Role of cytokine and nitric oxide in the inflammatory response produced by sulfur mustard (HD)†

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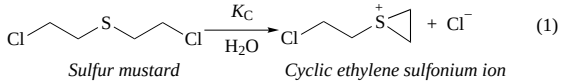
We have determined by immunocytochemistry the levels of interleukin-1 beta; (IL-1β) in cultured human epidermal keratinocytes (NHEK) following exposure to HD. Human skin keratinocytes release significant numbers of IL-1 cytokine as determined by the Quantikine™ Interleukin-1β kit, an enzyme-linked immunosorbent assay (ELISA) procedure. Exposure of NHEK [~10^6–10^7 cells, to HD (2 mM) and preincubation for 3 h at 37 °C] results in significant changes in IL-1 activation. In neonatal NHEK exposed to HD, IL-1β is decreased. Conversely, in adult breast NHEK exposed to HD, IL-1β is increased. Nitric oxide (˙NO) has been implicated as the effector molecule that mediates IL-1β. To confirm the involvement of ˙NO in the expression of the IL-1β, electron paramagnetic resonance (EPR) spectroscopy was employed. EPR detectable iron–nitrosyl complex in NHEK exposed to HD (18 h post exposure to 1 mM HD) were measured, and the generation of ˙NO and this induced complex was blocked by N^{ω}-nitro-L-arginine (L-NOARG), a competitive inhibitor of nitric oxide synthase (NOS). Our results show the release of nitric oxide during IL-1 cytokine expression when keratinocytes are exposed to HD. Based upon this work, it appears possible that IL-1 could be used as a specific marker for epidermal cytoxicity in mechanistic studies of the toxicity of HD and in defining interventive and therapeutic regimens against HD vesication.

The experiments described in this work deal with the effects of sulfur mustard (HD) on normal human epidermal keratinocytes (NHEK). Keratinocytes are the cells found in the outer layer of the skin and are a primary target of mustard exposure. Analysis of cultured normal human epidermis using specific enzyme-linked immunosorbent assays (ELISA) and bioassays for the cytokine interleukin 1 (IL-1) have led to the conclusion that the average adult harbors 20–60 μg (0.6–1.9 nmol) of IL-1 in his or her epidermis.[1] Taking the epidermis as a three-dimensional space with a thickness of 0.1 mm and an area of 1.5 m^2, the concentration of IL-1 in this space is 4–12 nmol l^{-1}. This concentration exceeds the concentration of IL-1 required to activate certain cells by three orders of magnitude (<10 pmol l^{-1}).

Most keratinocyte interleukin (*in vivo* and *in vitro*) is cell associated; relatively little is released from the cell. Keratinocytes can express higher numbers of IL-1 receptors than can any other cell type studied.[1,2] A variety of stimuli can enhance IL-1 receptor expression *in vitro*. These include phorbol esters, calcium and UV irradiation.[3] In this report, we have determined by immunocytochemistry levels of interleukin-1 beta (IL-1β) in cultured NHEK following exposure to sulfur mustard (HD).

HD is a known alkylating agent causing, among other things, DNA mutations which contribute to cell injury or death. However, the exact mechanism of alkylation remains unclear. What is known is that the action of alkylating agents, such as HD, proceeds through a mechanism involving, as a first step, formation of a cyclic alkylating agent (sulfonium ion) and the release of chloride [eqn. (1)]. The hydrolysis of HD in aqueous media



Sulfur mustard *Cyclic ethylene sulfonium ion* (1)

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at 37 °C is rapid, with $t_{\frac{1}{2}} \approx 2$ min.[4,5] Furthermore, the HD may affect or alter various cell pathways prior to reaching the DNA located in the nucleus. Therefore, the action of alkylation agents on target organ cells may proceed *via* three principal mechanisms: (*i*) the physical interaction of the alkylating agent with cellular receptors; (*ii*) the chemical reaction of the alkylating agent with these receptors; or (*iii*) either type of metabolite–receptor interaction following metabolism of the alkylating agent within the target organ or elsewhere.

Although direct alkylation of DNA and RNA has been widely described, we hypothesize additional alkylation events of potential importance in skin injury. We have determined by immunocytochemistry levels of interleukin-1 beta (IL-1β) in cultures of normal human epidermal keratinocytes (NHEK) following exposure to HD (3 h post exposure to 2 mM HD). This exposure time is sufficient to allow release of a significant quantity of IL-1β. However, it is not long enough for the cells (adults and neonatals) to go through a complete cycle and divide, thus eliminating possible errors due to differences in cell turnover time (~21 h for neonatal-NHEK and ~24 h for adult-NHEK). The HD concentration range was chosen because it is generally observed that exposure to approximately 1 mM HD causes the formation of microblisters, while full blister formation occurs after exposure to approximately 2 mM HD. The expression of IL-1β in NHEK was found to be related to cell culture donor age. EPR spectroscopy was used to show the formation of an EPR detectable, $g = 2.04$, feature characteristic of iron–nitrosyl complex formation, and the generation of this induced complex by NHEK exposed to HD (18 h post exposure to 1 mM HD) was blocked by N^{ω}-nitro-L-arginine (L-NOARG), a competitive inhibitor of nitric oxide synthase (NOS). Nitric oxide (˙NO) has been implicated as the effector molecule that mediates IL-1β.[6] Our results show the release of nitric oxide during cytokine expression, IL-1β, when keratinocytes are exposed to HD. The combination of the nitric oxide with the chloride (Cl^-) in the plasma which is approximately 0.10 M,[7]

and the one released from sulfur mustard ($[ClCH_2CH_2]_2S$) upon cyclization to the sulfonium ion may lead to the formation of nitrosyl chloride (NOCl), a known potent nitrosylating agent. If NOCl is formed, it may play a role in the skin injury.

In addition, EPR and ELISA results show the generation of nitric oxide (•NO), therefore suggesting that nitric oxide synthase (NOS) plays a role during IL-1 expression when keratinocytes are exposed to HD. Inducible nitric oxide synthase (iNOS) has been associated with inflammatory and autoimmune tissue injury.[8] Their findings show the presence of iNOS in human skin that can be localized to keratinocytes in the epidermal layer. This paper shows the possible involvement of active nitrogen species in the pathways of cell injury/repair following exposure to sulfur mustards. Active nitrogen species are those derived from nitric oxide generated in cells. The possible involvement of the known alkylating agent nitrosyl chloride (NOCl) is discussed. The thermodynamic considerations and possible *in vivo* production of NOCl by macrophages and neutrophils have been reported.[9]

Experimental

NHEK (adult and neonatal, Clonetics, San Diego, CA) were cultured to confluency and harvested for experiments. The culture medium used was keratinocyte basal medium, modified Molecular Cellular Developmental Biology 153 (MCDB 153), which was supplemented with bovine pituitary extract (7.5 mg ml^{-1}), human recombinant epidermal growth factor (0.1 μg ml^{-1}), hydrocortisone (0.5 mg ml^{-1}), bovine insulin (5 mg ml^{-1}), gentamicin sulfate (50 mg ml^{-1}) and amphotericin-B (50 μg ml^{-1}). The medium was changed after 2 days of culture. After 3 and 6 days, the cells were harvested. After harvesting, the NHEK were resuspended in phosphate medium (5×10^6–5×10^7 cells per 0.5 ml). IL-1β and EPR spectra were measured after exposure of the suspension to 1–2 mM HD, 0.1 mM N^{ω}-nitro-L-arginine (L-NOARG; $C_6H_{13}N_5O_4$, Sigma Chemical Co., St. Louis, MO), or a combination of these agents (see Fig. legends). IL-1β release was measured after 3 h exposure and the heme-NO EPR spectrum was recorded after 18 h of exposure. The difference in post exposure time between the ELISA and the EPR experiments is due to the sensitivity of the two techniques. The IL-1β release was measured using a commercially available kit for the ELISA technique (Quantikine™). This kit is specific for IL-1β and shows no cross reactivity with other cytokines (*e.g.* IL-1α) as stated in the Quantikine™ brochure for IL-1β.

The Quantikine™ human IL-1β Immunoassay (Catalog Number DLB50, R&D Systems, Inc., Minneapolis, MN) was used for the quantitative determination of human IL-1β concentration in the cell cultures. This assay employs the quantitative 'sandwich' enzyme immunoassay technique. A monoclonal antibody specific for IL-1β is coated onto the microtiter plate provided in the kit. Standards and homogenous cell suspensions (100 μl) were pipetted into the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution was added to the wells and color was developed in proportion to the amount of IL-1β bound in the initial step. The color development was stopped and the intensity of the color was measured. The absorbance of each well was read at 450 nm. By comparing the optical density of the samples to the standard curve, the concentration of the IL-1β in the unknown samples was then determined. The assays were run in triplicate, and statistical evaluation was carried out using the paired sample *t* test with significance defined as $p < 0.05$. Representative data are shown in Fig. 2.

EPR spectroscopy experiments were performed using cell suspensions containing at least 5×10^6 keratinocyte cells per 0.5 ml suspended in 1 ml of complete MCDB 153 media. The cells were incubated at 37 °C for an additional 18 h in the presence or absence of 1 mM HD or 50–100 μM of L-NOARG at which time the cells were isolated and frozen at −70 °C. EPR spectroscopy was performed at 77 K on the cell suspensions using a Varian E-109 spectrometer equipped with an X-band (9.5 GHz) microwave bridge. The instrumental parameters at which the EPR spectra were recorded are given in the legend to Fig. 1.

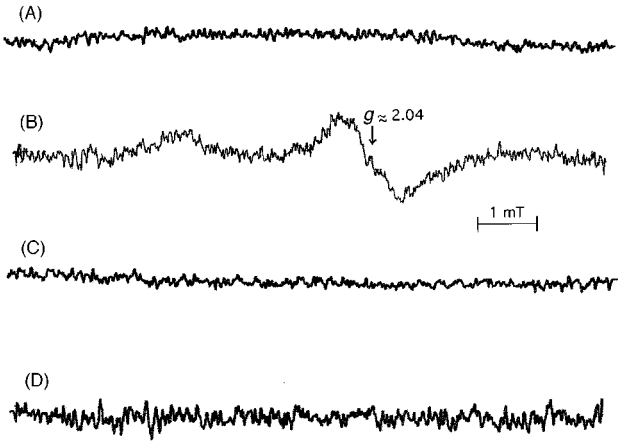

Fig. 1 Low temperature (liquid nitrogen, 77 K) EPR spectra recorded from NHEK suspensions (3.8×10^6 cells ml^{-1}). (A) Control, non-exposed adult-NHEK; (B) adult-NHEK exposed to 1 mM sulfur mustard (HD) collected 18 h post-HD exposure; (C) neonatal-NHEK exposed to 1 mM HD EPR spectrum obtained 18 h post exposure; (D) sample treated with N^{ω}-nitro-L-arginine (L-NOARG) to give a final concentration of 100 μM. EPR conditions: magnetic field, 334.5 mT; modulation frequency, 100 kHz; microwave frequency, 9.475 GHz; microwave power, 10 mW; receiver gain, 5×10^5; modulation amplitude, 0.5 mT and scan rate 0.62 mT s^{-1}.

Results and discussion

Adult-NHEK cells exposed to HD and incubated for 18 h at 37 °C induced the formation of EPR-detectable signals (Fig. 1). The EPR spectra in Fig. 1 were obtained by freezing the samples to 77 K in liquid nitrogen after incubation at 37 °C for 18 h. Fig. 1(A) is the control and represents the EPR spectrum obtained when adult-NHEK were incubated in the absence of HD and then frozen. However, adult-NHEK exposed to HD and incubated for 18 h generate the EPR spectrum shown in Fig. 1(B). This EPR spectrum has an approximate *g*-value of $g = 2.04$, which is similar to the *g*-values of known reported iron–nitrosyl complexes, suggesting the formation of nitric oxide (•NO).[10] The observed EPR spectrum may originate directly from the formation of •NO or one of the reactive nitrogen by-products (NO_x) generated in the biological decomposition of •NO. Neonatal-NHEK incubated in the presence and absence of HD generate the EPR spectrum as shown in Fig. 1(C). It is possible that if •NO is produced by neonatal-NHEK either in the unexposed controls or in the HD-exposed cells, this •NO may originate from another source and have a different function, thus rendering it unavailable to interact with iron to form an iron–nitrosyl type complex as observed in Fig. 1(B). It is also conceivable that low concentrations of •NO are always present in neonatal-NHEK. This concentration may be too low to detect by EPR spectroscopy, but high enough to cause other types of biological effects (*e.g.* cytokine release).

Since the spectrum in Fig. 1(B) suggests that adult-NHEK generates nitric oxide (•NO) during sulfur mustard (HD) exposure, the cells were incubated with HD in the presence of the specific nitric oxide synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NOARG)[11] to confirm the production of •NO. The result from this experiment is shown in Fig. 1(D). It can be seen that the EPR spectrum shown in Fig. 1(B) is not generated when the cells are incubated with HD in the presence of L-NOARG. This strongly suggests that •NO is produced when adult-NHEK are exposed to HD.

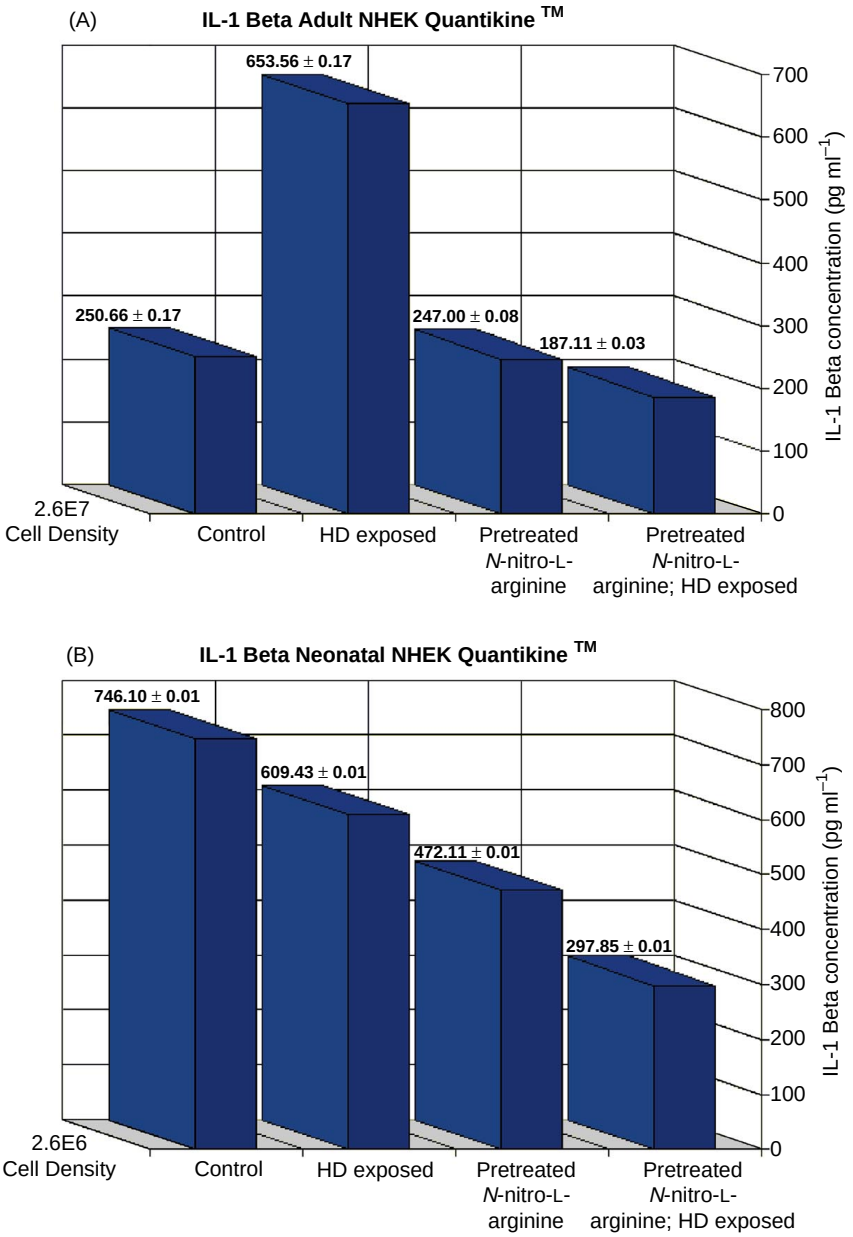

Fig. 2 IL-1β activation of adult- and neonatal-NHEK exposed to 2 mM HD. The IL-1β levels in cell suspensions (5×10^6–5×10^7 cells ml^{-1}) were measured using IL-1β Quantikine™ ELISA kit. (A) 100 μl aliquots of adult-NHEK (2.6×10^7 cells ml^{-1}) as a function of IL-1β (pg ml^{-1}) produced in HD-exposed and non-exposed controls. (B) 100 μl aliquots of neonatal-NHEK (7.5×10^6 cells ml^{-1}) as a function of IL-1β (pg ml^{-1}) produced in HD-exposed and non-exposed controls. The effect of N^{ω}-nitro-L-arginine (L-NOARG) alone and the combined effects of sulfur mustard and L-NOARG on IL-1β release by NHEK are also included in panel (A) and panel (B).

It is important to establish what role the production of $^{\bullet}$NO plays during exposure of NHEK to HD. Previous reports have shown that $^{\bullet}$NO is involved in immune/cytokine regulation.[12] Therefore, to establish the role $^{\bullet}$NO plays during HD exposure, the effect that HD exposure has on cytokine regulation must be determined. Initially the effect of HD on the production of interleukin 1β (IL-1β) was chosen because human skin keratinocytes express significant activation of IL-1 determined by the ELISA technique. For this reason, IL-1β was assayed in adult-NHEK and neonatal-NHEK (5×10^6–5×10^7 cells) exposed to HD (2 mM) and incubated for 3 h at 37 °C, using the ELISA technique. This exposure time is sufficient to allow release of a significant quantity of IL-1β. However, it is not long enough to allow the cells (adult and neonatal) to go through a complete cycle and divide, thus eliminating possible errors due to differences in cell turnover time (~21 h for neonatal-NHEK and ~24 h for adult-NHEK). The HD concentration range was chosen because it is generally observed that exposure to approximately 1 mM HD causes formation of microblisters while full blister formation occurs after exposure to approximately 2 mM HD (data not shown). The results are shown in Fig. 2. Adult-NHEK exposed to HD (2 mM) show a significant increase in the production of IL-1β [Fig. 2(A)]. When these cell suspensions are incubated with the specific NOS inhibitor L-NOARG, the production of IL-1β is decreased to the level obtained for the cells (controls) not exposed to HD. In addition, the combined effects of incubation with L-NOARG followed by exposure to HD slightly lowers the production of IL-1β when compared to the cell suspensions incubated with L-NOARG alone or to controls. For neonatal-NHEK exposed to HD (2 mM) the production of IL-1β is decreased when compared to the cells (controls) not exposed to HD [Fig. 2(B)]. Furthermore, incubation of the cell suspensions with L-NOARG alone also decreases the production of IL-1β. The combined effects of incubation with L-NOARG followed by HD exposure are also shown in Fig. 2(B). These show a further decrease in the production of IL-1β when compared to the controls and to the cells exposed to HD alone or cells incubated with L-NOARG alone. The concentrations of IL-1β per cell before and after exposure to HD or treatment with L-NOARG are given in

Table 1 Concentration of interleukin-1β per cell[a]

Cell type	n[b]	[Control]/ (pg/cell) × 10^6	[HD]/ (pg/cell) × 10^6	[L-NOARG]/ (pg/cell) × 10^6	[L-NOARG + HD]/ (pg/cell) × 10^6
Adult	3	9.64	25.14	9.50	7.20
Neonatal	3	99.48	81.26	62.95	39.91

[a] All columns are the average of a number of experiments. [b] n = number of experiments.

Table 1. The data show that the neonatal-NHEK contain a significantly large amount of IL-1β per cell when compared to the adult-NHEK.

The approximately 10-fold difference in IL-1β concentration per cell in the untreated control adult- and neonatal-NHEK can be attributed to two factors: (*i*) a continual higher level of IL-1β in neonatal-NHEK is required for the induction of growth factors for proliferation and other structural proteins required for the normal keratinocyte development; and (*ii*) the fully developed •NO-related immune pathways in adult NHEK differ from the developing •NO-related immune pathways in the neonatal-NHEK.[13] For instance, the data in Fig. 2 show that the production of IL-1β is directly linked to the production of •NO. Therefore, it is possible that the adult-NHEK contain a low concentration of the constitutive form of NOS (cNOS), but are capable of rapidly producing the inducible form of NOS (iNOS), which is activated immediately upon the presence of a foreign toxic substance.[11] This would mean that adult-NHEK produce iNOS on demand in the presence of a foreign toxic substance (HD in this case) generating the •NO which triggers the observed increase in IL-1β production. Alternatively, the neonatal-NHEK contain mainly the cNOS and thus require only small concentrations of the iNOS. However, neonatal-NHEK contain a steady concentration of cNOS which is continuously producing low levels of •NO which, in turn, triggers the observed higher level production of IL-1β. Although the adult- and neonatal-NHEK appear to contain different forms of the NOS enzyme, both types of cells are affected in the same manner by the specific NOS inhibitor L-NOARG even though each type of cell (adult and neonatal) reacts in an opposite manner when exposed to HD.

The lower yield of IL-1β [Fig. 2(B)] when neonatal-NHEK are exposed to HD as compared to unexposed controls is possibly due to the interaction of HD with the cell surface. It is possible that activation of cNOS and iNOS originates from different receptors on the cell surface. Therefore, since neonatal-NHEK appear to contain only the cNOS it is likely that the interaction of HD with the cell surface would interfere with the continuous production of •NO and IL-1β [Fig. 2(B)]. This is consistent with the fact that no EPR signal is observed for HD-exposed neonatal-NHEK when suspensions of these cells were run in the same manner as the adult-NHEK (Fig. 1). These observations are consistent with previous studies[14] that suggest that the surface characteristics of NHEK are continuously changing. These modulations reflect the stage of differentiation and activation of the NHEK. Thus, the NHEK in various stages of differentiation have distinct sets of surface moieties that are expressed in different manners (Fig. 2).

One thing is clear from the results in Fig. 2: the dependence of the production of IL-1β in adult- and neonatal-NHEK on the generation of •NO. This established •NO as an effector molecule in cytokine regulation, at least for IL-1β. This fact is supported by the observation that when the production of •NO is blocked by the specific NOS inhibitor (L-NOARG) the production of IL-1β is also decreased. The results suggest that the production of •NO by the NHEK serves as a direct interleukin-1 converting enzyme activator or that the •NO formed triggers a signal at the cell membrane which activates the interleukin-1 converting enzyme.

The fate of the •NO generated by NHEK after activating the IL-1β system remains to be addressed. Since chloride (Cl^-) is

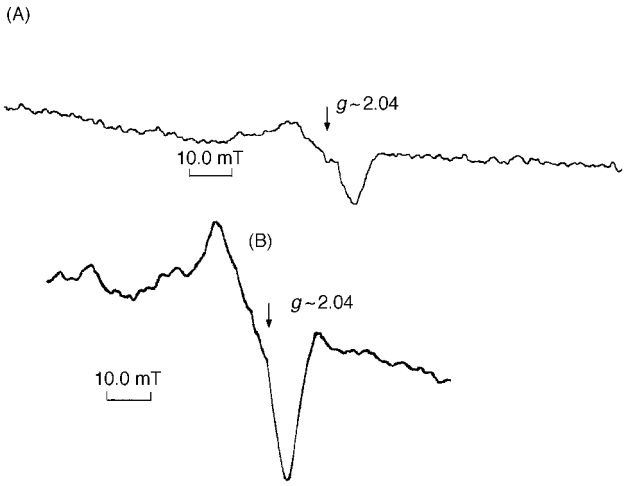

Fig. 3 Low temperature (77 K) EPR spectrum of hemoglobin (~10^{-4} M) after reaction with (A) chemically generated NOCl [nitrosyl chloride is generated *in situ* from alkylnitrite and titanium tetrachloride in DMF at 0 °C: $TiCl_4 + 4RONO \rightarrow Ti(OR)_4 + 4ClNO$]; and (B) dissolved •NO. EPR conditions: magnetic field, 334.5 mT; modulation frequency, 100 kHz; microwave frequency, 9.475 GHz; microwave power, 10 mW; receiver gain, 1.25×10^4; modulation amplitude, 1.0 mT and scan rate 0.833 mT s^{-1}

released upon dissolution of HD in aqueous environments, it is possible that the reactive nitrogen species formed is NOCl. NOCl is a nitrosylating agent that is consistent with the known biological action of HD. In addition, NOCl and •NO react with hemoglobin yielding the EPR spectra shown in Fig. 3. Fig. 3(A) represents the reaction of NOCl with hemoglobin and Fig. 3(B) represents the reaction of a solution of dissolved •NO with hemoglobin (1×10^{-4} M). Both these EPR spectra have similar *g*-values to the one observed in Fig. 1(B). However, in Fig. 3(A) the NOCl was generated chemically in a reaction vessel and then carried over with an inert gas (N_2) and bubbled through hemoglobin (1×10^{-4} M) solution. The hemoglobin solution containing the NOCl was rapidly frozen at 77 K in liquid nitrogen prior to obtaining its EPR spectrum. The chloride ion concentration (1–2 mM) in HD is small compared to the Cl^- concentration in plasma and cytosol (100 mM). However, HD interacts with the cells, ultimately generating nitric oxide and IL-1β. It is this increase of •NO over the base level in cells that is available for the production of NOCl. The Gibbs energy of formation of NOCl is energetically feasible.[9,15] Nitrosyl chloride can nitrosylate organic compounds directly, and therefore its presence poses two dangers: (*i*) it is a strong oxidant and (*ii*) nitrosylation leads to compounds that are often mutagenic or promutagenic (alkylation of DNA). Therefore, the EPR spectrum in Fig. 1(B) could originate from either the direct interaction of •NO with some type of porphyrin-containing molecule or the interaction of NOCl with the porphyrin-containing molecule.

However, because of the suggested difference in the NOS-type (inducible or constitutive) in these cells, their reaction to HD exposure is quite different. In terms of IL-1β production, keratinocytes in normal skin do not express significant numbers of IL-1 receptors. The normal keratinocyte cells can be in an 'activated' or 'refractory' state. The IL-1β receptor expression is associated with the active state of the keratinocyte and requires

an outside stimulus to express the receptor and generate IL-1β. Exposure to HD causes adult-NHEK to go into the activated state generating ˙NO, which in turn activates the IL-1β receptors causing the production of IL-1β. The ˙NO generated by the exposure of human keratinocytes to HD activates the interleukin-1 converting enzyme producing the observed increase in interleukin-1β.[16] The production of IL-1β triggers a series of other events in the keratinocyte ('effector' state). These observations are consistent with the results shown in Fig. 2(A). The neonatal-NHEK are continuously producing higher levels of ˙NO and IL-1β. However, when neonatal-NHEK are exposed to HD the cells go directly into the 'refractory' state either from the active state or the effector state.[17,18] In the refractory state the IL-1 receptors are downregulated consistent with the results in Fig. 2(B). In both cases NOCl could be produced leading to cell damage and DNA alkylation. These findings may provide guidance to the understanding of sulfur mustard toxicity and also suggest that L-NOARG has significant novel pharmacological effects other than the inhibition of NOS.

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