Induction of the 72-kilodalton heat shock protein and protection from ultraviolet B-induced cell death in human keratinocytes by repetitive exposure to heat shock or 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂

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Abstract It has been demonstrated that hyperthermia protects keratinocytes from ultraviolet B (UVB)-induced cell death in culture and in vivo. This effect is mediated by the antiapoptotic effect of heat shock proteins that are transiently induced after exposure to heat at sublethal temperatures. Consequently, induction of Hsp has been proposed as a novel means of photoprotection. However, in the face of daily UVB exposure of human skin in vivo, this approach would not be useful if keratinocytes become less sensitive to Hsp induction with repeated exposure to the inducing agent. The aim of this study was to investigate whether repeated exposure to hyperthermia or to the stress protein activating cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15dPGJ₂) leads to adaptation of the cells, attenuation of the heat shock response, and abrogation of the protective effect. Normal human epidermal keratinocytes (NHEK) and the carcinoma-derived cell line A431 were exposed to either 42°C or to 15dPGJ₂ for 4 hours at 24-hour intervals for 4 consecutive days. The intracellular level of the 72-kDa heat shock protein (Hsp72) was determined by enzyme-linked immunosorbent assay (ELISA). Cells were exposed to UVB from a metal halide source after the last heat or 15dPGJ₂ treatment, and survival was determined 24 hours after exposure by a MTT assay. Our results demonstrate that (1) heat shock and 15dPGJ₂ are potent inducers of Hsp72 expression and lead to increased resistance to UVB-induced cell death in human keratinocytes; (2) re-exposure to heat shock leads to a superinduction without attenuation of the absolute increase in Hsp72 and of its UVB-protective effect; (3) the UVB tolerance induced by 15dPGJ₂ is enhanced by repeated exposure without a further increase of Hsp72; (4) repeated heat shock and 15dPGJ₂ up to a concentration of 1 µg/mL have no influence on cell growth over a period of 4 days. We conclude that through repeated exposure to Hsp-inducing factors, stress tolerance can be maintained without additional toxicity in human keratinocytes. These results provide a basis for the development of nontoxic Hsp inducers that can be repeatedly applied without loss of effect.

INTRODUCTION

Ultraviolet (UV) radiation, electromagnetic radiation with wavelengths between 200 and 400 nm, represents one of the most abundant environmental factors affecting hu-

mans. According to wavelength and biological effects, UV radiation is basically divided into UVC (200–280 nm), UVB (280–320 nm), and UVA (320–400 nm). Solar UVC is not biologically relevant because it is completely absorbed in the atmosphere. In contrast, UVB and UVA are within the terrestrial solar spectrum (Trautinger 1999). Additional exposure comes from artificial sources that are used for skin tanning and in phototherapy. Sunburn, pigmentation, photoaging, and photocarcinogenesis are

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among the immediate and late consequences of the exposure of human skin to UV. The main initial molecular events that mediate these clinical responses are direct modification of DNA and formation of reactive oxygen species (ROS) through photodynamic processes (Freeman et al 1989; Tyrrell 2000). Cells react to these insults with the initiation of DNA repair and other protective mechanisms including the expression of Hsp (Gilchrest and Eller 1999; Trautinger et al 1999). Depending on wavelength, UV dose, and cell type, a certain percentage of UV-exposed cells will enter the apoptotic pathway. In the epidermis, these apoptotic cells appear at the microscopic level in the form of so-called sunburn cells within 24 hours after UV exposure. According to current understanding, UV-induced cell death aims to eliminate cells with severe DNA damage that cannot be repaired properly (Ziegler et al 1994).

Earlier studies have demonstrated that heat pretreatment reduces the rate of UVB-induced cell death in cultured keratinocytes and normal murine and human skin (Maytin et al 1993; Kane and Maytin 1995; Trautinger et al 1996b). The induction of heat shock proteins, particularly Hsp72, has been linked to the phenomenon of hyperthermia-induced UVB resistance. In addition, Hsp72 has been detected in human keratinocytes under non-stressful conditions, and downregulation of Hsp72 resulted in increased susceptibility to UV cytotoxicity (Trautinger et al 1993, 1995). Although it cannot be excluded that inhibition of UVB-induced cell death allows for the survival of cells that potentially harbor carcinogenic DNA lesions, there is emerging evidence indicating that heat preconditioning transiently improves DNA repair (McKay et al 1997; Jantschitsch and Trautinger 2003; Nadin et al 2003). As a consequence, therapeutic intervention with the aim of increasing the expression of heat shock proteins in the skin has been discussed as a means of photoprotection. Taking into account the daily exposure of human skin to UV in vivo, this would require that the heat shock response can be repeatedly induced in human skin cells without adaptation and/or attenuation. Because to date there is insufficient evidence on the response of mammalian tissues to repeated exposure to sublethal stress, this study was undertaken to investigate this question in human keratinocytes. For this purpose, we exposed keratinocytes in culture either to heat stress or to the heat shock protein inducing cyclopentenone prostaglandin 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15dPGJ₂) and determined cell growth, time course of Hsp72 expression, and resistance to UVB during repeated exposures.

MATERIALS AND METHODS

Cell culture

The epidermal carcinoma cell line A431 was cultured in Dulbecco's modified Eagle Medium (GIBCO, Invitrogen

Ltd., Paisley, UK) containing 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), penicillin (100 U/mL), streptomycin (100 µg/mL), and glutamine (2 mM). Normal human epidermal keratinocytes (NHEK) were grown in Epi-Life Medium supplemented with calcium chloride and human keratinocyte growth supplement (Cascade Biologies Inc., Portland, OR, USA).

For further treatment, cells were seeded in 35-mm petri dishes at about 75% confluency. Every 24 hours, cultures were exposed for 4 hours to either 42° in a humidified atmosphere or to 15dPGJ₂ (Sigma-Aldrich, St. Louis, MO, USA) dissolved in methyl acetate at final concentrations of 0.5, 1.0, and 2.0 µg/mL. Unheated cells otherwise handled in parallel and cells exposed to solvent alone were used as control ("mock-treated"). Four experimental conditions were studied for the effect of heat shock and 15dPGJ₂ on survival following UVB exposure: (1) 4 mock treatments; (2) mock-treated for 3 days, heat shock/15dPGJ₂ on the 4th day; (3) heat shock/15dPGJ₂ for 3 days, mock-treated on the 4th day; and (4) heat shock/15dPGJ₂ for 4 days. All conditions were followed by UV exposure after the last treatment.

UVB irradiation

Immediately after the last heat shock and 20 hours after the last exposure to 15dPGJ₂ (or the respective mock treatment), the cells were irradiated at a distance of 30 cm using a metal halide source (Mutzhas Supersun 5000 Solar simulator). We used a filter combination with a transmission between 290 and 320 nm. The output was monitored by a radiometer (IL 1700; International Light, Newburyport, MA, USA). Prior to UV irradiation, cells were washed twice with phosphate-buffered saline (PBS; with Ca²⁺ and Mg²⁺) and left in PBS. Following irradiation, PBS was replaced by fresh medium and the cells were incubated until further analysis. Control cells were treated identically but without UV exposure.

Preparation and analysis of cell extracts

Whole-cell extracts were prepared by repeated cycles of freeze-thawing. Protein content in the supernatants that were obtained after centrifugation was determined by a commercial assay (BCA Protein Assay kit; Pierce Biotechnology Inc., Rockford, IL, USA). Hsp72 was determined by enzyme-linked immunosorbent assay (ELISA) (StressGen Biotechnologies, Victoria, British Columbia, Canada) according to the manufacturer's instructions and expressed as nanograms per microgram of total cellular protein.

MTT assay

A MTT assay was used for the quantification of metabolically active, living cells. The mitochondria of living cells convert the yellow-colored tetrazolium compound to its red formazan derivate, which is measured photometrically at 450 nm (EZ4U; Biomedica, Vienna, Austria). The viability is calculated as percentage of absorbance relative to sham-treated control cultures.

Statistics

Results derived from replicate samples are indicated as mean $\pm$ SD. Data calculated from repeated independent experiments are given as mean $\pm$ SEM. For comparisons, paired/unpaired *t*-test was used as appropriate. Level of significance was $P < 0.05$.

RESULTS

Induction of Hsp72 by repetitive heat shock

In order to study the response of A431 and normal human keratinocytes to repeated heat stress, cells were exposed to 42°C for 4 hours every 24 hours for 4 consecutive days. Hsp72 was measured by ELISA prior to and after each heat shock. Baseline levels were 0.034 ± 0.012 ng/ μ g for NHEK and 0.894 ± 0.085 for A431 (Hsp72/total cellular protein; mean $\pm$ SD; $P = 0.0012$). As expected, Hsp72 was strongly induced 4 hours after a single heat shock in both cell types. In NHEK, the initial increase was 5.6 ± 1.3 times the baseline level, whereas in A431 it was lower at 1.9 ± 0.22 (mean $\pm$ SEM, $P = 0.01$) (Fig 1A,B). Hsp72 levels decreased in the intervals between hyperthermia but did not fall back to baseline. Upon subsequent heat exposures, the absolute increase in the amount of Hsp72 remained constant, indicating that with an identical trigger the response remains quantitatively stable, even after repeated challenge. This result is confirmed by the fact that it is observed in A431 and NHEK with nearly identical kinetics after correction for the different baseline expression and magnitude of induction.

Induction of Hsp72 by repeated exposure to 15dPGJ₂

Incubation of NHEK with 15dPGJ₂ for 4 hours resulted in a dose-dependent increase in Hsp72. In contrast to what has been observed with heat treatment, Hsp72 increased further even after 15dPGJ₂ was replaced by medium after 4 hours. At a concentration of 2.0 μ g/mL, peak levels were reached after 24 hours (11.7 ± 4.3 times basal expression). At 1.0 μ g/mL, peak levels were lower and achieved at 48 hours, ie, after 2 exposures (5.1 ± 1.3 times basal expression). No significant changes in Hsp72 were observed with 0.5 μ g/mL (Fig 1C).

Fig 1. Induction of Hsp72 by repetitive heat shock and 15dPGJ₂. Cells were exposed to 42°C for 4 hours (A,B) or to 15dPGJ₂ (C) every 24 hours for 4 consecutive days (indicated by bold sections on the x-axis). Hsp72 was measured by ELISA prior to and after each heat shock. Results are given as multiples of baseline expression and represent means of 3 (A431) and 4 (NHEK) independent experiments $\pm$ SEM.

Heat shock induced resistance to UVB

In accordance with earlier observations, we could confirm that exposure to heat induces increased resistance to UVB-induced cell death if the cells are exposed to UVB immediately after heat shock. The effect is UVB-dose de-

Fig 2. Heat shock induced resistance to UVB. Cells were exposed to single or repeated heat shock or 15dPGJ₂ prior to irradiation with UVB according to the experimental setup shown in D. Survival was determined 24 hours after irradiation by a colorimetric assay. (A) NHEK exposed to single and repeated heat shock; (B) A431 exposed to single and repeated heat shock; (C) NHEK exposed to

pendent and occurs in both cell types. We were further interested whether the effect still exists after repeated heat exposures and whether it is quantitatively changed. We found that there is no significant difference between the results obtained with cells that had been exposed to 1 heat shock compared to cells that had experienced repeated heat treatments, indicating that the heat shock response remains functionally active after repeated exposures and that attenuation does not occur (Fig 2A,B). The group that was irradiated immediately after the final heat shock, independent of whether there were multiple or single heat shocks, demonstrated increased survival when compared to either the control group (no heat shocks) or the group heat shocked 3 times and then sham-treated once before irradiation.

15dPGJ₂ induced resistance to UVB

A single exposure to 15dPGJ₂ at 1.0 and 2.0 µg/mL was able to induce increased resistance of NHEK to UVB. The protective effect could be maintained with repeated exposure, and even when 3 exposures were followed by a sham treatment the survival rate was significantly above control levels in all groups. The groups exposed 4 times to 15dPGJ₂ at 1.0 and 2.0 µg/mL showed higher survival rates compared to the groups exposed 3 times followed by a mock treatment, thus indicating that the last exposure in our experiment is still able to effectively reduce UVB-induced cell death. Neither the first nor the last exposure to 15dPGJ₂ at 0.5 µg/mL had a significant effect on survival after UVB when compared to its appropriate control. However, cells that had been treated 3 or 4 times showed higher survival rates than cells that had been treated only once or twice, indicating an accumulating protective effect in these groups (Fig 2C).

Influence of repeated heat shock and 15dPGJ₂ on cell growth

Cell proliferation continued during the 4-day period of the experiment and we were interested whether repeated heat shock or 15dPGJ₂ has an influence on cell growth. Total protein content in the cell extracts correlated directly with cell number, and for NHEK and A431 this parameter increased steadily during the time of the experiment irrespectively, whether the cells were heat treated or controls. Small variations that occurred during the heat shock incubation periods are attributed to the effect of medium change, because they were observed with

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single and repeated 15dPGJ₂; the UV dose in this experiment was 400 mJ/cm². Values indicate survival rates relative to unirradiated controls and represent mean ± SEM of 3 independent experiments.

both hyperthermia and sham treatment (Fig 3A,B). 15dPGJ₂ had a dose-dependent growth inhibitory effect. At 0.5 and 1.0 µg/mL, no significant difference to the solvent control could be observed. However, at 2.0 µg/mL, 15dPGJ₂ inhibited cell growth as total protein levels in the cell extracts remained constant throughout the observation period and did not show the steady increase that was observed in the other groups (Fig 3C).

DISCUSSION

With the discovery of heat shock proteins in human skin, research in experimental dermatology started its search for the function of these proteins in skin physiology and pathology (Trautinger et al 1996a). Ultraviolet radiation is among the most ubiquitous thoroughly investigated environmental factors that affect the skin, and it is reasonable to ask whether Hsp might be involved in the protection of keratinocytes from the potentially adverse effects of UV, ie, sunburn, photoaging, and photocarcinogenesis (Maytin 1995). DNA is the primary intracellular UVB-absorbing chromophore, and exposure to UVB leads to the formation of characteristic DNA photoproducts (mainly cyclobutyl dimers and [6-4] photoproducts). Although these lesions usually are repaired with high fidelity, error-prone repair mechanisms exist that can give rise to mutagenesis and eventually to the formation of skin cancer (Ziegler et al 1994). Formation of reactive oxygen species (ROS) is another consequence of UV exposure that has been linked with photoaging and photocarcinogenesis (Pourzand and Tyrrell 1999). At the cellular level, one response of keratinocytes to UV is apoptotic cell death. In histological sections of UV-exposed skin, this is easily visible in the form of so-called sunburn cells, and it can be reproduced easily and dose dependently in cell culture (Young 1987; Schwarz et al 1995; Schindl et al 1998). Activation of the "death receptor" CD95, formation of ROS, and DNA damage are among the reactions that have been described to initiate the apoptotic pathway (Kulms and Schwarz 2002). It has been demonstrated in various models that heat shock prior to irradiation can induce a state of increased resistance to UVB-induced cell death, a phenomenon that—in analogy to thermotolerance—has been called UV tolerance. The response can be induced in culture as well as in rodent and human skin *in vivo*, and Hsp72 has been demonstrated as one of its essential mediators (Kane and Maytin 1995; Simon et al 1995; Trautinger et al 1995, 1996b). It is a matter of current investigation as to whether heat-induced UV tolerance is associated with protection from carcinogenesis and photoaging and thus might have therapeutic potential. The available evidence has been recently reviewed (Jantschitsch and Trautinger 2003). Keratinocyte differentiation is another process for which the involvement of

Fig 3. Influence of repeated heat shock on total cellular protein. NHEK (A) and A431 (B) were exposed to 42°C for 4 hours every 24 hours for 4 consecutive days (solid lines) or sham treatments (dashed lines). (C) NHED were exposed to 15dPGJ₂ at various concentrations for 4 hours every 24 hours. Total protein content was determined from cell extracts prepared by repeated cycles of freeze-thawing. Results are given as mean $\pm$ SEM of 3 independent experiments.

Hsp has been recently demonstrated. During their transition from the basal cell layer to the stratum corneum, epidermal keratinocytes undergo a highly regulated process that transforms them from basal, "undifferentiated" keratinocytes into corneocytes that form the outermost protective layer of mammalian skin. This process involves the expression of specific differentiation-associated proteins that are cross-linked by isodipeptide and disulfide bonds to form the cornified cell envelope (CCE) (Hohl 1990). It is highly unlikely that the correct formation of the complex protein polymer structure of the CCE and of

the intermediate filament network of keratinocytes is possible without the assistance of molecular chaperones. Histological, ultrastructural, and biochemical data point to the fact that Hsp27 might be a promising candidate for that function (Jonak et al 2002). Various inflammatory and hereditary diseases (eg, psoriasis, the diverse group of congenital ichthyoses) are characterized with disturbance of this process, resulting in often grave consequences for the patient. As a consequence of these discoveries comes the idea of therapeutic modification of Hsp expression for photoprotection and treatment of skin diseases, particularly since pharmacological inducers and enhancers (also called co-inducers) of Hsp have been described (Vigh et al 1997; Morimoto and Santoro 1998).

An open question with regard to therapeutic Hsp induction is whether tissues are able to adapt to repeated or continuous exposure to a Hsp-inducing trigger. This is of particular interest in dermatology where the inducer could be used topically and should not lose its effect upon repeated application. Adaptation to the set point of the heat shock response has been described in yeast and fish, and in a *Drosophila* model the expression of a transgene under control of the *hsp70* promoter is reduced after repeated heat shocks (Dietz and Somero 1992; Hunt et al 1992; Carratu et al 1996). Very little information is available about the consequences of repeated stress exposure in mammalian cells. In rats, it has been shown that repeated whole-body hyperthermia for up to 5 times at 2-day intervals leads to accumulation of Hsp72 in the liver (Kimoto et al 2000). In a murine in vivo model, it was shown that heat shock protects from TNF-induced inflammatory shock and this protection is maintained after repeated heat treatments (Van Molle et al 2002). Regretfully, in neither study was skin examined. We have demonstrated here for the first time in a human tissue culture model that repeated heat stress of keratinocytes results in a molecular response that is quantitatively similar to that observed after a single exposure when expression of Hsp72 is taken as an end point. Moreover, the functional properties of the heat shock response with regard to UV tolerance are maintained and neither adaptation nor attenuation could be observed. Although Hsp72 levels did not fall back to baseline between heat shocks and thus pre-heat shock Hsp72 expression increased steadily throughout the experiment, this did not lead to a superincrease in UV tolerance (compared with 1 heat shock). In addition, no protective effect of heat shock could be demonstrated for the group heat shocked 3 times but then sham-treated immediately before irradiation. This corresponds with earlier data showing that the protective effect of heat shock disappears within 12 h after exposure (Trautinger et al 1995). The response to repeated heat shocks demonstrated to be very similar for tumor-derived and nor-

mal keratinocytes, irrespective of their baseline levels of Hsp72, that—in agreement with earlier observations in other malignancies—is significantly higher in carcinoma-derived cells (Jäättelä 1999).

15dPGJ₂ was used as an example of a pharmacological Hsp inducer that has been described to increase the expression of Hsp72 in various tissue culture models including transformed keratinocytes (Santoro et al 1989; Ikai et al 1992; Hamel et al 2000; Zhang et al 2004). 15dPGJ₂ is a cyclopentenone prostaglandin with anti-inflammatory and antiproliferative activities. It is a natural ligand of the peroxisome proliferator-activated receptor (PPAR)- γ , but various PPAR- γ -independent actions including activation of heat shock factor 1 (HSF1) have been described. According to a recent report, modification of protein thiols through direct interaction with its cyclopentenone ring structure is responsible for the activation of a stress response by 15dPGJ₂ (Zhang et al 2004). Here we show that incubation with 15dPGJ₂ for 4 hours followed by a 20-hour recovery period is a potent inducer of Hsp72 in NHEK with induction levels that are dose dependent and above that achieved by heat stress. This strong induction was observed with concentrations that inhibited cell growth upon repeated exposure (2 μ g/mL). At a nonotoxic concentration (1 μ g/mL), Hsp72 was still induced, although to a lesser degree. In contrast to what we found for repeated heat shock, Hsp72 induction by 15dPGJ₂ did not further increase after 1 to 2 exposures, indicating that this mechanism of induction is saturable. Similar to heat stress, a single exposure to 15dPGJ₂ increased the rate of cell survival after UVB. Interestingly, this protective effect continued to increase with repeated exposure, even with stable or falling Hsp72 levels, and was also observed at concentrations of 15dPGJ₂ that did not induce a measurable increase in Hsp72. This indicates that the UVB tolerance brought about by 15dPGJ₂ might be at least partly due to mechanisms that are independent of Hsp72. Among these might be the activation of PPAR- γ , because it has been recently shown that activation of PPARs inhibits UVB-induced inflammation and that induction of PPAR- γ agonistic agents is a consequence of UVB exposure of human keratinocytes (Kippenberger et al 2001; Zhang et al 2005).

Our results in normal and transformed human keratinocytes indicate that repeated exposure to sublethal stress in human keratinocytes is possible without measurable cytotoxicity and without loss of protective function. However, as shown for 15dPGJ₂, the response depends on the inducing agent and generalization is not possible. Furthermore, due to the growth kinetics of the cells, the study was limited to a period of 76 hours and a prediction for longer observation periods, a higher number of heat shocks, or prolonged exposures to 15dPGJ₂ cannot be made.

In summary, our results demonstrate for the first time that repeated induction of a heat shock response is possible in human keratinocytes and thus provide an experimental basis for further research into the feasibility and efficacy of Hsp induction in the skin by therapeutic intervention. For any Hsp-inducing substance that is considered for clinical use, evaluation of efficacy and toxicity of repeated exposure should be investigated in appropriate experimental and clinical models.

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