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Involvement of the Heme Oxygenase–Carbon Monoxide Pathway in Keratinocyte Proliferation

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It has been suggested that nitric oxide (NO), a small gaseous molecule with a multiplicity of cellular functions, plays an important part in the regulation of cellular proliferation. We have examined the effect of the NO donor sodium nitroprusside (SNP) on heme oxygenase-1 (HO-1) expression in human epidermal keratinocytes and investigated the contribution of the heme oxygenase pathway in the control of keratinocyte proliferation. Incubation of keratinocytes with 0.5 mM SNP resulted in a 2.5-fold increase in heme oxygenase activity which was reflected by a significant increase in HO-1 protein expression, as measured by Western blot. This effect was associated with a 200% increase in keratinocyte proliferation. The proliferative effect of the NO donor was totally abolished by coincubation of SNP with tin protoporphyrin IX, a potent inhibitor of heme oxygenase, or hydroxocobalamin, a NO scavenger. These results suggest that the heme oxygenase pathway is involved in keratinocyte proliferation mediated by NO.

During the course of wound healing in the skin, epidermal keratinocytes are exposed to a number of agents including inflammatory macrophage-derived free radicals which play a critical role in the control of microbial invasion of the skin. Nitric oxide (NO), a reactive nitrogen intermediate with a multiplicity of cellular functions is also produced by macrophages as well as granulocytes and is involved in host defence (1). NO is produced by NO synthase (NOS) when converting L-arginine to L-citrulline. The enzyme exists in two isoforms: a constitutively expressed protein (cNOS) and an inducible protein (iNOS). It has been shown that both constitutive and inducible proteins are expressed in human keratinocytes (2,3).

Physiological relaxation of vascular smooth muscle cells, inhibition of platelet aggregation and neurotransmission are examples of the effects of NO that is continuously synthesised by cNOS (4-6). In contrast, the inducible form of NOS can produce considerable amounts of NO (100-1000 times more than cNOS) and it is through this production pathway that NO appears to act in host defence (1).

Another recently discovered effect of NO is its ability to up-regulate the expression of vascular heme oxygenase-1 (HO-1), a small molecular weight heat shock protein (HSP32) (7,8). Heme oxygenase is the rate limiting enzyme responsible for the catabolism of heme to biliverdin, ferric iron and carbon monoxide (CO)(9). Heme oxygenase is found in a variety of mammalian cells and, like NOS, exists in two isoforms: a constitutive (HO-2) and an inducible protein (HO-1) (10). Heme oxygenase plays a key role in the maintenance of cell function, as biliverdin is a potent antioxidant and CO has been shown to elicit vasodilatation through the activation of guanylate cyclase (11,12).

HO-1 has been shown to be present in cultured human keratinocytes and recently our group has reported that NO donors increase HO-1 expression and activity in cultured transformed human keratinocytes (13,14). Since NO also has the ability to stimulate increased keratinocytes proliferation (15), the present study was designed to investigate a possible link between NO, HO-1 expression and keratinocyte metabolism. A potential physiological role for HO-1 in keratinocyte proliferation will be discussed.

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MATERIALS AND METHODS

Reagents. All reagents, unless otherwise stated, were obtained from Sigma Chemical Co. (CA, USA). Transformed human epidermal keratinocytes (SVK14 cell line) were donated by Dr Claire Linge (RAFT, Middlesex, UK). Polyclonal antibodies against rat and human HO-1 protein were obtained from Bioquote Ltd. (York, England).

Cell culture. SV40 transformed human epidermal keratinocytes (HEKs) were cultured in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated foetal calf serum, L-glutamine (2mM), cholera toxin (50 ng/ml), hydrocortisone (5 mg/ml), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). The cells were grown in a humidified chamber (5% CO_2, 95% air). For heme oxygenase activity assay, confluent monoloyers of HEKs in T-75 cell culture flasks were incubated for 6 hours in the presence of the NO donor sodium nitroprusside (SNP). The incubation was terminated by washing the cells with ice cold phosphate buffered saline (PBS) prior to harvesting for the assay.

Cell proliferation assay. Confluent HEKs in T-75 flasks were harvested with 0.25% trypsin - 1 mM EDTA and plated at a density of 2×10^5 cells per 35 mm tissue culture multi well plate (Costar, Cambridge, MA). The number of cells was determined by counting suspended cells in a haemocytometer using a light microscope. Cells were allowed to settle and grow for 48 h prior to incubation with SNP (0.25-1 mM) for 24 hours. In another set of experiments cells were treated with SNP in the presence of the heme oxygenase antagonist, tin protoporphyrin-9, (SnPPIX) (Porphyrin Products Inc., Logan, UT) or the NO scavenger hydroxocobalamin. Cells were washed with warm (37°C) PBS and removed form the plate using 1 ml 0.25% trypsin - 1 mM EDTA. Aliquots from each well were taken and the number of HEKs was counted as described above.

Cell metabolism assay. HEKs were grown to confluence in 96-well plates and were incubated for 6 hr with various concentrations of SNP (0.25-1 mM). Cell motabolism was assessed by using a cell viability assay (Promega, USA) based on the reduction of a tetrazolium salt. The MTS tetrazolium stock solution was diluted 1:6 (v/v) according to the protocol in supplemented RPMI 1640 medium, 200μl transferred into each well and left to incubate for 3 hr at 37°C. The absorbance at 490 nm was read using a plate reader (MODEL, MAKE). The assay uses a novel MTS tetrazolium compound (3-(4,5-demethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt) which is bioreduced by cells into a coloured formazan product that is soluble in medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The amount of formazan measured gives an indication of the metabolic activity of cells and the results are expressed as % increase in activity above control.

Heme oxygenase activity assay. Heme oxygenase activity was measured as described previously (8,16). Briefly, confluent HEKs in T75 flasks were incubated for 6 h in supplemented RPMI 1640 medium (final vol. 10ml, control group) or in the presence of SNP at various concentrations (0.5-1 mM). At the end of the incubation cells were washed with ice-cold PBS, scraped with a plastic policeman (Thomas Scientific, Swedesboro, NJ) and the cell suspension centrifuged at 10,000 *g* for 10 min at 4°C. The cell pellet was re-suspended in $MgCl_2$ (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen at −70°C, and freeze-thawed three times. The cell suspension (400 μl) was added to the reaction tube containing 0.8 mM NADPH, 2mM glucose 6-phosphate, 0.2 U glucose-6-phosphate 1-dehydrogenase, and 2 mg protein of rat liver cytosol as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4) and hemin (10μM) in a final volume of 1ml. The reaction was allowed to run for 1 hr at 37°C in the dark and terminated by the addition of 1 ml chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 $mM^{-1}cm^{-1}$ for bilirubin). Heme oxygenase activity was calculated as picomoles of bilirubin formed/mg protein/hour. The protein content of the cells was measured using a total protein assay kit (Bio-Rad, Herts, UK) by comparison with a standard curve obtained with bovine serum albumin.

Western blot analysis for HO-1. Confluent cells were incubated for 6 hr with supplemented RPMI medium alone (control) or medium containing 0.5 mM SNP. The incubation was terminated by washing the cells with ice-cold PBS. Cells were scraped and the cell suspension centrifuged at 10,000 *g* for 10 min at 4°C. The cell pellet was then re-suspended in phosphate buffer (100 mM, pH 7.4) containing 1% Triton X-100 and frozen at −70°C until Western blot was performed. Total protein was calculated as described above and 30 μg total protein was boiled in Laemmli buffer (17). Samples were separated on a 15% SDS-polyacrylamide resolving gel (Mini Protean II System, Bio-Rad, Herts, UK). Proteins were transferred onto a nitrocellulose membrane and non specific binding of antibodies was blocked with 3% non-fat dried milk in PBS (pH 7.4), for 2 h at room temperature. Membranes were then probed with a polyclonal rabbit anti-HO-1 antibody (Stressgen, Victoria, Canada) (1:1000 dilution in tris-buffered saline, pH 7.4) for 2 h at room temperature. Blots were visualised using an amplified alkaline phosphatase kit from Sigma (EXTRA-3A).

RESULTS

Effect of hemin and SNP on heme oxygenase activity and HO-1 expression in keratinocytes. Hemin is a potent inducer of heme oxygenase in a number of cell types in culture and was, therefore, used as a positive control in this study to show the ability of keratinocytes to express HO-1. Addition of SNP (0.5 mM final concentration) to the culture medium for a period of 6 h caused a 2.5 fold increase ($p<0.05$) in heme oxygenase activity (Fig. 1*A*). However, at a higher concentration of SNP (1 mM) heme oxygenase activity was significantly decreased ($p<0.05$) compared to 0.5 mM but was still significantly higher than control. The elevated heme oxygenase activity by SNP was reflected in a significant increase in HO-1 expression as shown by Western Blot analysis (Fig. 1*B*).

Effect of SNP on keratinocyte metabolism and proliferation. Keratinocyte cell metabolism, as determined by the MTS tetrazolium assay, was markedly increased in a concentration-dependent manner when cells were incubated with SNP (0.5, 0.75 and 1 mM) (Fig. 2). Similarily, incubations of cultured keratinocytes with 0.25 and 0.5 mM SNP for 24 h in culture medium resulted in 2.5 and 3-fold increase in cell proliferation, respectively ($p<0.05$). However, when incubated with a higher dose of SNP (1 mM) for 24 h, keratinocytes proliferation was not significantly different from control (Fig. 3).

Effect of hydroxocobalamin and SnPPIX on SNP-induced keratinocyte proliferation. There is evidence that hydroxocobalamin has NO binding properties (18). Therefore the use of hydroxocobalamin enabled us to

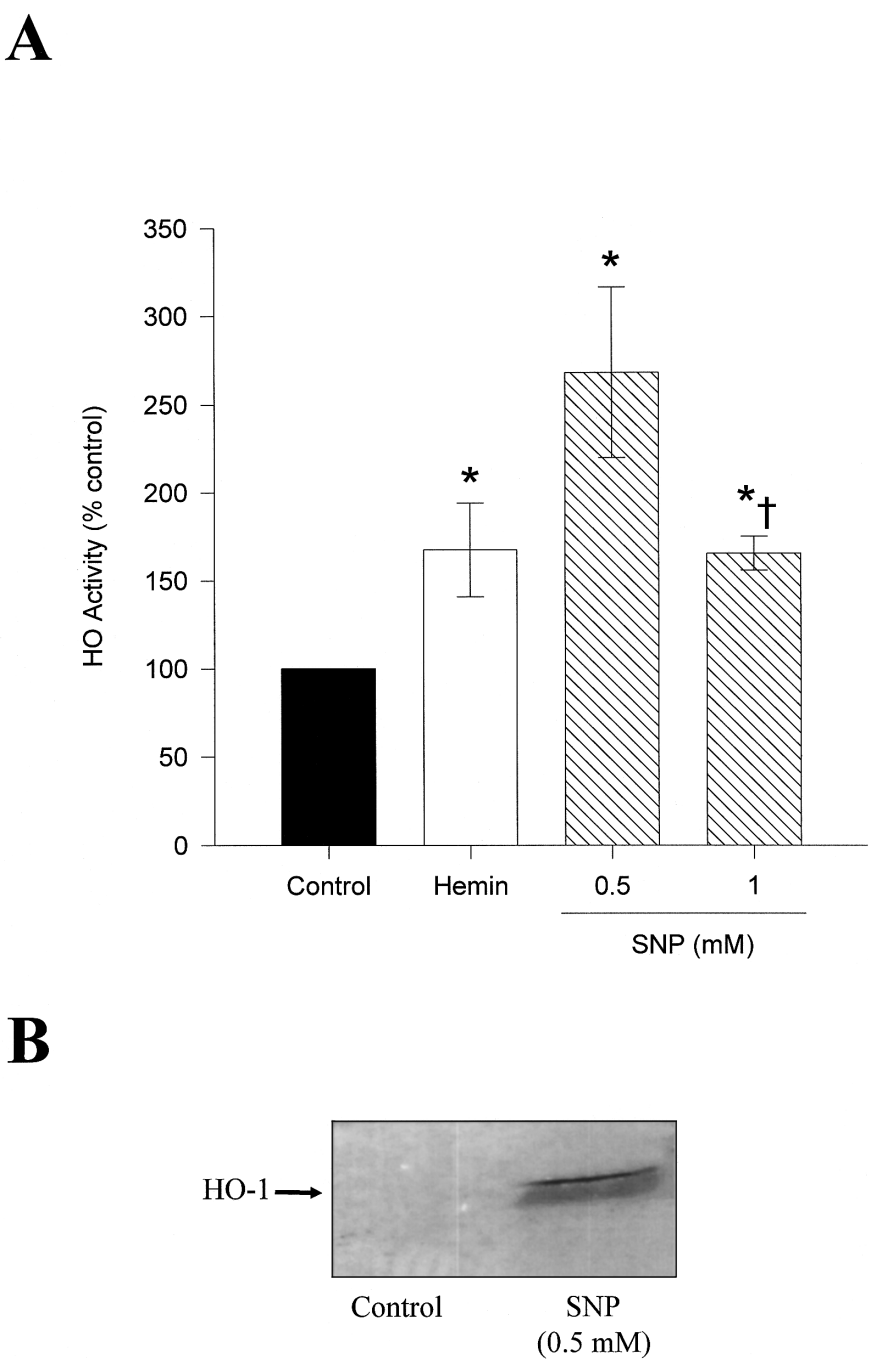

FIG. 1. Effect of hemin and sodium nitroprusside (SNP) on HO-1 induction in keratinocytes. **(A)** Heme oxygenase activity was measured after 6 h incubation of cells with 0.5 and 1 mM SNP or 300 μM hemin (positive control). Control experiments are represented by cells exposed to complete medium alone. Each value represents the mean ± SEM of 6 experiments. *, $P<0.05$ compared with control; †, $P<0.05$ compared with SNP 0.5 mM. **(B)** Samples of cells treated with SNP were also analysed for HO-1 expression by Western blot using a polyclonal antibody for HO-1.

determine the specific role of NO released from SNP in keratinocyte proliferation. As shown in Fig. 3, addition of hydroxocobalamin (0.5 mM final concentration) to the culture medium containing SNP resulted in a significant decrease in cell proliferation. In addition, incubation of keratinocytes with the heme oxygenase inhibitor SnPPIX (0.5 mM) also resulted in a significant reduction of the proliferative effect mediated by SNP.

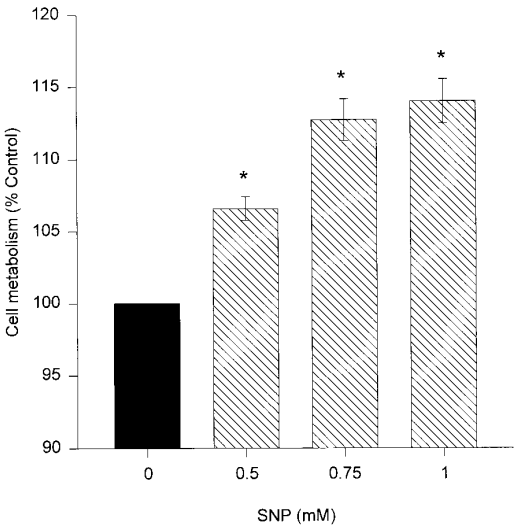

FIG. 2. Effect of sodium nitroprusside (SNP) on cellular metabolism in keratinocytes. Keratinocyte metabolism was determined by the MTS tetrazolium assay after 6h incubation with SNP (0.5-1 mM). Each value represents the mean ±SEM of 6 experiments. *, $P<0.05$ compared with control.

DISCUSSION

The main finding of this study is that the expression of HO-1 is involved in keratinocytes proliferation mediated by NO. Our data show that incubation of transformed human keratinocytes with SNP, a well-known NO donor, stimulates HO-1 expression and heme oxygenase activity resulting in increased cellular proliferation. Both blockade of SNP-derived NO with hydroxocobalamin and inhibition of the enzymatic action of heme oxygenase with SnPPIX were found to abolish the proliferative effect of NO.

The expression of HO-1 with the consequent increase in heme oxygenase activity has been described in a variety of tissues as a general response to stressful stimuli (19). It is believed that the products of this enzymatic pathway have a crucial biological role, since biliverdin is a potent antioxidant and CO has been shown to activate the cGMP pathway, thus being an important factor in the regulation of vascular tone. The induction of HO-1 in response to various NO donors has been recently reported both *in vitro* and *in vivo* systems, but the exact physiological significance of this effect has yet to be elucidated (8,20-22). In this study we found that increased heme oxygenase activity by SNP correlates with a higher proliferation of human keratinocytes in culture. The effect was more pronounced at relatively low concentrations of the NO donor (0.25-0.5 mM), whereas at a higher concentration of SNP (1 mM) heme oxygenase activity was reduced and cell proliferation significantly suppressed. Interestingly, the cell viability assay (MTS tetrazolium) indicated that the metabolic activity of keratinocytes gradually increased in a concentration-dependent manner following incubation with SNP (0.25-1mM). Taken together these results suggest that, under our experimental conditions, SNP does not promote cellular damage and that the decrease in heme oxygenase activation observed at higher concentrations of SNP can not be related to the potential cytotoxic action of NO. It can not be excluded that by increasing their metabolic activity, cells can catabolize NO much faster resulting in a higher decomposition of SNP over time. In fact, an increased formation of nitrite from SNP has been shown to correlate with a much lower induction of heme oxygenase by the NO donor (8).

In a previous report we have demonstrated that increased heme oxygenase activity in vascular endothelial cells by SNP was markedly suppressed in the presence of the NO scavenger hydroxocobalamin. The results of this study showing that hydroxocobalamin markedly reduced the number of growing keratinocytes support the hypothesis that NO itself is an important regulator of keratinocyte proliferation. The fact that SnPPIX suppressed the proliferative effect mediated by SNP suggests a direct involvement of HO-1 in keratinocytes metabolism. Although SnPPIX has been shown to partially inhibit heme-dependent proteins other than heme oxygenase (guanylate cyclase, NOS) (23), recent data from our laboratory indicate that

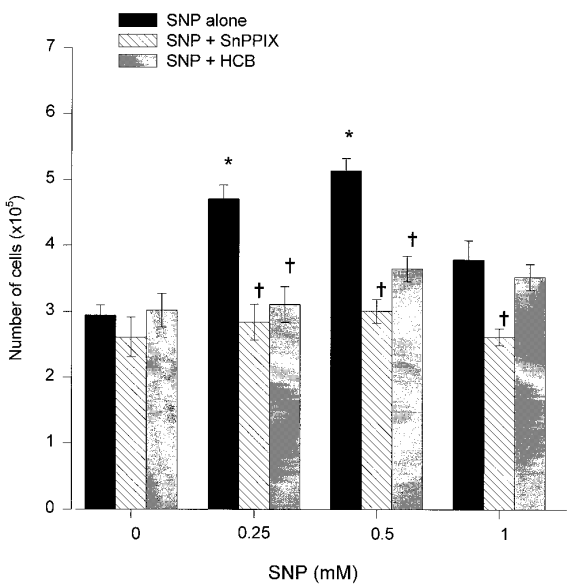

FIG. 3. Effect of hydroxocobalamin (HCB) and tin protoporphyrin IX on sodium nitroprusside (SNP) mediated keratinocyte proliferation. Keratinocyte proliferation was determined by counting the number of cells after 24 h incubation with SNP (0.25-1 mM), SNP + HCB (0.5 mM) and SNP + SnPPIX (0.5 mM). Each value represents the mean ±SEM of 6 experiments. *, $P<0.05$ compared with control; †, $P<0.05$ compared with SNP alone.

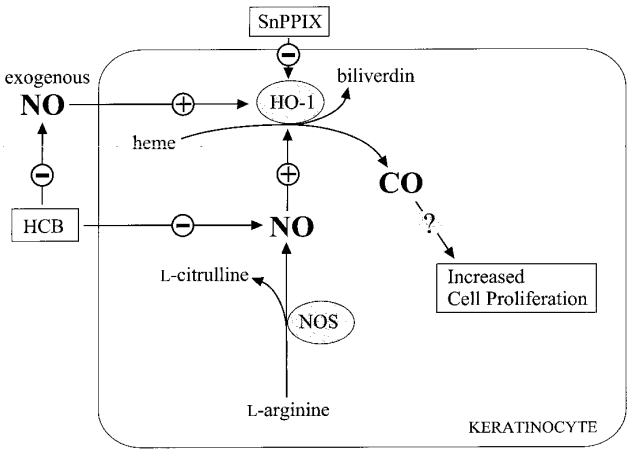

FIG. 4. Schematic diagram representing a possible mechanism of NO-mediated keratinocyte proliferation. NOS has been shown to be present in cultured human keratinocytes and NO can increase cell proliferation. In our experiments an exogenous source of NO was used (SNP). NO mediated HO-1 induction leads to an increase in the production of biliverdin and the messenger molecule CO. We propose that one of these molecules either directly, or through a secondary messenger system is responsible for mediating an increase in keratinocyte proliferation.

SnPPIX becomes a more selective and competitive inhibitor when the level of HO-1 is significantly increased (24). To our knowledge this study provides the first evidence that activation of the heme oxygenase pathway is associated with increased cellular metabolism and growth.

The exact mechanism by which HO-1 induction promotes keratinocytes proliferation remains to be established. It is possible that the NO/HO-1 system elicits cell proliferation by increasing the endogenous production of CO, a gaseous molecule that similarly to NO has been implicated in intracellular communication pathways (25). As represented in the schematic diagram of Fig. 4, increased HO-1 protein expression by NO would result in a higher catabolism of heme to CO, which either directly or via a second messenger (cGMP) could trigger the cascade of events required for the production of growth factors. The other product of heme oxygenase, biliverdin, has antioxidant properties but no data are available concerning the potential proliferative effect of this molecule. Janes et al (26) have shown that bilirubin, which is formed during the reduction of biliverdin by biliverdin reductase, decreases cellular proliferation in osteoblasts without affecting cell viability. These results were, however, obtained from jaundiced patients in conditions of extreme hyperbilirubinemia.

The inducible form of NOS (iNOS) has been found in skin cells including keratinocytes, langerhans cells and fibroblasts, and iNOS-derived NO is also produced in activated macrophages as part of their anti-microbial defense (2,27). As a consequence of this, a traumatic wound site should have high iNOS activity and, therefore, considerable levels of NO being released. Benrath and coworkers demonstrated that a topically administered NOS inhibitor (N^G-nitro-L-arginine methyl ester, L-NAME) to a UVB-induced wound area in rat skin resulted in increased necrosis (15). In addition, UVA/UVB radiations have been reported to stimulate NO release in human keratinocytes and to up-regulate HO-1 mRNA in skin fibroblasts (28,29). In view of the data presented here, we reason the elevated NO production might lead to increased keratinocytes proliferation resulting in acceleration of wound healing processes. Although in the present study we used an exogenous form of NO (SNP), the possibility that endogenously produced NO in the epidermis as part of the inflammatory response could influence the HO-1/CO pathway can not be excluded.

In summary, we have reported the ability of a NO donor to increase HO-1 expression in human keratinocytes postulating a possible mechanism that involves the heme oxygenase/CO pathway in cell proliferation.

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