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INACTIVATION OF RIBONUCLEOTIDE REDUCTASE BY NITRIC OXIDE

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SUMMARY: Ribonucleotide reductase has been demonstrated to be inhibited by NO synthase product(s). The experiments reported here show that nitric oxide generated from sodium nitroprusside, S-nitrosoglutathione and the sydnonimine SIN-1 inhibits ribonucleotide reductase activity present in cytosolic extracts of TA3 mammary tumor cells. Stable derivatives of these nitric oxide donors were either inactive or much less inhibitory. EPR experiments show that the tyrosyl radical of the small subunit of E. Coli or mammalian ribonucleotide reductase is efficiently scavenged by these NO donors.

The extracellular cytotoxicity of activated murine macrophages against tumor cells requires the expression of a NO synthase activity that is responsible for the controlled production of nitric oxide NO and citrulline from L-arginine (1-3). A specific pattern of metabolic perturbations in the tumor target cell characterizes this L-arginine-dependent cytotoxicity, including intracellular iron release, inhibition of mitochondrial functions and inhibition of DNA synthesis (4, for review). There is some evidence that nitric oxide itself is a mediator of the cytotoxic process since incubation of tumor cells with authentic NO gas reproduced the same pattern of biochemical alterations (2,3). The detection of nitrosyl-iron-sulfur complexes after induction of NO synthesis in cytotoxic macrophages (5,6) provided a molecular basis for the L-arginine-dependent mitochondrial dysfunction targeted at the level of electron-transfer 4Fe-4S enzymes (4). On the other hand, the mechanism of inhibition of DNA synthesis is unknown. Recent experiments have established that ribonucleotide reductase

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(EC.1.17.4.1) of mammary TA3 tumor cells was inhibited by unidentified product(s) of NO synthase metabolism (7). RNR activity controls DNA synthesis by providing the cell with deoxyribonucleotides. The β_2 subunit (named R2) of this $\alpha_2\beta_2$ enzyme contains a stable tyrosyl free radical generated by a μ-oxo-bridged binuclear iron complex (8). In an attempt to characterize the L-arginine-derived inhibitor(s) of RNR, we investigated the effect of NO on RNR activity in TA3 cells. NO was generated continuously in situ from chemical nitric oxide donors, under an aerobic atmosphere. These conditions were designed to mimic the physiological environment of tumor cells exposed to biosynthetic NO produced by cytotoxic macrophages. Using pure recombinant mouse and E. Coli R2 subunits, we also explored the reactivity of NO towards the tyrosyl radical and the diferric iron center, which are potential targets for L-arginine metabolites.

MATERIALS AND METHODS

Materials. SNP, potassium ferricyanide, DTE, ATP, CDP and SOD were obtained from Sigma. The sydnonimines SIN-1 and SIN-1C were synthesized by Cassella AG (Frankfurt, FRG) and kindly provided by Mrs Winicki (Laboratoires Hoechst France). GSNO was a generous gift of Pr. K. T. Douglas (University of Manchester, UK). Oxidized L-glutathione and XOD were purchased from Fluka. Oxyhemoglobin HbO_2 was prepared by reduction of hemoglobin with sodium dithionite as described in (9), and purified on a G25 Sephadex column. Radioactive CDP was purchased from Amersham France (specific activity: 680 GBq/mmol). The mammary adenocarcinoma TA3 cell line was obtained from Pr. L. Thelander (University of Umea, Sweden). Cytosols from the R2-overproducing TA3 subclone (10) were prepared as previously described (7). E. Coli and mouse protein R2 were prepared from overproducing strains of E. Coli (11, 12).

Assay of ribonucleotide reductase activity. RNR activity in TA3 cytosols was measured by reduction of [5-^{3}H]CDP, as previously described (7). Incubations contained 5 mM ATP, 200 μM CDP, 74 kBq of [5-^{3}H]CDP and cell extracts in 100 mM HEPES, pH 7.6, with Mg acetate 15 mM and DTE 10 mM. Solutions of nitric oxide donors were prepared extemporaneously and added at the beginning of the assay (no pre-incubation). Reactions were carried out in 90 μl for 50 min at 33 °C and stopped by heating at 90°C for 2 min. Samples were then incubated for 2 h at 37°C with 10 μl of C. adamanteus venom (200 mg/ml). Deoxycytidine was separated on a Zorbax ODS C_{18} column (Société Française Chromato Colonne) eluted with 10 mM sodium acetate buffer, pH 5.4, and 2% of the ion-pairing reagent pentanesulfonic acid (Waters). Radioactivity was quantitated using a flow-through scintillation detector (Berthold LB 506C-1). RNR activity in control samples varied from 150 to 300 pmol/min/mg protein.

E.P.R. measurements. Protein R2 (1-1.5 mg/ml) was prepared in 0.2 ml of 50 mM Tris buffer, 10% glycerol, pH 7.5, in an Eppendorf tube , on ice. In the case of mouse R2, 1 mM EDTA was also present in the buffer. The EPR spectrum of the solution was recorded and is taken as a reference. After addition of various concentrations of NO donors, the sample was incubated at 25 °C for 1, 5, 10 min, and then transferred to an EPR tube and frozen in liquid nitrogen. The amplitude of the signal was not

significantly affected by repeated cycles of freezing and thawing. EPR first derivative spectra were recorded at 80 K using a Varian E 102 EPR spectrometer operating at 9.2 GHz. The microwave power was set at 6 mW for E. Coli R2 and 30 mW for mouse R2 and the modulation amplitude was 3.2 G. The loss of the tyrosyl radical was quantitated from the amplitude (peak height) of the g= 2.00 signal.

UV-Visible spectroscopy. Light absorption spectra in the wavelength range 300-700 nm were recorded with a UVIKON 930 spectrophotometer. E. Coli R2, 1 mg/ml, was incubated for at least one hour with 1 mM desferrioxamine or ferrozine at room temperature, in a spectrophotometric cuvette containing 0.5 ml of 50 mM Tris, pH 7.5. Mobilization of the diferric center induced by the addition of 0.5 mM GSNO + 10 mM ascorbate was monitored from the appearance of the 428 nm band, characteristic of the Fe-desferrioxamine complex or that of the 562 nm band, characteristic of the Fe-ferrozine complex.

Statistical analysis. Data were compared by two-tailed Student's *t* test.

RESULTS

Nitric oxide donors. Sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO) and the molsidomine derivative SIN-1 belong to three unrelated groups of chemical NO donors which provide a continuous flux of NO (13-15) . SNP releases NO spontaneously. NO formation from SIN-1 is oxygen-dependent and is stimulated by alkaline pH. By following the decomposition of GSNO by UV-Visible spectroscopy at 332 nm and 545 nm in 50 mM Tris buffer, pH 7.5, we observed that the reaction required reducing equivalents provided by ascorbate or DTE, as previously mentioned by Park (16). That NO was a product of this decomposition was verified by EPR spectroscopy. When GSNO + ascorbate was incubated with ferrous ammonium sulfate, an EPR signal was observed at g=2.039, which is identical to signals characteristic of Fe-NO complexes (5,6).

Effect of nitric oxide donors on RNR activity. The addition of SNP, GSNO or SIN-1 to crude RNR preparations from TA3 cells resulted in a dose-dependent inhibition of RNR activity (Fig.1). GSNO was less inhibitory than SNP but more active than SIN-1. Means of IC_{50} were 0.27, 0.92 and 3.7 mM for SNP, GSNO and SIN-1, respectively. Table I shows that oxyhemoglobin and superoxide anions, generated by the xanthine-xanthine oxidase system, greatly decreased the inhibitory effects of 2.2 mM GSNO on RNR activity. Since oxyhemoglobin and superoxide react with NO very efficiently and thus inactivate it, these results suggest that NO or some species derived from NO was the actual inhibitor of RNR. Addition of SOD had no effect on the inhibition of RNR by GSNO probably because the endogenous synthesis of superoxide anion in TA3 cytosols was too low to interfere with NO production. On the other hand, addition of SOD resulted in an increased efficiency of 2.2 mM SIN-1 as an inhibitor of RNR (%RNR = 63.4 ± 6.8 with SIN-1 and 46.7 ± 3.6 with SIN-1 and SOD; $p<0.02$). This has to be related to the production of superoxide anion during decomposition of SIN-1 to NO (14).

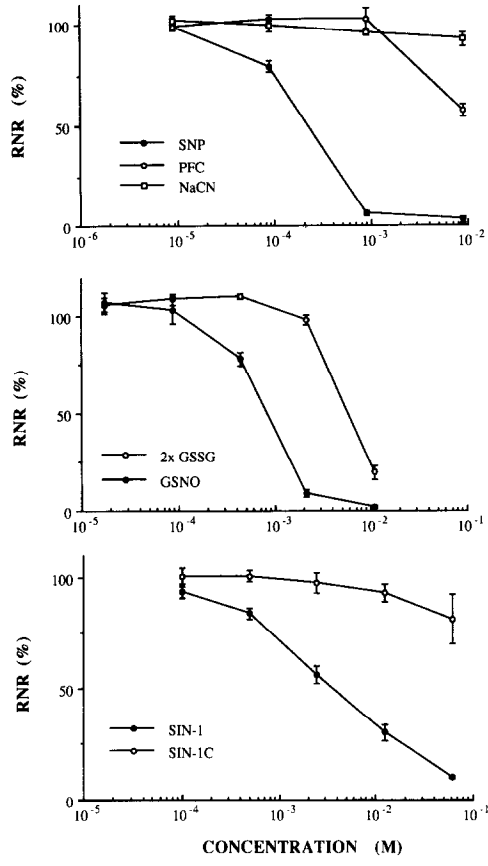

Figure 1. Inhibition of RNR by NO donors. Preparation of TA3 cell extracts and RNR assay were as described in "Materials and Methods". The values are mean ± SE of at least 3 experiments.

Table I. Inhibition of RNR by GSNO: implication of nitric oxide

Additives[a]	RNR activity[b] (% of control)	p [c]
GSNO	8.9 ± 2.1	
GSNO + HbO_2	50.1 ± 5.4	< 0.01
GSNO + SOD	12.7 ± 2.5	> 0.1
GSNO + XOD	23.1 ± 4.1	< 0.01

[a] When indicated, GSNO 2.2 mM, HbO_2 260 μM, SOD 646 U/ml and XOD 0.02 U/ml were added at the beginning of the assay. Furthermore, xanthine 500 μM was added with XOD, and catalase 260 U/ml was added with SOD and XOD.

[b] RNR activity was assayed as described in Materials and Methods. The RNR activity was not significantly modified by HbO_2, SOD and catalase, or XOD, xanthine and catalase. Data are mean ± SE of four experiments.

[c] Compared to GSNO alone.

Together with NO, the decomposition of SNP, GSNO and SIN-1 generates the more stable metabolites ferricyanide/cyanide (17), glutathione, in the oxidized and reduced form (16) and SIN-1 C (18), respectively. When tested against RNR activity, potassium ferricyanide PFC and oxidized glutathione GSSG exhibited no inhibitory effect, except at the highest concentration tested (Fig.1). Cyanide and SIN-1C did not significantly inhibit RNR activity. Nitrite and nitrate, which are end-products of NO oxidation, were not inhibitory for RNR at a concentration of 10 mM (nitrite: 98.9% ± 7.1; nitrate: 92.0% ± 1.2)

Effect of nitric oxide donors on the tyrosyl radical of protein R2. Ribonucleotide reductase activity is totally dependent on the presence of a tyrosyl radical in protein R2. The content of this radical can be accurately determined by EPR spectroscopy (8). In order to test whether the inhibition of ribonucleotide reductase by NO donors is due to a destruction of the tyrosyl radical, we studied the reaction of pure preparations of mammalian and bacterial R2 protein with GSNO, by EPR spectroscopy. As shown in Figure 2, GSNO, in the presence of 10 mM ascorbate, was an excellent scavenger of the free radical of both proteins. Ascorbate, nitrite, nitrate, glutathione had no effect on the EPR signal. Moreover, when ascorbate was omitted in the reaction mixture, the loss of the radical was drastically decreased. These results strongly suggest that NO is the active molecule. SIN-1 (Table II) and SNP were also tested on E. Coli protein R2 and found to be active, while SIN-1C (Table II) and ferricyanide were inactive. Again, that oxyhemoglobin protected the radical from SIN-1 and that the inhibitory effects of SIN-1 were increased at alkaline pH (Table II) is consistent with the actual scavenger being NO. The scavenging power of SNP

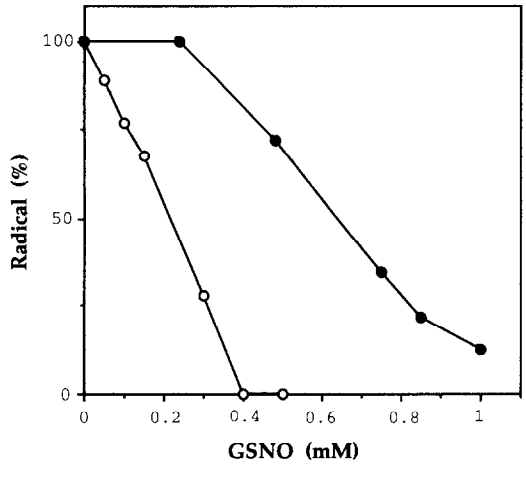

Figure 2. S-nitrosoglutathione scavenges the tyrosyl radical of protein R2 from E. Coli (open symbols) and from mouse (closed symbols). The reaction was carried out as described in "Materials and Methods" and initiated by addition of GSNO. The loss of the tyrosyl radical was monitored by EPR spectroscopy after 5 min. reaction.

Table II. Reduction of the tyrosyl radical by SIN-1

Additives[a]	pH	% radical[b]
SIN-1	7.5	76
SIN-1	9.0	16
SIN-1C	9.0	100
SIN-1 + HbO_2	9.0	42

[a] Reactions were carried out as described in Materials and Methods. Where indicated, SIN-1 or SIN-1C, 4 mM, HbO_2, 100 mM were added at the beginning of the assay. At both pH, Tris was used as a buffer. Incubation time: 15 min.

[b] The amount of radical was quantitated from the amplitude of the EPR signal.

was comparable to that of GSNO and much greater than that of SIN-1 (data not shown), in agreement with the inhibition of RNR in TA3 cell extracts reported above. Protein R2 also contains iron involved in the generation and stabilization of the tyrosyl radical. The observed loss of the radical could thus be due to a NO-dependent mobilization of iron. However, when pure E. Coli protein R2 was incubated with GSNO + ascorbate in the presence of strong iron chelators, desferrioxamine or ferrozine, and analyzed by UV-Visible spectroscopy, no Fe-desferrioxamine or Fe-ferrozine complexes could be detected at 428 and 562 nm respectively. This shows that iron was not removed from R2 during reaction with GSNO. On the other hand, addition of GSNO to R2 did not result in the formation of Fe-NO complexes since no new EPR signal, characteristic of iron nitrosyl moieties, could be detected.

DISCUSSION

We recently found a relationship between the L-arginine-dependent NO synthase pathway, inhibition of DNA synthesis and ribonucleotide reductase (7). Nitric oxide has been shown to be one of the possible cytostatic metabolites generated by the NO synthase activity (2,3). Here we demonstrate that NO donors are excellent inhibitors of ribonucleotide reductase in TA3 cells. It is very likely that these inhibitory properties are related to the strong reactivity of these molecules towards the tyrosyl radical of protein R2. Evidence is also presented pointing to NO itself, generated during decomposition of these molecules, as the active species. This is consistent with the recent observation from the X-ray three dimensional structure of E. Coli R2, that the radical is buried within the polypeptide chain and is accessible only by small molecules, O_2 in particular (19). Our data give thus a molecular basis for the inhibition of DNA synthesis in tumor cells induced by activation of the NO synthase pathway and may stimulate new strategies for antitumor chemotherapeutic

approaches based on NO. However, as far as ribonucleotide reductase is concerned, other targets for NO have to be tested. This is the case for the active thiols of the second subunit, protein R1, and the thiols of the redox transfer proteins such as thioredoxin or thioredoxin reductase. On the other hand, other metabolites, such as N-hydroxy-arginine, are potential inhibitors of ribonucleotide reductase.

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