. Mitochondrial reactive oxygen species specifically modulate the activation of NF-κB (Schreck *et al*, 1991) and are also involved in AP-1 activation (Devary *et al*, 1991; Ilnicka *et al*, 1993; Lee and Ilnicka, 1993). We have shown **(Figs 2–4)** that TBT induces a direct and concentration-dependent activation of NF-κB that could be modulated by antioxidants, such as PDTC or butylated hydroxyanisole, and by rotenone. Under our experimental conditions a variable NF-κB activation in control cells was observed; this may be explained by experimental variability and by a difference in the exposure time of autoradiography, as suggested by the free probe signal. Reactive oxygen species may combine with TBT to form radicals (Rivera *et al*, 1992), which may also account for the activation of transcription factors. For the short time examined (30 min), we can exclude autocrine induction of NF-κB activation by IL-1α itself, because adding a neutralizing antibody to murine IL-1α did not prevent NF-κB activation and early (4 h) IL-1α production induced by TBT (data not shown). Under the same experimental conditions, however, inhibition of the intracellular IL-1α increase was partial, which indicates that other transcription factors may be involved in TBT-induced IL-1α neosynthesis, most probably AP-1. Indeed, preliminary data show that 1 μM TBT is able to induce AP-1 activation after 1 h of treatment. Taken together, these data indicate that inhibition of the formation of free radicals modulates the activation of NF-κB and IL-1α neosynthesis induced by TBT.

To substantiate that TBT-induced IL-1α production and NF-κB activation in keratinocytes require functional mitochondria, we partially depleted cells of functioning mitochondria by a 5-d treatment with ethidium bromide, which selectively blocks the synthesis of mitochondrial DNA and RNA (Nass, 1972; Wiseman *et al*, 1978). The treatment reduced mitochondrial function as tested by MTT conversion by 50%, and TBT-induced IL-1α production was greatly reduced. This effect is not due to inhibition of protein synthesis, as judged by [^{3}H]leucine incorporation and by the observation that these cells actually had a higher constitutive level of intracellular IL-1α. It has been suggested (Kripke, 1991) that when skin is treated with DNA-damaging agents, epidermal cells are activated to release growth factors such as IL-1, IL-6, and granulocyte-macrophage colony stimulating factor in order to promote cell repair and development. EBr-treated cells can be

Table III. Mitochondrial Depletion Reduces TBT-Induced IL-1α Production

Treatment	Extracellular IL-1α (pg/ml)	Intracellular IL-1α (10^3 pg/mg)
Control	n.d.	0.73 ± 0.14
EBr, 200 ng/ml	n.d.	1.34 ± 0.14
TBT, 1 μM	463 ± 73	3.40 ± 0.67
EBr followed by TBT	164 ± 27[a]	1.20 ± 0.24[a]

HEL30 cells were treated with EBr for 5 d and then TBT or DMSO as vehicle control was added. After 24 h IL-1α release (extracellular IL-1α) and cell-associated (intracellular IL-1α) were measured by enzyme-linked immunosorbent assay. Mean ± SD of three to four determinations. Student's t test, [a] $p < 0.01$ *versus* TBT-treated cells. n.d., not detectable, below limit of detection.

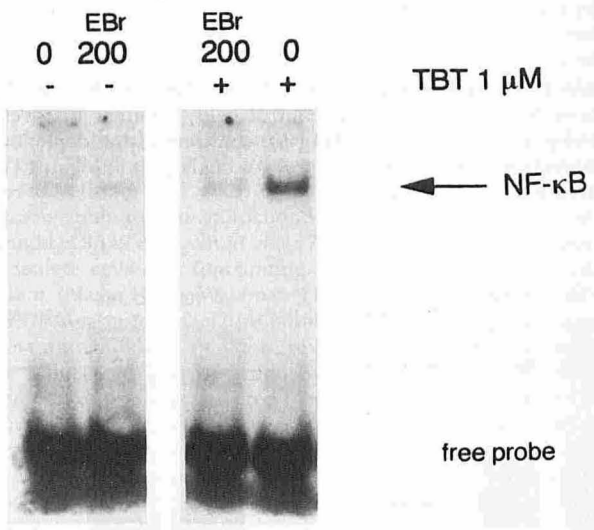

Figure 6. Mitochondrial depletion results in inhibition of TBT-induced NF-kB activation. Cells were treated for 5 d with EBr (200 ng per ml) in medium supplemented with 4.5 mg glucose per ml and 5 μg uridine per ml, after which TBT (1 μM) or DMSO vehicle control (0) was added for 30 min. Equal amounts of nuclear extracts (5 μg) were analyzed in EMSA with a ^{32}P-labeled DNA probe that detects binding activity of NF-κB.

further stimulated to produce IL-1α, however, e.g., by treatment with phorbol esters (data not shown), which bypass the mitochondrial intracellular signaling pathway. It still remains to be resolved which signaling pathway EBr utilizes in the induction of IL-1α. Mitochondrial impairment also results in a reduction of TBT-induced NF-κB activation. Taken together, these findings support the role of mitochondria as the source of second messenger molecules important for TBT-induced NF-κB activation and IL-1α neosynthesis.

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