Cellular glucose-6-phosphate dehydrogenase (G6PD) status modulates the effects of nitric oxide (NO) on human foreskin fibroblasts

Mei-ling Cheng[a,b,1], Hung-yao Ho[b,c,1], Chi-ming Liang[c,d], Yi-hung Chou[e], Arnold Stern[f], Fung-jou Lu[a,2], Daniel Tsun-yee Chiu[b,*]

[a] *Department of Biochemistry and Internal Medicine, College of Medicine, National Taiwan University, No. 1 Jen-Ai Road, Taipei, Taiwan*
[b] *Graduate Institute of Basic Medical Sciences and School of Medical Technology, Chang Gung University, 259 Wen-hua 1st Road, Kwei-san, Tao-yuan, Taiwan*
[c] *Graduate Institute of Life Sciences, National Defense Medical Centre, Taipei, Taiwan*
[d] *Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan*
[e] *Department of Neonatology, Chang Gung Children's Hospital, Kwei-san, Tao-yuan, Taiwan*
[f] *Department of Pharmacology, New York University Medical Centre, New York, NY, USA*

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Abstract Glucose-6-phosphate dehydrogenase (G6PD) plays an important role in cellular redox homeostasis, which is crucial for cell survival. In the present study, we found that G6PD status determines the response of cells exposed to nitric oxide (NO) donor. Treatment with NO donor, sodium nitroprusside (SNP), caused apoptosis in G6PD-deficient human foreskin fibroblasts (HFF1), whereas it was growth stimulatory in the normal counterpart (HFF3). Such effects were abolished by NO scavengers like hemoglobin. Ectopic expression of G6PD in HFF1 cells switched the cellular response to NO from apoptosis to growth stimulation. Experiments with 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one and 8-bromo-cGMP showed that the effects of NO on HFF1 and HFF3 cells were independent of cGMP signalling pathway. Intriguingly, trolox prevented the SNP-induced apoptosis in HFF1 cells. These data demonstrate that G6PD plays a critical role in regulation of cell growth and survival.

Key words: Nitric oxide; Glucose-6-phosphate dehydrogenase; Apoptosis; Growth

1. Introduction

The major function of glucose-6-phosphate dehydrogenase (G6PD) is to generate reducing equivalents in the form of NADPH to meet the cellular needs for reductive biosynthesis and maintenance of cellular redox homeostasis. The importance of G6PD is highlighted by clinical manifestation of its deficiency. G6PD deficiency, a common enzymopathy affecting over 200 million people worldwide, can cause neonatal jaundice, drug- or infection-induced hemolytic crisis, favism and, less commonly, non-spherocytic hemolytic anemia [1–3]. Until recently, most advances in the field have been made on the pathophysiology of G6PD-deficient erythrocytes and the molecular characterization of different G6PD variants. The chronic effects of G6PD deficiency on cells other than erythrocytes remain largely unexplored.

Several recent studies have shown that G6PD deficiency does affect the physiology of nucleated cells [4–7]. For instance, a number of G6PD-deficient fibroblasts grow at a reduced rate compared to the normal control [5]. Reduction in growth rate of these cells implies diminished cellular responses to mitogens. It is speculative that G6PD deficiency may alter cellular responses to growth factors and other signalling molecules. In the present study, nitric oxide (NO) has been found to exert different biological effects on normal and G6PD-deficient cells.

2. Materials and methods

2.1. Cell culture

The G6PD-deficient human foreskin fibroblast, designated HFF1, and its normal counterpart, designated HFF3, were isolated and maintained as previously described [5]. LGIN and LKGIN cells, which expressed G6PD from an exogenous human *G6PD* gene, were generated from HFF1 cells by transduction with G6PD-encoding retroviral vectors as described [5]. LEIN cells, which expressed enhanced green fluorescent protein (EGFP), served as control. These cells were cultured in Dulbecco's modified Eagle medium (Gibco Life Technology, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 U/ml of streptomycin and 0.25 mg/ml of amphotericin at 37°C in a humidified atmosphere containing 5% CO_2. The cell number was determined by the trypan blue dye exclusion method.

For cultures receiving NO treatment, about 2×10^5 cells were seeded on day −1 (i.e. 24 h before drug treatment), and treated with indicated concentrations of sodium nitroprusside (SNP; Calbiochem, San Diego, CA, USA) on day 0. The cell number and the extent of apoptosis were quantified on subsequent days. For delineation of NO signalling pathway, cells were pre-treated with selective inhibitor of NO-sensitive guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Calbiochem, San Diego, CA, USA) for 30 min before SNP addition, or incubated alone with 8-bromo-guanosine 3′,5′-cyclic monophosphate (cGMP) (Calbiochem, San Diego, CA, USA). For studies with trolox, cells were treated with 200 μM of trolox (Calbiochem, San Diego, CA, USA) for 30 min prior to addition of SNP.

*Corresponding author. Fax: (886)-3-3289540.
E-mail: dtychiu@mail.cgu.edu.tw

[1] These authors make an equal contribution to this study.
[2] Also corresponding author.

Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; EGFP, enhanced green fluorescent protein; G6PD, glucose-6-phosphate dehydrogenase; HFF1, G6PD-deficient human foreskin fibroblasts; HFF3, normal human foreskin fibroblasts; LEIN, cell line expressing EGFP; LGIN and LKGIN, cell lines expressing exogenous human *G6PD* gene; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; RNS, reactive nitrogen species; SNP, sodium nitroprusside; TUNEL, terminal dUTP nick end labelling

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2.2. Flow cytometric analysis and terminal dUTP nick end labelling (TUNEL) of apoptotic cells

Cells were rinsed with ice-cold phosphate-buffered saline (PBS), trypsinized and resuspended in 0.3 ml of PBS. They were then fixed and permeabilized by addition of 0.7 ml of ethanol. After a brief wash, the cells were gently resuspended in 1 ml of propidium iodide (PI) stain solution (40 μg/ml PI (Sigma, St. Louis, MO, USA), 100 μg/ml RNase A and 0.5% Triton X-100 in PBS), and incubated at room temperature for 30 min before analysis on a FACSCAN flow cytometer (Becton Dickinson, CA, USA). The sub-G1 fraction is considered as the apoptotic cells and taken as a measure of the extent of apoptosis [8]. The sub-G1 phase was quantified using Modfit software (Becton Dickinson, CA, USA). TUNEL was performed with in situ cell death detection kit (Roche, Germany), according to the instructions of the manufacturer. Finally, the samples were analyzed by flow cytometry, and the TUNEL-positive fraction was quantified by CELLQuest software (Becton Dickinson, CA, USA).

3. Results

3.1. Exogenous NO donor evoked different responses in normal and G6PD-deficient human fibroblasts

The HFF1 cells were isolated from foreskin of G6PD-deficient neonate, who had been identified to carry the Taiwan-Hakka variant ($G6PD^{1376T}$). The control HFF3 cells were derived from foreskin of normal, sex- and age-matched infant. The G→T transversion caused a significant reduction in G6PD activity in HFF1 cells (0.11 ± 0.005 U/mg of cell lysate) as compared with that of HFF3 cells (0.3 ± 0.01 U/mg of cell lysate) [5]. Upon exposure to NO donor SNP, HFF1 and HFF3 cells showed entirely different growth patterns. Addition of 50 or 100 μM of SNP enhanced growth of HFF3 cells as compared to the control (Fig. 1A). Such an effect became prominent on day 5 (i.e. ∼120 h after SNP addition), but was less conspicuous as the cultures reached confluence on day 7. At concentrations ranging from 10 to 50 μM, the growth stimulatory effect of NO donor displayed dose-dependence (Fig. 1C). The number of HFF3 cells (counted on day 5 after SNP addition) increased from $(44.16 \pm 1.45) \times 10^5$ (at 10 μM) to $(60.4 \pm 1.98) \times 10^5$ (at 50 μM). On the contrary, as compared to that of untreated control, the number of SNP-treated HFF1 cells decreased progressively with time (Fig. 1B). Such reduction in cell number showed dose-dependence (Fig. 1C), and was due to an increased rate of cell death.

3.2. The cell death induced in HFF1 cells by SNP was typical of apoptosis

SNP induced apoptosis in HFF1 cells, which exhibited convoluted outline and characteristic membrane blebbings (unpublished observation). The extent of apoptosis was quantified by two approaches. First, the appearance of cells with a low DNA stainability (sub-G1 fraction) in cell population has been considered as a marker of apoptosis [8]. As evident in Fig. 2B, the extent of apoptosis in HFF1 cells significantly increased on day 3 after SNP addition. Treatment with 50 and 100 μM of SNP for 7 days resulted in about 35.12 ± 4.12% and 53.06 ± 5.07% of apoptosis, respectively. Beginning with the SNP concentration of 10 μM, the degree of apoptosis increased significantly with concentration (Fig. 2C). In contrast, SNP did not induce apoptosis in HFF3 cells at any SNP concentration from 10 to 100 μM (Fig. 2A,C).

TUNEL labelling of the free DNA ends formed by fragmentation of genomic DNA, followed by flow cytometric analysis, gives another reliable means for quantification of apoptosis [9]. Incubation of HFF1 cells SNP caused an increase in mean relative fluorescence, indicating incorporation of fluorescein-dUTP onto ends of nuclear DNA fragments in apoptotic cells (Fig. 3A). This was supported by fluorescence microscopic examination of TUNEL-labelled, SNP-treated

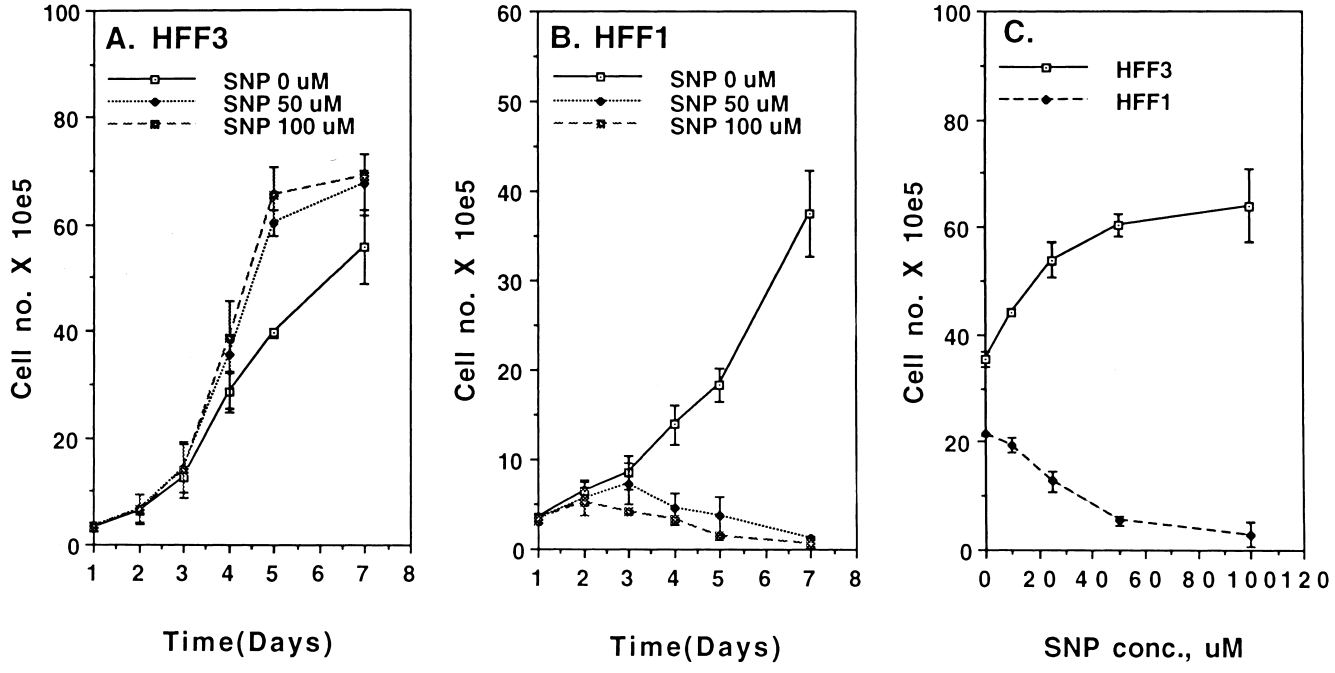

Fig. 1. Effects of SNP on growth kinetics of normal (HFF3) and G6PD-deficient (HFF1) human foreskin fibroblasts. Twenty-four hours before SNP addition (i.e. day −1), about 2×10^5 HFF3 (A) or HFF1 (B) cells were seeded, and treated with 0, 50 and 100 μM of SNP on day 0. Cell number was determined at daily intervals. (C) Effects of SNP on HFF3 and HFF1 cells showed dose-dependence. About 2×10^5 HFF3 or HFF1 cells were treated with indicated concentrations of SNP, and the cell number was determined 5 days after treatment. Results are expressed as mean ± S.E.M. of five determinations.

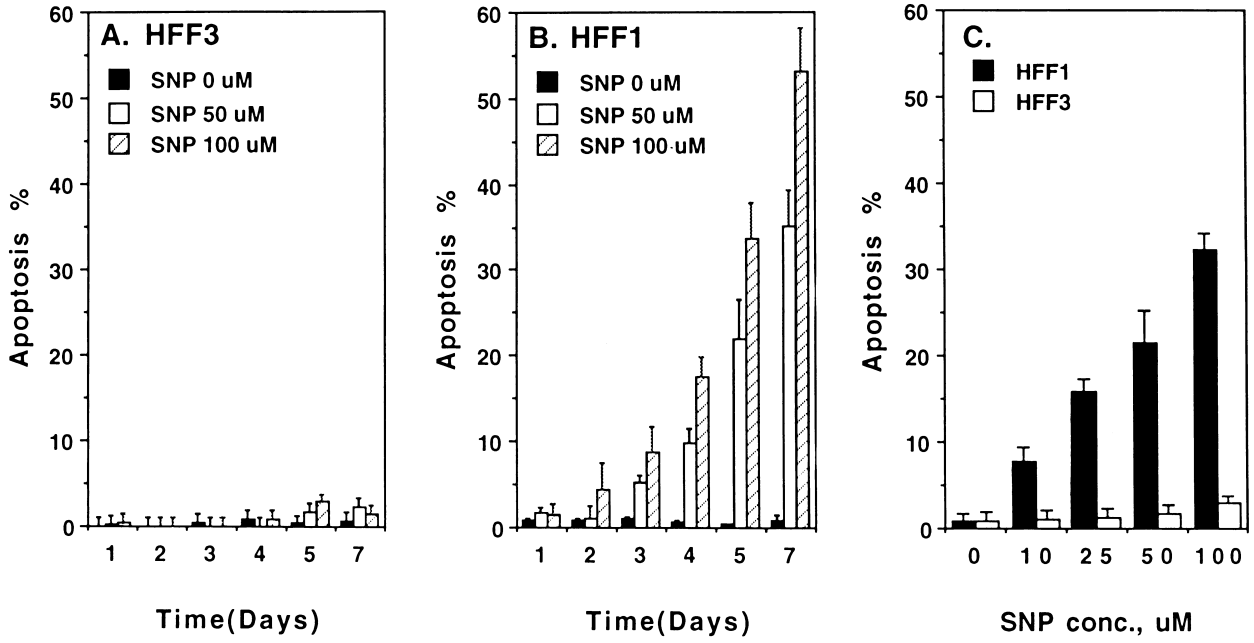

Fig. 2. SNP induced apoptosis in HFF1 cells. HFF3 (A) and HFF1 (B) cells were treated as described in Fig. 1. The extent of apoptosis was measured by PI staining and flow cytometric analysis. (A) and (B) show the temporal course of SNP-induced apoptosis. (C) SNP induced apoptosis of HFF1 cells in a dose-dependent manner. The level of apoptosis in HFF1 and HFF3 cells incubated with indicated concentrations of SNP was determined 5 days after treatment. The data are presented as mean ± S.E.M. from five separate experiments. ↑

HFF1 cells (data not shown). Addition of SNP to HFF1 cells caused a significant increase in the percentage of TUNEL-positive cells in a dose-dependent manner (Fig. 3B). Meanwhile, an insignificant level of apoptosis was detected in SNP-treated HFF3 cells.

3.3. The observed effects of SNP are most likely to be mediated by NO

When HFF3 cells were co-incubated with 50 μM of SNP and 50 μM of hemoglobin or freshly prepared erythrocytes, both of which are well known NO scavengers, the growth stimulatory effect of SNP was completely inhibited (data not shown). Similarly, treatment with hemoglobin or freshly prepared erythrocytes rescued HFF1 cells from SNP-induced apoptosis (data not shown). Furthermore, potassium ferrocyanide ($K_4Fe(CN)_6$) or potassium ferricyanide ($K_3Fe(CN)_6$), the analogues of SNP but devoid of NO, did not mimic the effects of SNP at concentrations ranging from 10 μM to

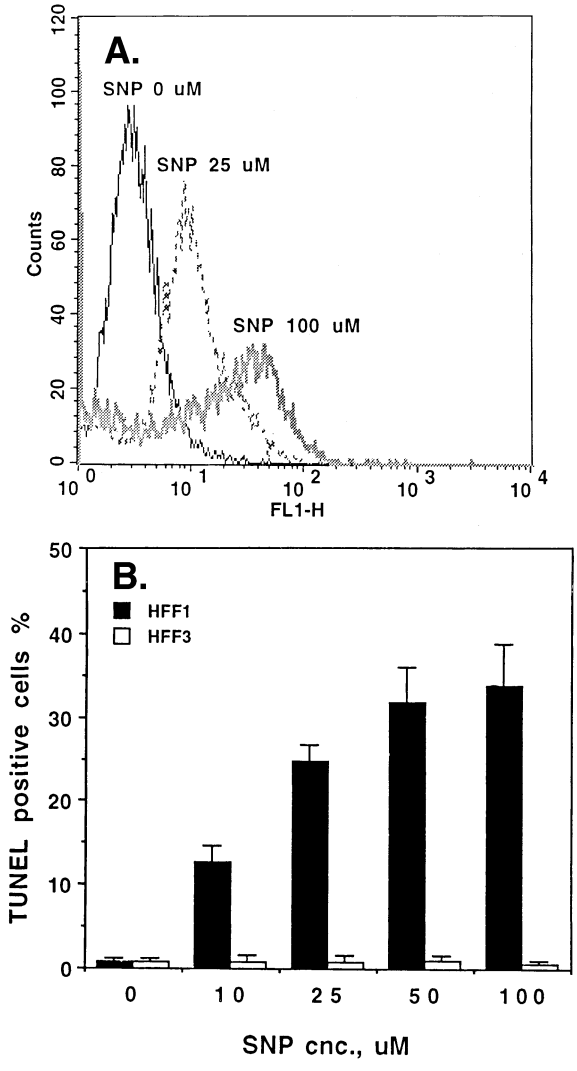

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Fig. 3. Quantification of the extent of apoptosis in HFF1 and HFF3 cells by TUNEL assay. (A) HFF3 and HFF1 cells were treated with varying concentrations of SNP, and the level of apoptosis was determined 5 days after treatment by TUNEL assay and flow cytometric analysis. Representative histograms of HFF1 cells treated with 0, 25 and 100 μM of SNP are shown here. (B) The extent of apoptosis with varying SNP concentrations for HFF1 and HFF3 cells is shown here. Results are expressed as mean ± S.E.M. from five separate experiments.

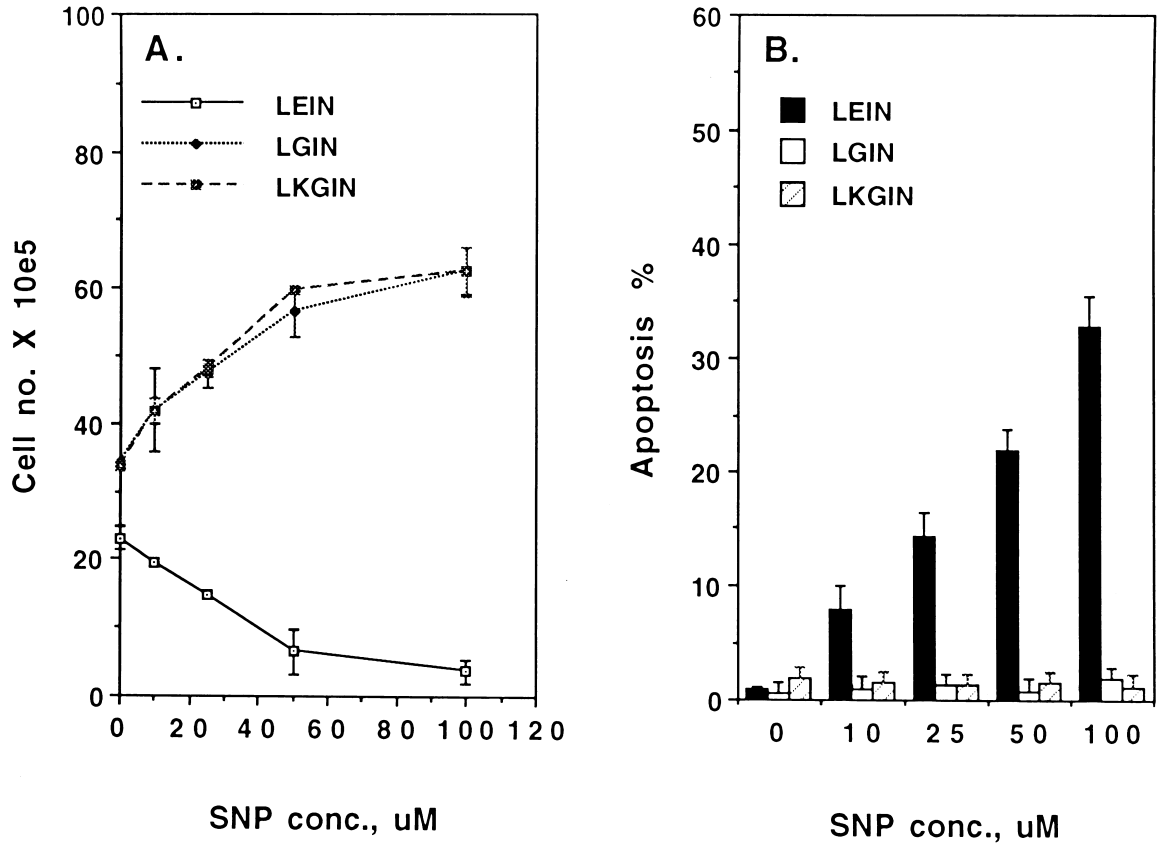

Fig. 4. Ectopic expression of G6PD changed the cellular response of HFF1 cells to SNP from apoptosis to stimulated proliferation. The HFF1 cells stably transduced with control retroviral vector (LEIN cells) or G6PD-expressing retroviruses (LGIN and LKGIN cells) were treated with various concentrations of SNP for 5 days. The cell number determined is shown here (A). The percentage of apoptosis in LEIN, LGIN and LKGIN cells was determined as described in Fig. 2 (B). The data are expressed as mean ± S.E.M. from five separate experiments.

100 μM (data not shown). All these findings advocate that SNP brings about its effects via NO.

3.4. *Replenishment of G6PD changed biological responses of HFF1 cells to NO from apoptosis to growth stimulation*

To test the possibility that G6PD per se was held responsible for different biological responses of fibroblasts to NO, we investigated the effect of ectopic expression of G6PD in HFF1 cells. Cell lines stably carrying exogenous human *G6PD* gene, LGIN and LKGIN, were generated by infection of HFF1 cells with recombinant retroviral vector; LEIN cells, which expressed EGFP, served as control [5]. Addition of SNP caused a decrease in cell number of LEIN cells, while it stimulated growth of LGIN and LKGIN cells (Fig. 4A). As in the case of HFF1 cells, apoptosis accounted for the loss of LEIN cells from SNP-treated cultures (Fig. 4B). It was clear that expression of G6PD in LKGIN and LGIN cells protected them from NO-induced apoptosis.

3.5. *cGMP pathway is not involved in transduction of growth stimulatory and apoptotic signals of NO in HFF3 and HFF1 cells*

One of the ways by which NO elicits downstream biological events is via activation of guanylate cyclase [10]. To examine the potential role of the NO–guanylate cyclase signalling pathway in transduction of growth stimulatory or apoptotic signals in our fibroblasts, two approaches were taken. First, HFF1 and HFF3 were pre-treated with 10 μM of ODQ, the inhibitor of guanylate cyclase, for 30 min before addition of 50 μM of SNP. As shown in Fig. 5A, ODQ did not affect the growth stimulatory effect of NO donor on HFF3 cells. Moreover, it did not have any protective effect on HFF1 cells undergoing SNP-induced apoptosis (Fig. 5C,D). In the second approach, a cell permeable cGMP analog, 8-bromo-cGMP, was used to test whether it could mimic effects of NO donor. HFF1 and HFF3 cells were incubated with 1 mM of 8-bromo-cGMP, 50 μM SNP, or both for 5 days. No significant difference in cell number between the cGMP analog-treated HFF3 cells and untreated control was observed (Fig. 5A). Nor did 8-bromo-cGMP affect the extent of apoptosis in HFF1 cells (or SNP-treated HFF1 cells) (Fig. 5C,D). These data indicate that the cGMP pathway is not involved in the action of NO donor on our human fibroblasts.

3.6. *NO-induced apoptosis, but not growth stimulation, involves generation of oxidative stress*

NO released from donor can form other reactive nitrogen species (RNS), which can bring about oxidative or nitrosative stress [11]. Recent study has shown that trolox can protect leukemic cells from NO-induced apoptosis, implying the involvement of oxidative stress [12]. To test the possibility that RNS-evoked oxidative stress is involved in NO signalling in our model system, we pre-incubated either HFF3 or HFF1

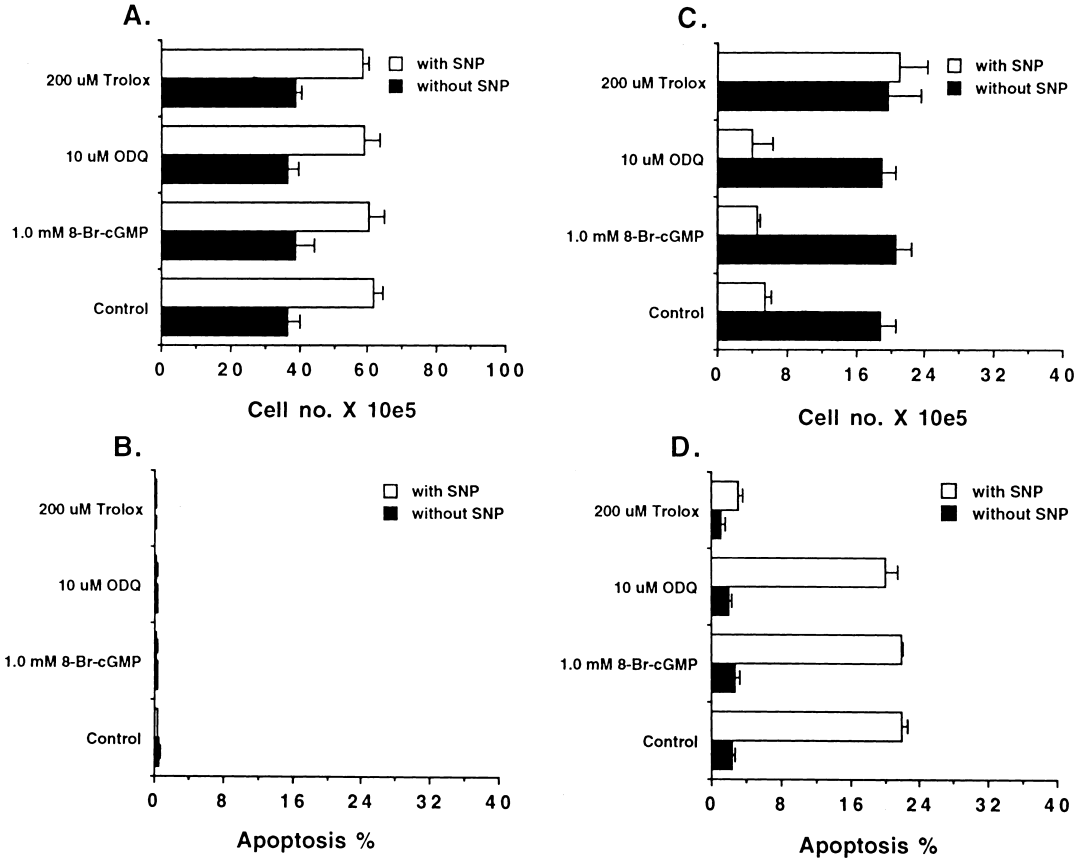

Fig. 5. Guanylate–cGMP pathway did not mediate the effects of SNP on HFF1 and HFF3 cells. HFF1 and HFF3 cells were pre-incubated with 10 μM of ODQ or 1 mM 8-bromo-cGMP for 30 min before treatment with (or without) 50 μM of SNP. For treatment with trolox, HFF1 and HFF3 cells were pre-incubated with 200 μM of trolox prior to SNP addition (or without SNP treatment). Cell number (A and C) and level of apoptosis (B and D) of HFF3 (A and B) and HFF1 (C and D) were determined 5 days after drug treatment. Results are expressed as mean ± S.E.M. from five separate experiments.

cells with trolox, and examined its effect on the biological outcomes of SNP treatment. As evident in Fig. 5A, trolox did not change the growth stimulatory effect of SNP on HFF3 cells. The cell number did not vary significantly between the SNP-treated group ($61.75 \pm 2.63 \times 10^5$) and the trolox- and SNP-treated group ($58.54 \pm 1.89 \times 10^5$). On the contrary, trolox treatment completely protected HFF1 cells from SNP-induced apoptosis (Fig. 5C,D). However, such protection, unlike the effect of ectopic G6PD expression, was not associated with NO-induced growth stimulation. These findings suggest that oxidative stress may be involved in NO-induced apoptosis of HFF1 cells.

4. Discussion

In the present study, we show that G6PD is critical for modulation of cellular responses to NO. SNP stimulated growth of normal HFF3 cells (Fig. 1), whilst it caused apoptosis of G6PD-deficient HFF1 cells (Figs. 2 and 3). All these effects were nullified upon co-incubation with hemoglobin or erythrocytes (data not shown). Additionally, potassium ferrocyanide or potassium ferricyanide, molecules structurally related to SNP but unable to generate NO, had no effect on HFF1 and HFF3 cells (data not shown). These findings indicated that NO released from SNP was held responsible. Replenishment of G6PD in HFF1 cells instigated them to respond to NO by growth enhancement (Fig. 4), suggesting that G6PD per se modulates the cellular responses to signalling molecules. Moreover, our findings demonstrate that NO did not effect via the classical NO–cGMP pathway (Fig. 5). The biological effects of NO appear to be mediated by the roles of NO or other RNS as oxidative and nitrosative agents. Consistent with this, trolox, a potent antioxidant of lipid peroxidation, could protect the HFF1 cells from SNP-induced apoptosis.

NO can exist in different chemical forms like NO^+, $NO^\bullet$, NO^-, NO_2, N_2O_3, $ONOO^-$ and $ONOOCO_2$. These RNS have different chemical reactivities (i.e. oxidation, nitrosation, nitration etc.) towards multifarious biological molecules, and hence different effects on cellular functions [11,13–15]. For instance, S-nitrosylation of a critical cysteine residue of Ras stimulates guanine nucleotide exchange and downstream signalling [16]. The exact identities, concentrations, half-lives and reactivities of RNS in a cell, or more precisely in different subcellular compartments, may depend on the local condition, such as the redox status.

The ability of G6PD to alter the cellular responses to NO is probably related to its indispensable role in production of NADPH and sustenance of an adequate redox balance [17]. For example, G6PD-null mutants of *Saccharomyces cerevisiae* and murine embryonic stem cells are extremely sensitive to oxidizing agents [7,18]. In addition, our G6PD-deficient HFF1 cells have an NADPH/$NADP^+$ ratio reduced by 50%, as compared with HFF3 cells (unpublished observation).

Apparently, G6PD deficiency changes the intracellular milieu to a more oxidizing environment.

Redox homeostasis is critical to numerous physiological processes, and modulates cell growth and survival signals [19,20–23]. A shift in redox balance towards formation of more reactive oxygen species in G6PD-deficient cells causes alteration in cell physiology [5,24]. In the current study, we demonstrate that G6PD status determines the cellular responses to NO. It is probable that the regulatory molecules, such as signal transducers, or the effector molecules within growth or apoptotic pathways are targets of redox control. Changes in redox states of these molecules, such as reversible oxidation of -SH groups of cysteine residues, may modulate their biological activities as well as their reactivities towards RNS [15,25]. For example, the cysteine residues of *N*-methyl-D-aspartate receptor react with NO^+ only if they are in the free sulfhydryl state [15]. Hence, depending on the redox status, these regulators or effectors may determine the cellular responses to NO: a reducing cellular environment in HFF3 cells favors proliferation, whereas a more oxidizing environment predisposes HFF1 cells to apoptosis. Consistent with this, ectopic expression of G6PD in HFF1 cells prevented apoptosis and restored the growth response to NO. Additionally, trolox protected G6PD-deficient cells from SNP-induced apoptosis, suggesting RNS-evoked oxidative stress and lipid peroxidation as critical events in NO-induced apoptosis. Peroxidation of mitochondrial lipids, in particular cardiolipin, has been shown to play a crucial role in apoptosis [12]. Interestingly, there was a lack of effect of trolox on SNP-stimulated growth of HFF3 cells. Two plausible explanations exist. First, NO or RNS may still stimulate proliferation of HFF3 cells via oxidant signalling, but lipid peroxidation is not involved in mitogenic signalling. Second, different activities of RNS, such as nitration of tyrosine residue, may be involved in regulation of the growth signalling pathway. Experiments are under way to test these possibilities. Taken together, the present study demonstrates that G6PD plays an important role in proper functioning of the cell's growth and survival machinery.

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