UVB Radiation-Mediated Expression of Inducible Nitric Oxide Synthase Activity and the Augmenting Role of Co-Induced TNF-α in Human Skin Endothelial Cells

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Nitric oxide (NO) plays a pivotal role in ultraviolet radiation-induced inflammation in human skin. We had earlier reported on the inducible nitric oxide synthase (iNOS) inducing activity of UVA radiation. We now demonstrate that UVB-exposure induces expression of the iNOS in vessel endothelia of normal human skin and in cultured human dermal endothelial cells (HUDEC), although by a molecular mechanism different from UVA-mediated induction. With HUDEC, UVB induces iNOS expression and leads to significant enzyme activities, although at app. 5-fold lower levels than can be achieved with proinflammatory cytokines. In contrast to our earlier observation with UVA, cytokine-challenge combined with simultaneous UVB-exposure had no additive effects on iNOS expression nor activity. Interestingly, a time-delay between UVB-irradiation and cytokine-challenge enhances endothelial iNOS enzyme activity 2.5-fold over cytokines activation only. This time-dependent effect strongly correlates with UVB-induced endothelial TNF-α expression. In HUDEC addition of TNF-α results in enhanced expression of the inducible arginine transporter system CAT-2 essential for substrate supply and thus iNOS activity. In summary, UVB induces iNOS mRNA and enzyme activity in HUDEC. Moreover, UVB augments CAT-2 expression through a TNF-α-dependent mechanism which essentially contributes to increased iNOS activity.

Key words: cationic amino acid transporter/cytokines/endothelial cells/inducible nitric oxide synthase/nitric oxide/TNF-α/UVB
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Prolonged exposure of the human skin to ultraviolet radiation (UVR) can result in inflammation including erythema and edema formation, and also in premature aging, immune suppression and skin cancer (Jagger, 1985; Gange, 1987). Erythema formation is the result of local increases in blood flow in both the superficial and deep vascular plexus of the dermis (Greaves, 1986). UVB-induced erythema were shown to be significantly reduced by inhibitors of nitric oxide synthases (NOS) indicating the involvement of nitric oxide (NO) in regulation of local vascular tone and blood flow in normal human skin (Goldsmith *et al*, 1996).

Endothelial-derived NO plays a physiological role in blood pressure regulation and in inhibition of platelet aggregation and platelet adhesion and is also held responsible for erythema and edema formation through vascular leakage as a marker for local inflammation but also modulates leukocyte adhesion (Langrehr *et al*, 1993).

During inflammatory processes, cytokines are known modulators of endothelial cell functions (Pober and Cotran, 1990). One of the prominent effects that cytokines can exert on endothelial cells is the induction of the expression of the inducible isoform of the NOS-family, the iNOS, ensuing high-output NO synthesis (Suschek *et al*, 1993; Suschek *et al*, 1994; Hoffmann *et al*, 1999).

High-output NO production may serve for local defense against viruses, bacteria, protozoa, and helminths (Liew and Cox, 1991), but is also involved in toxic effects on neighboring cells (Mills, 1991; Corbett *et al*, 1993; Holt *et al*, 1993), in immune-mediated tissue destruction, as for instance in autoimmune diabetes (Kolb and Kolb-Bachofen, 1992) and in the pathogenesis of septic shock and cytokine-induced hypotension (Kilbourn and Belloni, 1990).

Although numerous cytotoxic effects on a variety of mammalian cells were described to be associated with iNOS-derived high-output NO formation, more recently, many publications point to a powerful protective activity of iNOS-generated NO during oxidative stress resulting from exposure to reactive oxygen species (ROS) such as superoxide, hydrogen peroxide or alkyl peroxides (Wink *et al*, 1995; Wink *et al*, 1996) all of which are known intracellular mediators of UVR-induced cytotoxicity (Danpure and Tyrrell, 1976; Black, 1987). Indeed, we could show recently that endogenously produced as well as exogenously applied NO effectively protects from UVA-induced and ROS-mediated damage and apoptosis of endothelial cells (Suschek *et al*, 1999, 2001a).

We had previously shown that UVA irradiation, capable of penetrating far into the dermal layers of human skin, will induce human dermal endothelial iNOS expression *in situ* in

Abbreviations: CAT, cationic amino acid transporters; HUDEC, human dermal endothelial cells; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor α; UVA/B/C, ultraviolet A/B/C

the absence of proinflammatory cytokines. Moreover, UVA irradiation significantly augments iNOS expression and activity induced by the addition of Th-1-type cytokines (Suschek *et al*, 2001b).

Here, we now examine the effects of UVB radiation on the expression of the iNOS in specimens of normal human skin maintained in organ culture and on iNOS expression and activity in cultures of human dermal endothelial cells. We here show that UVB radiation induces iNOS mRNA expression similar to UVA-mediated effects. In contrast to UVA, however, UVB irradiation modulates endothelial iNOS activity by a mechanism involving endogenous TNF-α production and also increases the expression of the L-arginine transport system CAT (cationic amino acid transporters)-2.

Results

Effects of UVB irradiation on iNOS expression *in situ* and *in vitro* UVB irradiation (50 mJ per cm^2) of normal human skin specimens maintained in organ culture results in *de novo* iNOS protein expression in small vessel-lining endothelia within 24 h post irradiation. As positive controls, specimens were incubated with proinflammatory cytokines resulting in the identical positive signal for iNOS protein (Fig 1*A*).

With human dermal endothelial cell (HUDEC) cultures, UVB irradiation in the absence of cytokines, concentration dependently (1.25–10 mJ per cm^2), leads to the induction of expression of iNOS mRNA (Fig 1*C*) as well as protein (Figs 1*B* and *C*). Again, cytokine incubation of HUDEC (interleukin (IL)-1β + interferon-gamma (IFN-γ), each 500 U per mL) was used as a positive control and led to iNOS mRNA and iNOS protein expression as previously shown (Suschek *et al*, 2001b).

The impact of cytokine challenge on UVB-induced iNOS expression and activity and the role of cationic amino acid transporter on endothelial iNOS activity The expression of iNOS mRNA parallels iNOS enzyme activity (Fig 2*A* and Table I). In contrast to our observation with UVA (Suschek *et al*, 2001b), the combination of UVB irradiation and subsequent cytokine addition do not augment iNOS expression or activity above cytokine treatment only. Interestingly, a delayed addition of cytokines 18 h after UVB irradiation leads to a strong and significant increment in iNOS expression and activity as compared to cytokines only.

Since iNOS activity depends on the availability of its substrate L-arginine transported via the CAT (Closs, 2002) we also searched for an impact of UVB on CAT-2 expression. As shown in Fig 2*B*, HUDEC constitutively express CAT-2. UVB irradiation (10 mJ per cm^2) alone does not influence the CAT-2-mRNA expression levels, whereas activation with proinflammatory cytokines IL-1β + IFN-γ (each 500 U per mL) strongly augments CAT-2 mRNA expression as expected. Delayed (18 h) cytokine challenge post-UVB irradiation leads to a significant additional enhancement of CAT-2 mRNA expression over the increment by cytokines alone. Again, UVB irradiation immediately followed by cytokine challenge does not increase CAT-2 expression. Thus, the pattern of CAT-2 mRNA expression completely parallels that of iNOS expression and activity (Table I).

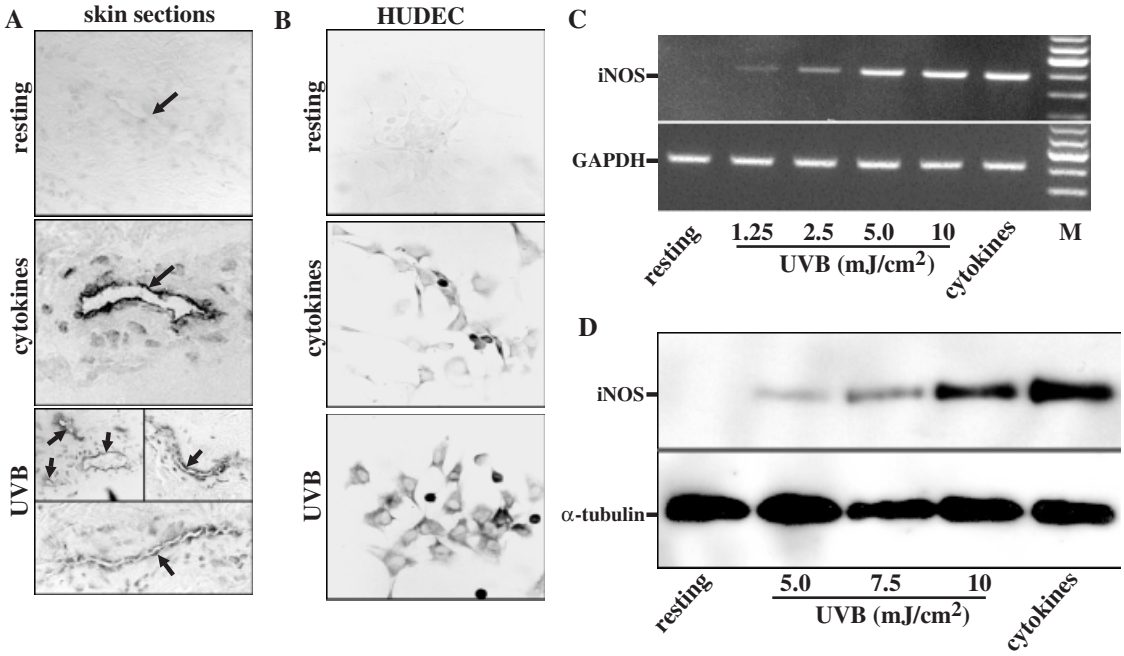

Figure 1
UVB irradiation induces the expression of iNOS mRNA and protein in vessel endothelia and human dermal endothelial cell (HUDEC) cultures. Specimens of human skin or HUDEC cultures were exposed to UVB radiation (50 mJ per cm^2) and 24 h after irradiation immunohistochemistry was performed using a monoclonal anti-iNOS antibody. As a positive control, specimens were incubated for 24 h with proinflammatory cytokines (IL-1β + TNF-α + IFN-γ, 1000 U per mL each). (*A*) Cryostat sections of human skin with positive vessel endothelia after cytokine activation or after UVB irradiation. No signal was found in untreated skin. *Scale bar* = 100 μm. (*B*) HUDEC cultures also stain positively after UVB challenge or incubation with proinflammatory cytokines (IL-1β + TNF-α + IFN-γ, 500 U per mL each). UVB-induced iNOS expression occurs dose dependent in the tested range (2.5–10 mJ per cm^2) as shown here by PCR (*C*) or western technique using iNOS-specific monoclonal antibodies (*D*). Positive control: cytokine challenge (IL-1β + TNF-α + IFN-γ, 500 U per mL each). Shown are representative photomicrographs of one out of three individual experiments with identical results. *Scale bar* = 100 μm.

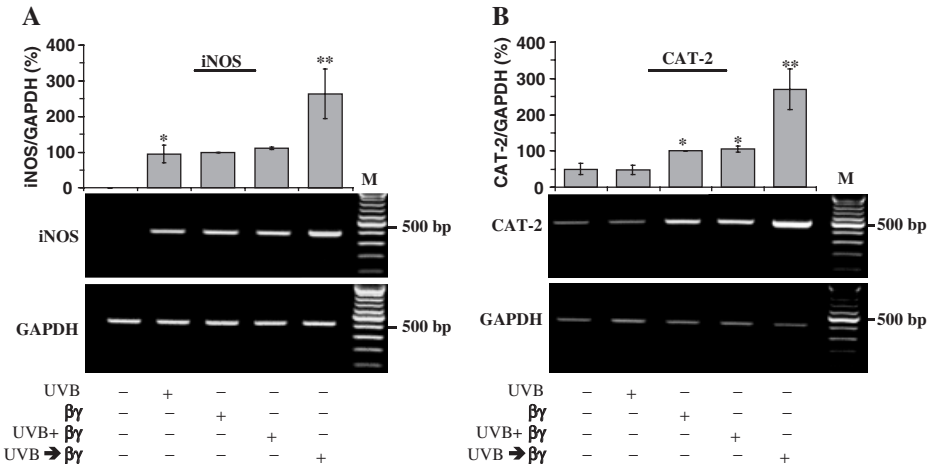

Figure 2
Effects of UVB on iNOS and CAT-2 mRNA expression in HUDEC. UVB irradiation (10 mJ per cm^2) or cytokine challenge ($\beta\gamma$, IL-1β + IFN-γ, 500 U per mL each) of resting HUDEC leads to *de novo* iNOS mRNA expression (*A*), but has no influence on the low level of constitutive CAT-2 mRNA expression (*B*), whereas cytokine-mediated activation significantly augments CAT-2 mRNA levels. Combining UVB irradiation immediately followed by cytokine activation (UVB + $\beta\gamma$) has no effects on iNOS or CAT-2 expression relative to UVB treatment or cytokine-challenge alone. In contrast, a delayed cytokine challenge of HUDEC starting 18 h after UVB irradiation (UVB $\rightarrow$ $\beta\gamma$) leads to a strong and significant increase in both, iNOS and CAT-2 expressions. M, DNA size marker. Values represent the mean ± SD of four to six individual experiments. *$p < 0.001$.

Competition for arginine transport by excess lysine + ornithine (10 mM each) or lack of exogenous arginine both completely inhibit the cytokine and/or UVB-induced iNOS enzyme activity to the same degree as does incubation with the NOS inhibitor L-NIO (L-N^5-(1-Iminoethyl)-ornithin) (0.25 mM) (Table I).

Examination of CAT-1 mRNA expression reveals a constitutive expression unchanged by UVB or cytokine treatments (data not shown).

Impact of endogenously produced TNF-α on UVB-induced endothelial iNOS induction and activity TNF-α is a potent costimulatory factor for iNOS expression and activity (Suschek *et al*, 1993). As UVB radiation is known to induce TNF-α expression in dermal cells (de Kossodo *et al*, 1995; Strickland *et al*, 1997), we next examined whether the UVB-induced effects or parts of it are under the influence of endogenous endothelial TNF-α production.

Indeed, UVB irradiation induces TNF-α mRNA expression after 30 min (Fig 3*A*) and after 4 h significant amounts of TNF-α protein (Fig 3*B*) are detected in culture supernatants. When cell cultures were grown in the presence of thalidomide (20 μg per mL), TNF-α synthesis was effectively blocked (Fig 3*B*).

Furthermore, in UVB-irradiated cell cultures the time delay for cytokine-mediated increases in iNOS activity can be explained by the UVB-induced endogenous TNF-α expression and the time needed for protein secretion (Fig 3*B vs C*) and indeed neutralizing anti-TNF-α-antibodies completely block the increment in NO-synthesis. Neither UVA irradiated nor non-irradiated HUDEC produce TNF-α (not shown), demonstrating the UVB specificity of this response.

Next, we examined the role of endogenous TNF-α production on iNOS and CAT-2 mRNA expression or iNOS activity, respectively, and find indeed that TNF-α production is a crucial factor, since inhibition of endogenous TNF-α formation by the presence of thalidomide (20 μg per mL) effectively blocks the cytokine-mediated increments of both, iNOS and CAT-2 mRNA expressions (Fig 4) and also of iNOS activity (Table I). Furthermore, addition of recombinant human TNF-α completely reverses these thalidomide effects (Fig 4 and Table I). Neither human IL-6 nor IL-8, which represent two proinflammatory endothelial cytokines that may also be affected by thalidomide were able to reverse the thalidomide-mediated suppression (Table I). Thalidomide, however, does not block the UVB-mediated *de novo* iNOS expression in resting cells nor does it interfere with IL-1β/IFN-γ-induced iNOS or CAT-2 activity or expression, respectively. These data give strong evidence for UVB-induced endogenous endothelial TNF-α production as an important enhancing factor in UVB-induced iNOS expression and activity.

Discussion

Recently, we have demonstrated that UVA irradiation induces a *de novo* expression of the iNOS in human dermal endothelia in the absence of cytokines and significantly augments endothelial iNOS activity in the presence of proinflammatory stimuli (Suschek *et al*, 2001b). The timing of the *de novo* expression of this NO-synthase isotype as induced by UVA or as demonstrated here by UVB strongly correlates with the appearance of erythema formation which peaks 24 h after UV-irradiation (Greaves, 1986). Thus, it appears that in addition to the neuronal NOS activity in dermal keratinocytes, as hypothesized earlier (Deliconstantinos *et al*, 1995), iNOS expression in endothelia might contribute to erythema formation.

Although both, UVA (Suschek *et al*, 2001b) and UVB radiation are potent inducers of endothelial iNOS expression and activity, the molecular basis of this response is not identical. Simultaneous challenge of endothelial cells with UVA + cytokines significantly increases iNOS expression and activity relative to cells treated with cytokines only. In contrast with UVB, such increases are not observed, rather here only a time-delay between UVB irradiation and cytokine challenge increase strongly iNOS-mRNA expression as well as enzyme activity. This indicates the involvement of an additional UVB-inducible event serving as modulator or enhancer of iNOS expression and activity.

One obvious candidate for such a factor is TNF-α, since this gene is responsive to UVB and UVC but not to UVA (Bazzoni *et al*, 1994; de Kossodo *et al*, 1995). UVB-induced TNF-α expression in known to occur in keratinocytes (Köck *et al*, 1990) and dermal fibroblasts (de Kossodo *et al*, 1995). For endothelial iNOS expression and activity, TNF-α is not sufficient for *de novo* induction (Suschek *et al*, 1993), but represents a strong costimulator, a finding confirmed by the

Table I. Impact of cationic amino acid transport and TNF-α on endothelial iNOS enzyme activity

	Nitrite (nM)							
Treatment	**No additives**	**+lys/orn**	**ARG depr.**	**+L-NIO**	**+anti-TNF-α**	**+thal**	**+thal +TNF-α**	**+thal +IL-6**	**+thal +IL-8**
Resting	111 ± 14	93 ± 45	76 ± 37	26 ± 8	121 ± 7	88 ± 24	88 ± 22	110 ± 32	91 ± 12
UVB	250 ± 30*	77 ± 18	81 ± 26	36 ± 25	247 ± 22	236 ± 7	191 ± 21	231 ± 31	201 ± 18
βγ	612 ± 90**	94 ± 9	88 ± 16	20 ± 12**	590 ± 32	660 ± 67	521 ± 62	563 ± 22	624 ± 27
UVB + βγ	769 ± 82**	86 ± 10	68 ± 22	11 ± 9	544 ± 41	602 ± 109	623 ± 44	547 ± 21	603 ± 41
UVB → βγ	1361 ± 139***	85 ± 31	75 ± 23	14 ± 8	517 ± 33****	794 ± 51****	1221 ± 119*****	644 ± 34	635 ± 38

In resting or cytokine activated (βγ, IL-1β + IFN-γ, each 500 U per mL) and/or UVB-challenged (10 mJ per cm^2) human dermal endothelial cells (HUDEC) nitrite formation and its dependence on exogenous arginine or on CAT-mediated arginine-transport or on TNF-α function was determined. Values represent the mean ± SD of three to six individual experiments.
iNOS, inducible nitric oxide synrhase; UVB, ultraviolet B; lys/orn, 10 mM lysine + ornithine; ARG depr., arginine deprivation of the growth medium; L-NIO, NOS inhibitor L-NIO (0.25 mM); anti-TNF-α, anti-human TNF-α antibodies; thal, thalidomide (20 μg per mL) alone or simultaneously with IL-6 or IL-8. UVB + βγ, UVB-irradiation and cytokine-challenge were performed simultaneously; UVB → βγ, HUDEC were irradiated by UVB and 18 h later activated by the respective cytokines.
* $p < 0.001$ as compared to the resting controls.
** $p < 0.001$ as compared to resting or UVB-irradiated only cell cultures.
*** $p < 0.001$ as compared to all other cell cultures.
**** $p < 0.001$ as compared to respective cell cultures cultivated in the absence of any additives.
***** $p < 0.001$ as compared to the respective cultures treated with thalidomide only.

Figure 3
UVB-induced endothelial TNF-α formation parallels the increases in iNOS activity of irradiated endothelial cells. The effects of UVA (18 J per cm^2) or UVB (10 mJ per cm^2) radiation on endothelial TNF-α formation and nitrite accumulation were compared. Human dermal endothelial cells cultures (HUDEC) were irradiated with UVA or UVB and at time points indicated TNF-α mRNA expression or TNF-α concentration in culture supernatants was determined using TNF-α-specific RT-PCR or ELISA. UVB irradiation after 30 min leads to the appearance of TNF-α mRNA expression (*A*) and after 4 h significant amounts of TNF-α in culture supernatants (*B*, △), whereas non-irradiated or UVA-irradiated cultures (*B*, ○ or ●) do not form TNF-α. Addition of thalidomide (*B*, ▲, 20 μg per mL) suppress the TNF-α production after UVB challenge. (*C*) Non-irradiated (○) as well as UVB-irradiated (at t = 0 h) HUDEC cultures maintained in the absence (●) or presence (▲) of TNF-α-neutralizing antibodies were cytokine-challenged (IL-1β + IFN-γ, each 500 U per mL) at the indicated time points after the light stimulus (○, βγ; ●, UVB → βγ; ▲, UVB + anti-TNF-α → βγ), and 24 h after the last challenge nitrite accumulation was determined. Shown are the relative increases (cytokine activated non-irradiated cells at 22 h = 100%). Values represent mean ± SD of three to four individual experiments.*$p < 0.001$.

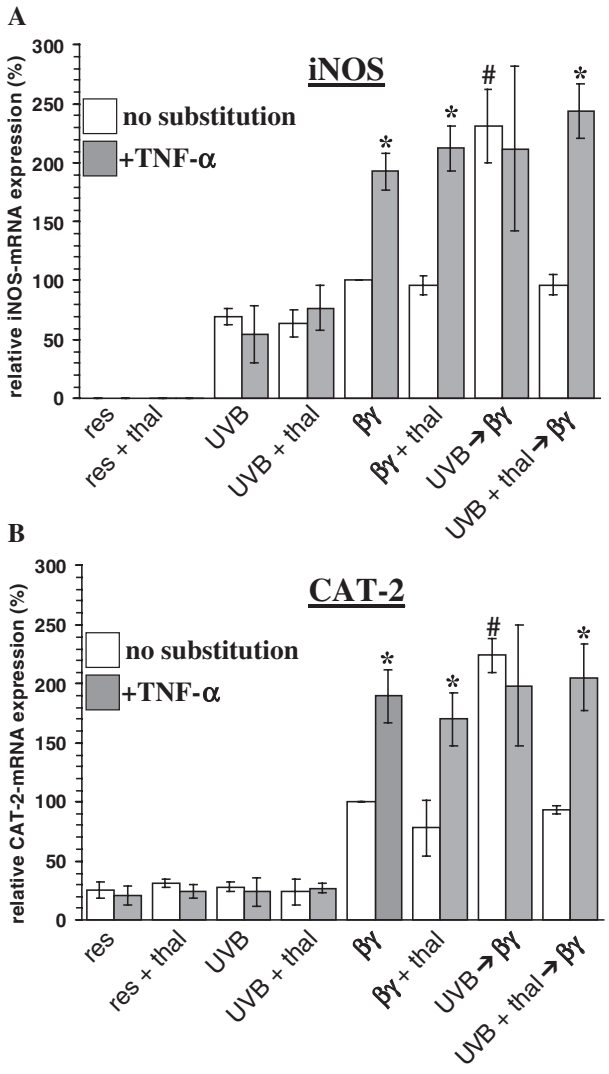

Figure 4
TNF-α plays a pivotal role in enhancing UVB-induced iNOS- and CAT-2-mRNA expression. Human dermal endothelial cells (HUDEC) were treated as in Fig 2 with and without thalidomide (thal) addition (20 μg per mL). The relative iNOS- or CAT-mRNA expression represents the product/GAPDH ratio obtained by the densitometric analysis of the amplification products on agarose gels. The significant increases in iNOS- (*A*) or CAT-2-mRNA expression (*B*) following an delayed cytokine challenge (βγ, IL-1β + IFN-γ, 500 U per mL each) 18 h post-UVB irradiation (10 mJ per cm^2; UVB → βγ) are blocked by thal (UVB + thal → βγ) and are restored by adding human recombinant TNF-α (1000 U per mL, gray bars). Bars represent the mean ± SD of three individual experiments. $^*p < 0.01$ as compared to the respective cultures incubated in the absence of TNF-α. $^\#p < 0.001$ as compared to all other not TNF-α-challenged cultures.

present data. We here show that UVB induces endothelial TNF-α formation, but that this event is not involved in UVB-induced *de novo* iNOS expression. On the other hand, endothelial TNF-α protein production reaches a maximum 18–24 h after UVB challenge and serves as a potent enhancer of iNOS expression and enzyme activity. This effect becomes apparent when UVB-irradiated cells are subsequently challenged with proinflammatory cytokines where the necessary the time-delay matches the time needed for maximal endogenous TNF-α formation.

In addition, TNF-α has also been shown to represent a key signal for the induction of the "inducible" cationic amino acid transporter CAT-2 responsible for the influx of higher L-arginine concentration (Closs *et al*, 2000; Closs, 2002) as an essential prerequisite for maximal iNOS activity (Schnorr *et al*, 2003; Suschek *et al*, 2003). Indeed, the pivotal role of TNF-α on CAT-2 expression and the impact of CAT-2 on iNOS enzyme activity is highlighted by our findings that iNOS-mediated NO formation after UVB challenge parallels TNF-α-dependent CAT-2 expression, and that blocking TNF-α formation or activity abolishes increases in CAT-2 expression and in iNOS activity. In our experiment, we intend to block TNF-α expression by addition of thalidomide which is not TNF-specific but also unspecifically affects the expression of a variety of other cytokines (Matthews and McCoy, 2003). Here, the inhibitory effects of thalidomide on iNOS or CAT mRNA expression can be completely restored by addition of TNF-α but not IL-6 and IL-8, two other cytokines known to be affected by thalidomide. Furthermore, regarding iNOS activity we used TNF-α specific monoclonal antibodies and could completely block the mentioned UVB-induced increases in endothelial nitrite formation. Again, this effect was completely restored by addition of recombinant TNF-α but not IL-6 or IL-8. Therefore, although thalidomide is not a specific TNF-α inhibitor, the experiments presented here give a strong evidence for endogenous TNF-α as mediator of UVB action on CAT-2 expression and thus NO formation in human dermal endothelial cells.

In conclusion, we here demonstrate that UVB radiation induces iNOS mRNA, protein, and function in HUDEC. Further, cytokines strongly augment UVB-induced iNOS and CAT-2 expression in a delayed pattern and through a TNF-α-dependent mechanism, and also iNOS activity and NO generation. Thus, TNF-α is required for maximal increases in iNOS mRNA expression and activity following UVB exposure under inflammatory conditions, although it is not sufficient in itself for iNOS induction. This effect may also contribute to the known time-delay in UV-induced erythema formation.

Materials and Methods

The study was approved by the ethical committee of the Heinrich-Heine-University of Düsseldorf and conducted according to Declaration of Helsinki Principles. Patients gave written informed consent.

Reagents Recombinant human IL-1β was purchased from HBT (Leiden, Netherlands), recombinant human IFN-γ, and recombinant human TNF-α from Genzyme (Cambridge, Massachusetts). The lipopolysaccharide (LPS) content in this cytokine batches never exceeded 0.1 ng per μg protein. The polyclonal anti-human-TNF-α-antiserum, monoclonal anti-human-TNF-α-antibody, the human TNF-α ELISA kit, IL-6 and IL-8 were purchased from R&D Systems (Wiesbaden, Germany), Neutral Red (3% solution), the oligo dT16-primer, and anti-α-tubulin antibody from Sigma (Deisenhofen, Germany), the monoclonal anti-iNOS and anti-eNOS antibodies from Transduction Laboratories (Lexington, KT, USA), peroxidase-conjugated goat anti-mouse IgG from Zymed Laboratories (San Francisco, CA, USA), trypsin, EDTA from PAA (Graz, Austria), the monoclonal anti-CD31 and anti-CD34 antibodies from Dako (Glostrup, Denmark), Taq-polymerase from Gibco Laboratories (Eggenstein, Germany), the iNOS inhibitor L-NIO, the fluorescence based (DAN) nitrite assay from Alexis Biochemicals (Läufelfingen, Switzerland), and 3,3′-diaminobenzidine (DAB) from Serva GmbH (Heidelberg, Germany). Light source used was an UV-800 unit (Waldmann, FRG) emitting the UVB spectrum from 280 to 320 nm

(0.6 mW in a distance of 35 cm) with an intensity peak at 311 nm. The UVA source (4000 W lamp emitting the UVA spectrum from 340 to 400 nm) as well as the dosimeter was purchased from Sellas Medizinische Geräte (Gevelsberg, Germany).

Cells Human dermal microvascular endothelial cells (HUDEC, four separate batches) were purchased from Promo Cell and were maintained for up to 6 passages in endothelial cell basal medium supplemented with 5% fetal calf serum (FCS) 2 mM L-glutamine, 2 mM L-arginine, 100 μg per mL streptomycin, 100 U per mL penicillin, 250 ng per mL amphotericin B, but without hydrocortisone, epidermal growth factor (EGF), and endothelial cells growth supplement (ECGS).

Organ culture experiments Human skin specimens were derived from mammoplastic surgery (Department of Plastic Surgery, Florence Nightingale Hospital, Düsseldorf, Germany), cut into 4-mm squares and placed in 24-well culture plates with RPMI/20% FCS supplemented with 0.1 mg per mL penicillin, 0.1 mg per mL streptomycin, and 0.25 μg per mL amphotericin B. It is known that during UVB irradiation of human skin, only 20%–30% of the light penetrate the epidermis and reach the dermal vasculature (Bruls *et al*, 1984a, b; Meunier, 1999). Thus, we calculated that 50 mJ per cm^2 represents a non-toxic dose of UVB radiation sufficient for iNOS induction in vessel endothelia in human skin specimens. Skin specimens were irradiated with UVB (50 J per cm^2) and cultured for 24 h. As positive controls, specimen were also incubated with proinflammatory cytokines for 24 h. Here, we used a combination of IL-1β, TNF-α (1000 U per mL each), and 200 U per mL IFN-γ that was found to be effective in iNOS induction in primary human keratinocytes (Bruch-Gerharz *et al*, 1996). Specimens were then embedded in Tissue-Tek (Reichert-Jung, Vienna, Austria) and snap frozen in liquid nitrogen for immunohistochemical characterization.

Experiments with HUDEC were performed with cells from passages 2–6. Endothelial cells (1×10^5) were cultured in 24-well tissue culture plates. Resident or cytokine activated HUDEC were then irradiated with UVB at doses indicated. Viability of endothelial cells was routinely controlled at the beginning and end of each experiment by neutral red staining and trypan blue exclusion.

Cell culture experiments In HUDEC, iNOS expression and activity were induced by incubation with the cytokines IL-1β, IFN-γ (500 U per mL each) and/or TNF-α (1000 U per mL), applied in the combinations mentioned (Hoffmann *et al*, 1999; Suschek *et al*, 2001b). HUDEC cultures were either treated with UVB (approx. 1.7 s for 1 mJ per cm^2) followed immediately by cytokine challenge or HUDEC were activated by cytokines 2–24 h after the UVB challenge. Probes for analysis always were collected 24 h after the last treatment.

Immunohistochemistry Cryostat sections from skin specimens (7 μm) were fixed with 0.2% glutaraldehyde in Tris-buffered solution (TBS), pH 7.0, followed by three washing steps in TBS. After blocking of unspecific binding with 0.5% bovine serum albumin (BSA) in TBS for 30 min and rinsing, sections from resting skin were incubated overnight with anti-vWF antiserum or monoclonal anti-CD31, anti-CD34 (diluted each 1:30) or anti-ecNOS (diluted 1:100) antibodies in a moist chamber. Additionally, sections from cytokine-activated skin specimens (IL-1β + TNF-α: each 1000 U per mL) as well as skin specimens irradiated with UVB (50 mJ per cm^2) were incubated with a monoclonal anti-iNOS antibody (diluted 1:100). Slides were washed three times with TBS for 5 min. As secondary antibody peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG was used in a final dilution of 1:30 for 1 h in TBS. All steps were performed at 4°C. After washing in TBS sections were incubated with 0.05% DAB + 0.015% H_2O_2 for 5 min at room temperature. For light microscopy, sections were dehydrated, cleared with xylene and embedded in Eukitt. The phenotype of small vessel skin endothelial cells was positive for vWF, CD31, CD34, and ecNOS, as expected (data not shown).

Immunocytochemistry For immunocytochemistry of HUDEC, cells were grown on sterile glass coverslips, washed with phosphate-buffered saline (PBS) and fixed with acetone at −20°C for 10 min. Blocking of unspecific binding, endothelial cell characterization, and iNOS-specific staining was performed as described above.

HUDEC cultures exhibited the identical antigenic phenotype for vWF, CD31, CD34, and ecNOS (data not shown). Labeling experiments also showed that the cell cultures consisted of pure endothelial cells since the respective staining patterns with the endothelial specific markers were found in all cells (data not shown).

Nitrite determination After 24 h of incubation, nitrite was determined in culture supernatants using the fluorometric DAN (2,3-diaminonaphtalen) test kit following the manufacturer's instructions and using $NaNO_2$ as standard (Marzinzig *et al*, 1997). Measurements were performed at excitation wavelength of 365 nm and the emission wavelength of 415 nm.

Detection of endogenous endothelial TNF-α production HUDEC (7×10^6) were cultured in 10 cm diameter culture dishes in a humidified incubator at 37°C in a 95% air/5% CO_2 atmosphere in 7 mL RPMI 1640 with 10 % FCS. Resting or cytokine activated (IL-1β + IFN-γ, each 500 U per mL) HUDEC were irradiated with UVB (10 mJ per cm^2). After 24 h of incubation, secreted TNF-α was determined in culture supernatants using the human TNF-α-specific ELISA kit exactly as recommended by manufacturers.

Inhibition of endogenous endothelial TNF-α expression or activity Endogenous endothelial TNF-α production was inhibited using thalidomide (20 μg per mL) (Sastry, 1999). Alternatively, TNF-α activity was neutralized by addition of monoclonal anti-human-TNF-α antibodies (150 μg per mL with a neutralizing dose ND_{50} of 0.2–0.04 μg per mL in the presence of 0.25 ng per mL of rhTNF-α using the murine L-929 cytotoxicity assay). Effectiveness of thalidomide treatment or specificity of the monoclonal anti-rat-TNF-α antibody was characterized prior to its use (Table II).

Polymerase chain reaction (PCR) Total cellular RNA (1 μg each) prepared from UVB-irradiated (10 mJ per cm^2) resting or cytokine activated (IL-1β + IFN-γ, each 500 U per mL) HUDEC was used for cDNA synthesis using the dT16-oligonucleotide as primer. Reverse transcription was carried out at 42°C for 60 min. The cDNA (500 ng each) was used as template for PCR primed by using the oligonucleotides and cycle conditions given in Table III. Prior to PCR analysis, we routinely determine the relative amount of the respective specific amplification product at different PCR cycles and thus ensure that amplification conditions are always within the linear phase. Aliquot of amplification products were subjected to electrophoresis on 1.8% agarose gels. Bands were visualized by ethidium bromide staining. Densitometric analysis of the visualized amplification products was performed by using the KODAK 1D software (KODAK, Stuttgart, Germany).

Western blot analysis of the iNOS protein Resting or cytokine-activated HUDEC (7×10^6) were irradiated with UVB (10 mJ per cm^2). After 24 h, endothelial iNOS protein expression was examined exactly as described by us previously (Suschek *et al*, 2001a) using the NuPAGE electrophoresis system (Invitrogen, Karlsruhe, Germany). Briefly, cultures were washed, scraped from the dishes, lysed by the LDS sample buffer (4×), transferred to a microcentrifuge tube, and boiled for 5 min. Proteins (40 μg per lane) were separated by electrophoresis in a 12%-Bis–Tris NuPAGE Novex pre-cast polyacrylamide gel using the MOPS-SDS running buffer system under reducing conditions (500 mM dithiothreitol) and transferred to nitrocellulose membranes (Invitrogen) using the NuPAGE transfer buffer (25 mM Bis–Tris, 25 mM Bicine, 1 mM EDTA, 20% methanol, pH 7.2) following the manufacturer's instructions.

Table II. Endothelial NO formation and the impact of thalidomide and neutralizing anti-human-TNF-α antibodies

	Nitrite (nM)		TNF-α (pg per mL)		
Treatment	−	+ **anti-TNF-α**	−	+ **anti-TNF-α**	+ **Thalidomide**
Resting	106 ± 12	94 ± 26	52 ± 9	43 ± 22	45 ± 10
βγ	554 ± 39*	553 ± 106	167 ± 18*	62 ± 14****	51 ± 16****
TNF-α	98 ± 41	73 ± 26	nt	nt	nt
βγ + TNF-α	1433 ± 172**	682 ± 148***	nt	nt	nt

Human dermal endothelial cells (HUDEC) were incubated for 24 h with a cytomix (βγ) consisting of interleukin (IL)-1β + interferon (IFN)-γ (each 500 U per mL) and/or TNF-α (500 U per mL) with or without addition of 150 μg per mL of a neutralizing monoclonal anti-human-TNF-α antibody. Nitrite concentrations were determined in culture supernatants using the DAN (2,3-diaminonaphtalen) assay. Additionally, cells were cultured in the presence or absence of 150 μg per mL of the monoclonal anti-human-TNF-α antibody or thalidomide (20 μg per mL) and the concentration of human TNF-α in culture supernatants was measured by ELISA. Both, anti-TNF-α antibodies and thalidomide was added to culture supernatants at the time of cytokine-challenge. Values represent the mean ± SD of three individual experiments.

*$p < 0.001$ as compared to resting cells.

**$p < 0.001$ as compared to IL-1β + IFN-γ only activated cell cultures.

***$p < 0.001$ as compared to the respective cultures without anti-TNF-α.

****$p < 0.001$ as compared to TNF-α-concentrations in the respective cultures without anti-TNF-α antibodies or thalidomide.

NO, nitric oxide; nt, not tested.

Table III. Human primer sequences and cycling conditions

Product/GeneBank accession no.	Sequence (bases) cycle protocol	Product size (bp)
iNOS	Sense: 5′-TGCCAGATGGCAGCATCAGA-3′ (1021–1040)	
L09210	Antisense: 5′-TTTCCAGGCCCATTCTC CTGC-3′ (1394–1413)	393
	Cycle protocol: 40 × (30″ at 94°C, 30″ at 60°C, 30″ at 72°C) + 5′ 72°C	
TNF-α	Sense: 5′-CAGGCAGTCAGATCATCTTCTC-3′ (1398–1407 and 1595–1606)	
M26331	Antisense: 5′-TCACCCTTCTCCAGCTGGAA-3′ (2261–2280)	296
	Cycle protocol: 33 × (30″ at 94°C, 60″ at 58°C; 60″ at 72°C) + 5′ at 72°C	
CAT-1	Sense: 5′-GCCATTGTCATTCCTTCCTGA-3′ (bases 340–361)	
NM_003045	Antisense: 5′-CTTCCCTTCTTTTGTGTCATT-3′ (bases 850–870)	531
	Cycle protocol: 34 × (30″ at 94°C, 30″ at 60°C, 30″ at 72°C) + 5′ at 72°C	
CAT-2	Sense: 5′-ATGGTTGCTGGGTTTGTGAAAG-3′ (bases 616–637)	
D29990	Antisense: 5′-CAACCCATCCTCCGCCATAGC-3′ (bases 1096–1116)	501
	Cycle protocol: 35 × (30″ at 94°C, 60″ at 58°C; 60″ at 72°C) + 5′ at 72°C	
GAPDH	Sense: 5′-CAACTACATGGT TTACATGTTCC-3′ (bases 153–175)	
M17851	Antisense: 5′-GGACTGTGGTCATGAGTCCT-3′ (bases 549–568)	416
	Cycle protocol: 22 × (30″ at 94°C, 30″ at 60°C, 30″ at 72°C) + 5′ at 72°C″	

iNOS, inducible nitric oxide synthase.

Further incubations of the blots were: 2 h with blocking buffer (2% BSA, 5% non-fat milk powder, 0.1% Tween 20 in PBS buffer), 1 h at 37°C with a 1:2000 dilution of the monoclonal anti-iNOS antibody, and 1 h with a 1:2000 dilution of the secondary horseradish peroxidase-conjugated rabbit-anti-mouse-IgG-antibody. Finally, blots were incubated for 5 min in ECL reagent (Pierce, Rockford, IL, USA), and exposed to an autoradiographic film. To control equal loading of total protein in all lanes, blots were stained with a 1:2000 solution of the mouse anti-α-tubulin antibody. Otherwise conditions were as described above. Densitometric analysis of the visualized iNOS protein or α-tubulin were performed by using the KODAK 1D software.

Statistical analysis Values were reported as mean ± standard deviations (SD). For statistical analysis we used ANOVA followed by an appropriate *post hoc* multiple comparison test (Tukey method). A p of < 0.05 was considered significant.

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