Dinitrosyl iron complexes with thiol-containing ligands and *S*-nitroso-D,L-penicillamine as inductors of heat shock protein synthesis in H35 hepatoma cells

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Abstract The concentration-dependent effect of various nitric oxide donors on synthesis of different heat shock proteins was evaluated in Reuber H35 hepatoma cells and their heat shock protein-inducing ability was compared with the effect of a heat shock. A 6 h incubation of H35 cells with the dimeric (diamagnetic) form of dinitrosyl iron complex with glutathione or *N*-acetyl-L-cysteine activated synthesis of various heat shock proteins, heat shock protein 28, 32, 60, 70, 90 and 100. Synthesis of these proteins was evaluated by [^{35}S]methionine and [^{35}S]cysteine labelling with subsequent separation of proteins by polyacrylamide gel electrophoresis. The dinitrosyl iron complex with glutathione appeared to be the most efficient inductor of heat shock protein synthesis and initiated the synthesis of heat shock protein 28 even more efficiently than a 30 min heating of cells. In the same experiments, *S*-nitroso-D,L-penicillamine exerted a considerably lesser effect on the synthesis of heat shock proteins. It was suggested that the active moiety of dinitrosyl iron complexes as inductors of heat shock protein synthesis is represented by their $Fe^{+}(NO^{+})_2$ groups which move to thiol groups of the proteins participating in the initiation of heat shock protein synthesis.

Key words: Nitric oxide; Heat shock protein

1. Introduction

Using the method of Western blot analysis, it has previously been shown that exogenous nitric oxide (NO) donors, such as dinitrosyl iron complexes with glutathione (DINC-GS) or *S*-nitroso-D,L-penicillamine (SNAP), are able to initiate synthesis of heat shock protein 70 (HSP70) in cultured hepatocytes [1,2]. Subsequently, a similar activity of SNAP was demonstrated on smooth muscle cells from rat aorta [3] and mucosa cells from guinea pig intestine [4], which supported the idea that NO could induce HSPs in a wide variety of cell types. At the same time, two questions remained unanswered, (1) are NO donors also able to induce other HSPs? and (2) is there any specificity in the pattern of HSPs that different NO donors are able to induce?

The present study addresses these questions by comparing the efficiency of DNIC-GS and DNIC with *N*-acetyl-L-cysteine (NAC) and S-nitrosothiol (SNAP) as inductors of HSP synthesis in Reuber H35 hepatoma cells.

2. Materials and methods

2.1. Chemicals

Reduced GS, NAC, *N*-acetyl-D,L-penicillamine (NAP) and ATP (Sigma, St. Louis, USA) were used in experiments. Gaseous NO was synthesized in the reaction of $FeSO_4$ with $NaNO_2$ in 0.1 M HCl with subsequent purification by the method of fractional low-temperature in an evacuated system as described before [5]. Cell culture media were purchased from Gibco, Life Technologies (Alphen a/d Rijn, The Netherlands).

2.2. DNIC and SNAP synthesis and treatment

Dimeric forms of DNIC-GS or DNIC-NAC were synthesized in 15 mM HEPES buffer by treatment with NO of a Fe^{2+}-thiol (1:2) solution according to the method described in [5]. SNAP was synthesized by mixing 50 mM NAP and $NaNO_2$ solutions at pH 1 with a subsequent increasing pH to neutral values. The concentration of the synthesized SNAP was measured spectrophotometrically by the intensity of the absorbance band at 340 nm. After preparation, the DNIC and SNAP solutions were frozen and kept in liquid nitrogen. The solutions were thawed immediately before use. The DNIC-GS solution was added to the culture medium to obtain final concentrations of 50, 100, 250 and 500 μM, the DNIC-NAC solution at 50, 100, 250, 500 and 750 μM and the SNAP solution at 250, 500 and 750 μM. The concentration of DNIC in the cell was calculated from a ratio of the peak height of the EPR signal at $g = 2.04$ to the peak height of the EPR signal obtained from DNIC solutions that were measured at a known concentration (500 μM). It was verified that the EPR linewidth did not depend on the sample mixture. During the incubation period with NO donors, cells were kept at their normal growth temperature (37°C).

2.3. Cell culture

Reuber H35 hepatoma cells were routinely grown at 37°C in subconfluent monolayers in plastic flasks (Greiner, Frickenhausen, Germany). Standard growth medium consisted of Leibovitz (L15) medium, pH 7.4 (Flow/ICN Laboratories, CA, USA), supplemented with 10% fetal calf serum (Gibco, Life Technologies, Alphen a/d Rjin, The Netherlands) and the antibiotics potassium penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). For experiments, cells were grown as a monolayer in 8 cm^2 dishes or in 25 cm^2 plastic flasks as described before [6].

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Abbreviations: DNIC-GS, dinitrosyl iron complex with glutathione; DNIC-NAC, dinitrosyl iron complex with *N*-acetyl-L-cysteine; SNAP, *S*-nitroso-D,L-penicillamine; HSP, heat shock protein

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2.4. Protein synthesis, gel electrophoresis and analysis of labelled proteins

Protein synthesis was determined by incorporation of [^{35}S]methionine and [^{35}S]cysteine (specific activity of both amino acids 1300 Ci/mmol, Amersham, Bristol, UK). For incorporation studies, L15 medium without methionine and cysteine was used, to which 5 μCi of the radioactive tracers was added per ml of medium. Labelling was carried out continuously during 6 h. After this procedure, cells were lysed and solubilized in sample buffer (125 mM Tris-HCl, pH 6.8, 5% SDS, 10% β-mercaptoethanol, 15% glycerol). Radioactivity incorporated into protein was determined as hot trichloroacetic acid-precipitable material. Separation of proteins from samples containing equal amounts of radioactive proteins was performed by polyacrylamide gel electrophoresis (acrylamide 10%, bisacrylamide 0.8%).

2.5. Heat shock

Cells were exposed to heat shock by immersion of sealed culture dishes in a water bath in which the temperature was regulated within 0.1°C with a Thermomix 1420 (Braun AG, Melsungen, Germany). The temperature of the cell culture was stabilized within 1 min after immersion of the dishes.

2.6. EPR experiments

Cells were sedimented by centrifugation at $10\,000 \times g$ for 5 min at 4°C and the pellet was diluted with 0.3 ml supernatant. The suspension obtained was placed in glass ampoules and frozen in liquid nitrogen. EPR spectra were recorded on an X-band EPR radiospectrometer ESP-300 (Bruker, Germany) at a microwave power of 6.3 mW. The samples were located in a rectangular cavity (Bruker ER4102ST, operating in TE_{102} mode with unloaded $Q = 2500$) equipped with a liquid nitrogen quartz finger dewar. The microwave frequency was ca. 9.62 GHz. The magnetic field was modulated at 100 kHz and a modulation amplitude of 0.5 mT.

3. Results and discussion

In order to determine the effect of different concentrations of NO donors on possible induction of specific heat shock proteins, we analyzed the overall pattern of newly synthesized proteins using gel electrophoresis. The patterns were compared with the effect of a short exposure (30 min) to increasing temperatures. As can be observed in the autoradiograms presented in Fig. 1, heating of cells at 41.5, 42.0, 42.5 and 43.0°C resulted in a standard heat shock response, namely induction/enhanced synthesis of major HSPs: HSP28, HSP60, HSP70, HSP90 and HSP100. The identity of these HSPs has been confirmed with immunoblot assays (not shown). Treatment of the cells with the dimeric form of DNIC-GS for 6 h also induced synthesis of all these HSPs (Fig. 1). A concentration-dependency was observed based on the intensity of autoradiographic bands. An obvious induction of HSP synthesis was already observed at a concentration of 50 μM DNIC-GS. Maximum activation of HSP synthesis was observed at 100 and 250 μM DNIC-GS. When the DNIC-GS concentration was increased to 500 μM, the effect of DNIC-GS on HSP synthesis decreased and did not differ from that at 50 μM DNIC-GS. Therefore, DNIC-GS activated most members of the HSP family with a cupola-shaped concentration-effect curve. This might be due to a small inhibition of the overall protein synthesis during the time period that cells were incubated in the presence of the highest concentration of NO donors. In general, the overall protein synthesis was hardly affected (not shown). Only at the highest concentrations of DNIC-GS (500 μM) and DNIC-NAC (750 μM), an inhibition of about 20% was detected. This may cause a retardation in the full synthesis of all HSPs at the highest concentrations leading to an apparent activation of most members of HSP families with a cupola-shaped concentration-effect curve in the time period studied. DNIC-NAC and SNAP resulted in a less pronounced accumulation of HSPs as compared with DNIC-GS.

Although the synthesis of HSP60, HSP70, HSP90 and HSP100 was somewhat lower in comparison with the effect of a heat shock, the synthesis of HSP28 induced by different concentrations of DNIC-GS was more pronounced. It is interesting to note that a 60 min incubation with DNIC-GS, followed by extensive washing of the cells to remove DNIC, and a subsequent incubation for 6 h with labelled amino acids (as distinct from the 6 h incubation in the presence of DNIC-GS) did not induce a visible activation of HSP synthesis. Therefore, the initiation of HSP synthesis requires a prolonged exposure of the cell culture to DNIC-GS.

A 6 h incubation of the cells in the presence of SNAP hardly induced the synthesis of HSPs (Fig. 1). A notable amount of HSP28 and HSP32 was detected only at a concentration of 750 μM. In the study of Kim et al. [2], accumulation of HSP70 was observed in rat hepatocytes as late as 12 h after addition of SNAP at the same concentration to the cell culture. In smooth muscle cells from rat aorta and mucosal cells from guinea pig intestine, a notable amount of HSP70 was found only 8 h after the addition of 1 and 0.3 mM SNAP, respectively [3,4]. It cannot be excluded that the lack of induction of HSP70 by SNAP in this study is due to an insufficient duration of the SNAP exposure (though the duration of incubation with SNAP was the same as that with DNIC-GS, 6 h).

Substitution of GS for NAC in DNIC sharply attenuated the efficiency of DNIC as inductor of HSP synthesis (Fig. 1). The decrease in accumulation of HSP70 and HSP100 was especially pronounced. This decrease may result from the ability of NAC, which is released by DNIC-NAC, to enter the cells and thereby to increase the intracellular concentration of thiol groups. According to Xu et al. [3] and Byrne et al. [4], this can attenuate the synthesis of HSPs by some yet unknown mechanism. Nevertheless, in comparison with the effect of

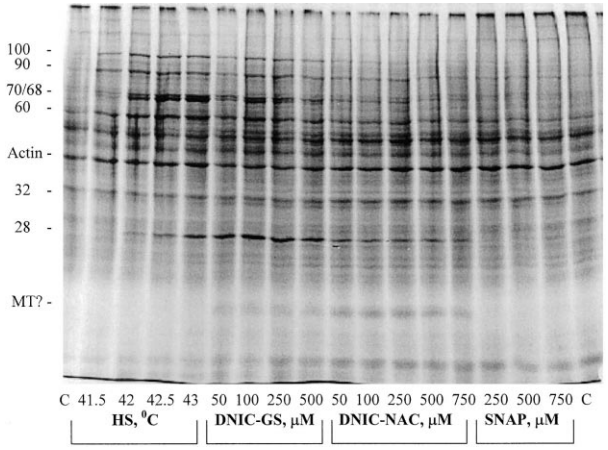

Fig. 1. Autoradiographs showing the patterns of newly synthesized proteins by H35 hepatoma cells in response to different temperatures and to different concentrations of NO donors. C, control. Heat shock (HS) was applied for 30 min at 41.5°C, etc. The different NO donors were present for 6 h during which period incorporation of ^{35}S-labelled amino acids into proteins was monitored. The different HSPs are indicated by their molecular weight in kDa (Mt, possibly metallothioneine). Concentrations of NO donors are given under respective electrophoretic lanes. Results of a representative experiment are shown.

SNAP, DNIC-NAC was able to induce HSP synthesis (especially HSP28) to a higher extent. On the whole, the presented results show that DNICs with thiol-containing ligands are able to induce efficiently not only the synthesis of HSP70 as was demonstrated before [1] but also other major HSPs in hepatoma cells.

Up to now, the DNIC complexes have been used infrequently in studies on the effects of NO on various biochemical and physiological processes. However, it is interesting to note that a number of studies have shown that DNICs can exert a more pronounced effect on these processes than *S*-nitrosothiols (RS-NO) or sodium nitroprusside (SNP), the compounds that are commonly used as NO donors. For instance, DNICs with diverse anionic ligands decrease the blood pressure in animals and inhibit platelet aggregation more efficiently than SNP [7–10]. Na,K-ATPase and GS-reductase are inactivated more efficiently by DNIC-GS in comparison with the inactivation induced by nitroso GS, when applied at the same concentration [11,12]. Finally, low-molecular weight DNICs can destroy the active center of adrenodoxin, an iron-sulfur protein, an effect which is not observed with different forms of RS-NO [13]. We believe that this comparison in favor of DNIC will be continued in further investigations.

The question arises which factor(s) may be responsible for the higher efficiency of HSP synthesis induced by DNIC-based NO donors in comparison with more conventional NO donors (SNAP). It is known that DNICs can release NO and *S*-nitrosylate proteins as well as low-molecular weight compounds [5,14,15]. This property is in principle also characteristic for RS-NO compounds including SNAP [16,17]. However, low-molecular weight DNICs, as distinct from RS-NO compounds, are able to serve as donors of $Fe^{+}(NO^{+})_2$ groups. These groups can move to thiol groups of proteins and low-molecular weight compounds with the formation of correspondent endogenous DNICs [5,14,15]. This transfer can be considered as analogue of a *S*-transnitrosylation reaction in which the NO^{+} cation travels between thiol groups of proteins and low-molecular weight compounds with the formation of various RS-NOs [16,17].

The capacity of DNIC to donate/release $Fe^{+}(NO^{+})_2$ groups to free thiols predominates over its *S*-nitrosylating activity [5,14,15]. In our experiments, the dimeric form of DNIC was used, which facilitated the transfer of $Fe^{+}(NO^{+})_2$ groups to proteins. We suggested that the activating effect of low-molecular weight DNICs on HSP synthesis might result from the ability of the complexes to donate their $Fe^{+}(NO^{+})_2$ groups to thiol groups of proteins. This may either directly or indirectly cause the induction of HSPs. The direct effect of NO donor may be related to modification of the structure of the heat shock transcription factor (HSF1). Blockade of HSF1 thiol groups may result in exposure of leucine-isoleucine amino acid residues and thus provide trimerization of HSF1 monomers with subsequent HSF1 activation [18]. Likewise, heavy metals are also able to block thiol groups, thereby changing the conformation of HSF1 monomers and inducing metallothioneins in hepatocytes [19]. In this study, metallothioneins appeared to be induced by DNIC, as could be deduced from the position on the autoradiograms presented in Fig. 1.

The indirect effect of NO donor may be related to modification of the structure of various proteins due to blockade of their thiol groups. These abnormally shaped proteins may induce HSPs, since abnormal or damaged proteins have been recognized as the non-specific trigger signal for *hsp* induction.

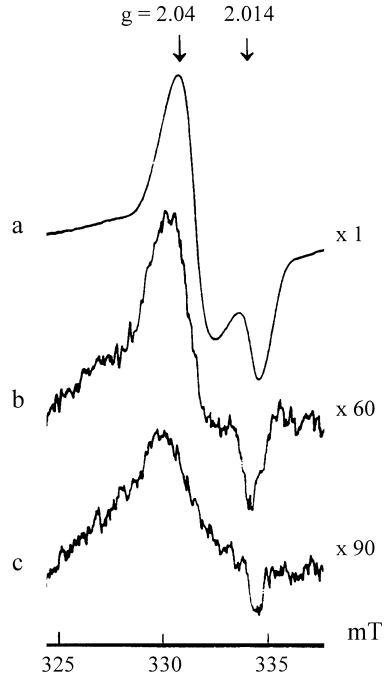

Fig. 2. EPR spectra from a frozen solution of DNIC with cysteine (500 μM) (a) and DNIC in hepatoma cells after a 3 h incubation with DNIC-GS (500 μM) (b) or SNAP (500 μM) (c). The spectra are recorded at 77 K. On the right, relative amplification of the radiospectrometer. The relative amplification factor is amplification of the radiospectrometer conventionally assumed as 1 in recording the EPR spectrum from a frozen solution of DNIC with cysteine (500 μM) (a). When the EPR spectrum was recorded from DNIC in hepatoma cells after a 3 h incubation with DNIC-GS (500 μM) (b) or SNAP (500 μM) (c), the radiospectrometer amplification was increased 60 and 90 times, respectively. Therefore, to obtain b and c signals with a factor 1, the signals should be decreased 60 and 90 times, respectively. We used different amplifications of the signal to demonstrate more distinctly the triplet structure of EPR signals of DNIC.

Therefore, we suggest that the initiating effect of SNAP on HSP synthesis in cell cultures observed in this and other studies [2–4] could result from the RS-NO transformation to the paramagnetic (monomeric) form of DNIC. Therefore, the HSP synthesis could be induced not by SNAP itself but by the DNIC originating from SNAP. The transformation of SNAP or other RS-NOs occurring on their contact with animal cells has been observed by many investigators [14,20–22]. The transformation can occur through RS-NO binding to Fe^{2+}. The Fe^{2+} ions catalyze mutual oxidation-reduction of two RS-NO molecules by the one-electron mechanism with subsequent decay of RS-NO [5]. The NO^{+} ion and NO molecule remain bound to the Fe^{2+} ion forming DNIC. We cannot exclude another mechanism of DNIC formation from RS-NO with the participation of endogenous reducers destroying RS-NO with the release of thiols and neutral NO molecules therefrom. The NO molecules enter cells and form DNIC through interaction with endogenous Fe^{2+} and thiol-containing ligands. The decomposition of RS-NO under the action of a number of reducers has been demonstrated [14,23–25].

In order to study whether incubation of cells with either DNIC-GS or SNAP would give rise to emergence of intra-

cellular DNIC, we performed some EPR measurements. Our EPR measurements showed that a 3 h incubation of hepatoma cells with 0.5 mM SNAP indeed resulted in the emergence of paramagnetic DNIC displaying a characteristic EPR signal at $g_{\perp} = 2.04$ and $g_{\parallel} = 2.014$ (Fig. 2c). For comparison, the same figure demonstrates the EPR signal from a frozen solution of monomeric DNIC with cysteine (Fig. 2a) and the signal observed in cells that were incubated for 3 h in the presence of DNIC-GS (0.5 mM) (Fig. 2b). Comparison of the intensity of the EPR signal from this solution at a known DNIC concentration (500 μM) (Fig. 2a) with the intensity of the EPR signal from DNIC in the cells incubated with SNAP (Fig. 2c) showed that the DNIC content in the cells did not exceed 1 μM. This concentration was considerably lower than the concentration of DNIC formed inside hepatoma cells after incubation in the presence of dimeric (diamagnetic) DNIC-GS (1:2) (10 μM) (Fig. 2b). (It is interesting to note that washing of cells resulted in washing-out of DNIC therefrom and this effect resulted in suppression of HSP synthesis in the cells as it has been mentioned above.) Therefore, we observed parallel changes in the concentration of DNIC emerging in cells on their contact with DNIC-GS or SNAP and in the HSP accumulation initiated by these agents. The data confirm once more that the modifying effect of SNAP and other RS-NOs on thiol groups of cellular proteins, which results in various alterations of biochemical and physiological processes and has been observed by many investigators [16,26–32], can be due to DNICs originating from RS-NO rather than due to a direct effect of RS-NO itself.

In conclusion, in this paper, we demonstrated that NO donors (mainly DNIC-GS) were able to induce other HSPs in addition to HSP70. DNIC-GS was able to induce HSP100, HSP90, HSP70, HSP68, HSP60 and small HSPs such as HSP32 and HSP28 as well as metallothioneins. The capacity of DNIC-GS to activate synthesis of numerous HSPs suggests that the NO- or $Fe^{+}(NO^{+})_2$-dependent link is a common one for activation of these HSPs.

With respect to the pattern of HSPs induced by different NO donors, we reported here a NO donor-specificity. DNIC-GS induced all HSPs that were also induced by heat shock. DNIC-NAC and SNAP only induced the low-molecular weight HSPs. HSP32, HSP28 and metallothioneins were induced by all studied concentrations of DNIC-NAC, whereas SNAP was only able to induce HSP28 and HSP32 at the highest concentrations. Thus, the previously demonstrated stressor-specific induction of HSPs [6,33] manifests itself again as exemplified by effects of different NO donors.

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