Inhibition of Glutathione Reductase by Dinitrosyl-Iron-Dithiolate Complex*

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The biological signal molecule nitric oxide (NO) exists in a free and carrier-bound form. Since the structure of the carrier is likely to influence the interaction of NO with macromolecular targets, we assessed the interaction of a dinitrosyl-iron-dithiolate complex carrying different thiol ligands with glutathione reductase. The enzyme was irreversibly inhibited by dinitrosyl-iron-di-L-cysteine and dinitrosyl-iron-di-glutathione in a concentration- and time-dependent manner (IC50 30 and 3 μM, respectively). Evaluation of the inhibition kinetics according to Kitz-Wilson yielded a Kj of 14 μM, and a k3 of 1.3 × 10−6 s−1. A participation of catalytic site thiols in the inhibitory mechanism was indicated by the findings that only the NADPH-reduced enzyme was inhibited by dinitrosyl-iron complex and that blockade of these thiols by Hg2+ afforded protection against irreversible inhibition. This inhibition was not accompanied by formation of a protein-bound dinitrosyl-iron complex and/or S-nitrosation of active site thiols (Cys-58 and Cys-63). However, one NO moiety exhibiting an acid lability similar to a secondary N-nitrosothiols was present per mol of inhibited homomeric enzyme. These findings suggest specifically N-nitrosation of glutathione reductase as a likely mechanism of inhibition elicited by dinitrosyl-iron complex and demonstrate in general that structural resemblance of an NO carrier with a natural ligand enhances NO+ transfer to the ligand-binding protein.

Nitrosyl transfer from endogenous nitric oxide (NO) carriers such as S-nitrosothiols (1) and dinitrosyl-iron complex (DNIC) (2) to macromolecular targets is regarded as one major mechanism of biological NO signaling (3). Evidence has been provided that endogenous low mass S-nitrosothiols (predominantly S-nitrosothiophosphate) and proteinaceous S-nitrosothiols (S-nitroso-hemoglobin, S-nitrososerum albumin, and other yet unidentified proteins) exist in human erythrocytes (4), plasma (5) and bronchial secretion (6). A further NO adduct with GSH, GSNOH, was recently postulated as another transport form of NO (7). S-Nitrosation of protein thiols or subsequent reactions such as ADP-ribosylation (8), formation of protein disulfide (9), and cysteine sulfenic acid (10) may influence protein function by allosteric mechanisms. Furthermore, reversible S-nitrosation of cell membrane-bound proteins may be involved in transmembrane NO transport (11).

A concept has been derived from established chemistry to account for the influence of the redox state of the NO moiety on biological NO transfer reactions. According to this concept nitrosation of nucleophilic targets occurs by attack of nitrrosium (NO+)−like species assumed to be present in “NO carriers” such as N2O3, S-nitrosothiols, and certain iron-nitrosyl complexes (3, 12). Less attention has been paid to the influence of the carrier structure on the interaction of NO with macromolecular targets. However, it is conceivable that the NO carrier will direct the NO moiety specifically to macromolecules recognizing the carrier structure, provided the NO adduct is sufficiently stable and the binding kinetics between the carrier and the macromolecules are rapid enough to outbalance the decomposition of the NO carrier adduct. There is also evidence that NO adducts may exhibit intrinsic bioactivity independent of NO release. Thus, the L-stereoisomer of S-nitrosocysteine was found to exhibit a significantly higher blood pressure lowering activity compared with the D-isomer, suggesting the existence of stereospecific S-nitroso-L-cysteine receptors in the cardiovascular system (13).

To assess the influence of the carrier structure on the interaction of NO with a given macromolecule we chose glutathione reductase (GR; EC 1.6.4.2) as a model target, and low mass dinitrosyl-iron complexes with L-cysteine and glutathione ligands as NO carriers. These complexes exhibit S-nitrosating activity toward serum albumin in vitro (2), and protein-bound forms exist in vivo in animal tissues expressing inducible NO synthase activity (14). The flavoenzyme GR catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to maintain a high intracellular level of GSH. GR carries a redox-active disulfide (Cys-58–Cys-63) in its active site which is reduced by electron transfer from NADPH via the flavin (15). Recently it has been shown that GR is inhibited by certain NO carriers (S-nitrosothiogluthathione, sodium nitroprusside, S-nitroso-N-acetyl-DL-penicillamine) in millimolar concentrations (16), suggesting that GR is a potential target for nitrosation reactions.

We show here that low mass dinitrosyl-iron complexes in concentrations that may be present under pathophysiological conditions irreversibly inhibit GR, possibly via N-nitrosation. We furthermore demonstrate that the inhibitory potency of the dinitrosyl-iron moiety increases with structural resemblance of the NO carrier to the natural GR substrate, GSSG.

**EXPERIMENTAL PROCEDURES**

*Materials—* GR from bovine intestinal mucosa, fatty acid-free bovine serum albumin (BSA); 2,3-diaminonaphthalene, L-cysteine, glutathione...
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(oxidized and reduced), diethyl pyrocarbonate (DEPC), and Sephadex G-25 were supplied by Sigma, Deisenhofen, Germany. 1-Nitroso-2-hydroxynaphthalene-3,6-disulfonic acid and 1-nitrosopyrrolidione were obtained from Aldrich, Deisenhofen, Germany. NO gas was prepared by reaction of FeSO₄ (Fluka, Buchs, Switzerland) with NaNO₂ in 5 M HCl and was purified by low temperature vacuum (p = 0.01 mm Hg) distillation (2).

Paramagnetic dinitrosyl-iron complexes of the type ([NO]FeRS₂)₂ × 18 RSH (RS = l-cysteine, or GSH) were synthesized by mixing evacuated (5 min high vacuum) solutions of FeSO₄ (5 mg/ml) and neutralized thiols (72 mM) in a Thunberg-type reaction vessel under pure NO gas (PNO, 500 kPa) for 3 min before mixing. The solution immediately turned dark green and was evacuated after 1 min for a further 2 min to remove excessive NO. The solution bearing ([NO]FeRS₂)₂ in >98% yield with respect to iron was immediately frozen and stored in liquid nitrogen.

S-Nitroso-l-cysteine and S-nitroso-BSA were prepared at 4 °C by mixing either l-cysteine (100 mM) or fatty acid-free BSA (2 mM) for 10 min with an equimolar amount of sodium nitrite dissolved in 0.5 M H₂SO₄. The S-nitrosothiols were frozen and stored in liquid nitrogen. The yield of both S-nitrosothiols was >90% with respect to free thiol added (BSA-thiol/BSA = 0.4 ± 0.04), using molar absorption coefficients of S-nitroso-BSA (ε₅₃₀ = 870 M⁻¹ cm⁻¹, 1) and S-nitroso-l-cysteine (ε₅₃₀ = 16.7 M⁻¹ cm⁻¹) (17).

Determination of S-Nitrosothiols and Nitrite—At concentrations >1 μM S-nitrosothiols were assessed by diazotization of sulfanilamide and azocoupling with N,N-ethylendiamine in the presence and absence of Hg²⁺ ions (3 mM) according to Saville (18) as described recently (2).

In the nanomolar range nitrite and S-nitrosothiols were quantified by an acid-catalyzed intramolecular diazotization reaction of 2,3-diaminonaphthalene with nitrite forming the highly fluorescent product 2,3-diaminonaphthotriazole (19). The sample (570 μl) was mixed with 30 μl of 0.1 M potassium P buffer, pH 7.4, and 84 μl of freshly prepared 2,3-diaminonaphthalene (0.05 mg/ml in 1 M HCl). To release NO from S-nitrosothiols the buffer contained 20 mM HgCl₂, Hg₂Cl₂ did not interfere with the fluorescent assay. After 10 min of incubation at 20 °C in the dark the reaction was terminated by addition of 42 μl of 4.5 M NaOH to maximize the intensity of the fluorescent signal (19). The fluorescence was measured with excitation at 375 nm and emission at 415 nm (Deltascan, Photon Technology). Emitted light was detected by a photon-counting photomultiplier (D-104, Photon Technology) and the photomultiplier digital output was collected by an IBM-compatible computer. The content of S-nitrosothiol was calculated by the difference of emission readings of Hg²⁺-containing versus Hg²⁺-free samples. A calibration curve was established in each experiment with freshly synthesized S-nitroso-l-cysteine and sodium nitrite as standards (0.02–2 μM).

The detection limit was 20 nm.

EPR Spectroscopy—EPR spectra were recorded on a Bruker EPR 300E spectrometer at 20 °C on solutions (25 μl) filled in a quartz capillary tube (1 mm, inner diameter). The measurements were performed with a modulation amplitude of 1 Gauss, a microwave frequency of 9.6 GHz, a microwave power of 20 mW, and a time constant of 0.2 s.

The concentration of dinitrosyl-iron complex was calculated by comparison with the EPR signal of a standard low molecular mass dinitrosyl-iron complex based on double integration of the first derivative EPR signals.

Chemical Modifications of Glutathione Reductase—Histidine residues were carbamoylated by adding a 100-fold molar excess of DEPC and subsequent incubation for 5 min at 20 °C (20). Thiol groups were blocked by incubation of the protein (5 μM protein in 0.1 M potassium phosphate buffer, pH 7.4) for 5 min at 20 °C with HgCl₂ (15 μM) in the presence of NADPH (1 mM). To further demonstrate the involvement of catalytic site thiols in DNIC-induced inhibition of GR the enzyme (5 μM) was preincubated with HgCl₂ (15 μM; 5 min at 20 °C) prior to incubation with DNIC-GSH (30 μM; 10 min at 37 °C). Controls were performed without HgCl₂ and in the absence and presence of DNIC. The solutions (100 μl) were deaslated at 5 °C by passing over a Sephadex G50 Nick® column (Pharmacia) equilibrated with assay buffer (see below) and then incubated with dithiothreitol (DTT) (10 mM, 37 °C) for up to 70 min. After 10, 30 or 70 min of DTT-treatment the activity of GR was assessed in 1:250 diluted aliquots as described below.

Inhibition of Glutathione Reductase Activity—The reduction of GSSG by GR was determined at 20 °C by monitoring the oxidation of NADPH at 340 nm (ε₃₄₀ = 6200 M⁻¹ cm⁻¹) (21). The enzyme was diluted in assay buffer (200 mM potassium chloride, 1 mM EDTA, 50 mM potassium phosphate, pH 6.9). To avoid an interference by NADPH-oxidase activity both reference and sample cuvettes contained NADPH (0.38 mM) and GR (3.5–20 mM) in a final volume of 1 ml. The reaction was started by addition of GSSG (1 mM) to the sample cuvette. The enzyme activity was calculated from the initial rate of the absorbance decrease at 340 nm during 3 min of incubation. For establishment of concentration-response relationships, 1–5 μM GR was preincubated with inhibitors for 30 min and then diluted 300–1000-fold in the final assay mixture. For characterizing individual residues of the bovine GR, the numbering system of the well studied human enzyme (15, 16) was used; the active site thiols are Cys-58 and Cys-63, and the catalytic imidazole is His-467.

Inhibition of GSH Reductase by DNIC—To study the influence of DNIC on the activity of isolated GR, the enzyme was incubated with different concentrations of DNIC-l-cysteine and DNIC-GSH in the presence of NADPH and substrate (GSSG). The GSSG-consumption was quantitated by monitoring continuously by recording the decrease in absorbance at 340 nm (Fig. 1). During 3 min of reaction at 20 °C, samples containing DNIC-l-cysteine exhibited a nearly constant rate of decrease in absorbance (data not shown), which was inversely related to the concentration of DNIC used. In contrast, NADPH consumption in DNIC-l-cysteine containing reaction mixtures initially exhibi...
The redox state of the enzyme, GR (catalytic site, which is reduced after binding of NADPH. To assess whether DNIC influenced the aggregation state of the homodimeric form is essential for catalytic function of GR (15). We analyzed the reversibility of Hg\(^{2+}\)-elicited inhibition of GR by DNIC. Hg\(^{2+}\) exhibits a high affinity for thiols. GR (5 \(\mu\)M) was inhibited by the treatment with Hg\(^{2+}\) (15 \(\mu\)M) within 60 s at 20 °C. DTT and dimercaptopropanol regenerated the initial activity (5 mM, 10 °C) of the enzyme, indicating that the Hg\(^{2+}\)-bound thiol groups at the active center were accessible to both agents (see also Williams (15)). Therefore the dithiols used should be able to interact with the thiols in the complex-inhibited enzyme without sterical hindrance. Altogether these findings show that inhibition of GR by DNIC is irreversible.

To assess the involvement of cysteine-thiols within the catalytic site in DNIC-induced inhibition of GR we examined whether or not pretreatment of GR by Hg\(^{2+}\), which inhibits GR in a thiol-reversible manner (see above), affords protection against the irreversible inhibition of GR by DNIC-GSH. Since GR contains 3 cysteine thiols per subunit, NAPDH-reduced GR was pretreated with a 2-fold molar excess of Hg\(^{2+}\) prior to incubation with a maximally inhibitory concentration of DNIC-GSH (30 \(\mu\)M). Half of the original GR activity was restored within 10 min followinc incubation of Hg\(^{2+}\)/DNIC- and Hg\(^{2+}\)-treated GR with DTT. DTT, however, failed to restore catalytic activity to GR treated with DNIC only. The degree of inhibition after 10 min of DTT treatment was: DNIC-treated GR, 85 ± 10%; Hg\(^{2+}\)-treated GR, 45 ± 4%; Hg\(^{2+}\)/DNIC-treated GR, 52 ± 7% (mean ± S.E.; \(n = 3\)). This inhibition was not significantly altered after either 30 or 70 min of treatment with DTT. Thus, Hg\(^{2+}\)-pretreatment protects GR from inhibition by DNIC. Consequently, thiols within the catalytic site of GR appear to be the main targets of DNIC and are involved in the irreversible inhibition.

**Kinetics of Inhibition**—To study the kinetics of GR inhibition by DNIC the enzyme (1 \(\mu\)M) was exposed at 20 °C to 0, 6, 10, 15, 30, 60, 120, and 240 min. Original tracings are representative of three similar experiments.
were replotted according to Kitz-Wilson (1/
the inhibition by the corresponding DNIC concentration and
Ki constant of the reversible complex (Fig. 5
S.E. from three experiments.

Therefore DNIC-inactivated enzyme (5
covalent modification accounts for inhibition of GR by DNIC.
by low molecular weight DNIC (2), we assessed whether this
mechanism of inhibition of GR by DNIC. The dinitrosyl-iron
mixture exhibited exclusively the EPR signal of the low mo-
polar mass DNIC (Fig. 5
bound DNIC (serum albumin-DNIC; Fig. 5
inhibitor (5
assess whether or not the dinitrosyl-iron group was attached to
the inhibited protein, GR (5
assessed for
say. Under the mildly acid conditions (0.1 M HCl) of this assay
neither nitrite nor S-nitrosothiol could be detected. However
S-nitrosothiol moiety, a
S-nitroso group.

Chemical Characterization of DNIC-modified GR—
The following experiments were performed to reveal the molecular
mechanism of inhibition of GR by DNIC. The dinitrosyl-iron
moiety of low molecular DNIC binds to free thiol groups of
proteins due to a thiol-ligand exchange reaction (2, 26). To assess
whether or not the dinitrosyl-iron group was attached to
the inhibited protein, GR (5
was incubated with different
centrifugation and then centrifuged reversed inactivation of GR. In the presence of
Hg2+ 0.76 ± 0.04 mol nitrite/mole inactive GR was detected (n =
array, a histidine residue (His-467) is located in the
vertical axis.

FIG. 3. Kinetics of the GR inhibition by DNIC-GSH. The enzyme
(1 mM) was incubated with 6 (●), 10 (■), 50 (□), or 200 (▲) μM DNIC-
GSH in assay buffer at 20 °C. Aliquots were removed and assayed for
activity at different time points (0, 2, 6, 12, 18, 24, and 30 min). The
natural logarithm of the ratio of the remaining activity (E) and the
initial activity (E io) is plotted versus the time of incubation. The slopes
of the lines representing apparent rate constants of inhibition k app were
determined by linear regression analysis. Values represent means ±
S.E. from three experiments.

20, 35, 50, or 200 μM DNIC-GSH in assay buffer containing 1
mM NADPH. Aliquots were taken and tested for GR activity
after different time intervals (0, 2, 6, 12, 18, 24, and 30 min).
The time course of inhibition displayed first order kinetics at all
DNIC concentrations used. In Fig. 3 the natural logarithm of
the ratio from the remaining activity (E) and the initial
activity (E io) is plotted versus the time of incubation yielding
straight lines according to Equations 2–4 (see “Experimental
Procedures”). The slopes represent the rate constants k app for
the inhibition by the corresponding DNIC concentration and
were replotted according to Kitz-Wilson (1/k app versus 1/I) as
shown in Fig. 4. A linear relationship was apparent which was
used to calculate the kinetic constants by linear regression
analysis (r = 0.95). The constant for the conversion of the
reversible enzyme-inhibitor-complex to the irreversibly inhib-
ited enzyme (k i) amounted to 1.3 × 10 −6 s−1, the
dissociation constant of the reversible complex (K i) was 14 μM.

Chemical Characterization of DNIC-modified GR—The follow-
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FIG. 4. Kitz-Wilson replot of GR inhibition by DNIC-GSH. Double
reciprocal plot of k app (Fig. 3) versus DNIC concentration. Values for
15, 20, and 35 μM DNIC were taken from separate experiments. The
dissociation constant K i of the reversible enzyme-inhibitor complex was
derived from the intercept of the graph obtained by linear regression
analysis with the horizontal axis. The rate constant for irreversible
inhibition k i is represented by the reciprocal value of the intercept with
vertical axis.

remove excess inhibitor. The protein was concentrated by cen-
trifugation (Ultrafree 30-kDa cut-off, Millipore) and assayed
for NOX and S-nitrosothiol (18). Neither chromatography nor
centrifugation reversed inactivation of GR. In the presence of
Hg2+ 0.76 ± 0.04 mol nitrite/mole inactive GR was detected (n =
array, a histidine residue (His-467) is located in the
vertical axis.

Hence it was investigated whether a N- or C-nitrosation of
GR by DNIC accounts for inhibition. N-Nitrosopyrrolidine, a
nitrosamine of a cyclic secondary amine, also released NO
independently of Hg2+ under the acid conditions of the Griess
reaction (0.25 M HCl). After 30 min 50 ± 4 μM nitrite was
generated by this agent (100 μM). In contrast, the C-nitroso
compound 1-nitroso-2-hydroxynaphthalene-3,6-disulfonic acid
(50 μM, 60 min incubation), failed to give a positive Griess
reaction, either in the presence or absence of Hg2+ . To
increase the sensitivity of the Griess reaction the inhibited GR was also
assessed for S-nitrosothiols by the 2,3-diaminonaphthalene
assay. Under the mildly acid conditions (0.1 M HCl) of this assay
neither nitrite nor S-nitrosothiol could be detected. However
when the inhibited GR was preincubated in 0.25 M HCl for 30
min at 37 °C and then neutralized with NaOH, 0.83 ± 0.05 mol
nitrite/mole enzyme were found by the 2,3-diaminonaphthalene
assay. This finding reveals that the NO moiety bound to GR
exhibits a peculiar acid lability. A similar acid lability was
exhibited by N-nitrosopyrrolidine. The N—NO bond was split in
0.25 M HCl but not in 0.1 M HCl.

To further substantiate that the NO moiety is firmly bound
to DNIC-inhibited GR under neutral conditions, purified soluble
guananyl cyclase (GC) was used as a sensitive NO detector.
DNIC-inhibited GR was desalted and then incubated with GC
for assessment of cGMP formation. The basal GC activity
was not influenced by DNIC-inhibited GR (400 nM). In contrast, the
reference S-nitroso protein S-nitroso-serum albumin at 10-fold
lower concentration (40 nM) stimulated GC activity 17-fold.
N-Nitrosopyrrolidine (4 μM) and 1-nitroso-2-hydroxynaphthalene-
3,6-disulfonic acid (4 μM) did not enhance GC activity
(data not shown). These findings tend to exclude the possibility
that DNIC-inhibited GR carries a labile S-nitrosothiol moiety,
but favor the concept of the formation of a stable N-nitroso
group.

According to the tertiary structure of GR derived from x-ray
diffraction analysis, a histidine residue (His-467) is located in the
direct vicinity of Cys-58 (35). During catalysis of GSSG
reduction His-467 takes a proton from Cys-58, thereby facili-
were recorded at 20 °C. The relative signal gain was 1 in
Horizontal bars

Inhibition of GR by DNIC.

by that covalent modification of a histidine, presumably His-467,
for inhibition of the enzyme. This finding supports the notion
indicating that the modification of histidine residues accounted
for 3 min (20 min) at 20 °C. DEPC treatment completely inactivated GR
by DEPC could be observed, and a rate constant of the conversion of the
reversible enzyme-inhibitor complex to the irreversibly inhibited
enzyme \( k_3 \) of \( 1.3 \times 10^{-3} \) sec\(^{-1} \) were derived from the
Kutz-Wilson replot. Thus DNIC-GSH was bound with high affi-

ity in a rapid equilibrium preceding the irreversible inhibi-
tory reaction.

Chemical Characterization of DNIC-inactivated GR—The irre-
versible inhibition of GR suggests a covalent modification of the
enzyme by DNIC. A likely target site is the catalytic center of GR,
which carries a redox-active disulfide (Cys-58–Cys-63).

The requirement for reduced thiols at the catalytic site was
 evidenced by the finding that DNIC led to inhibition of GR only
in the presence of the coenzyme NADPH, and that GR pre-
treated with \( \text{Hg}^{2+} \) was protected against inhibition by DNIC.

Other agents which inhibit GR by carbamoylation (1,3-bis(2-
chloroethyl)-1-nitrosourea) or alkylation (1-(2-chloroethyl)-3-
(2-hydroxyethyl)-1-nitrosourea) of Cys-58 also influence the en-
zyme activity only after two-electron reduction of the enzyme
by NADPH (28, 29). Therefore inhibition of GR by DNIC could
involve a stable attachment of the dinitrosyl-iron moiety to one
or both cysteines via a ligand exchange reaction (30) or a
S-nitrosation of one or both cysteines, as has been shown for the
interaction of DNIC with serum albumin (2).

Low molecular weight DNIC did not react with GR to form a
protein-bound DNIC, as assessed by EPR spectroscopy (Fig. 5).
This finding excludes that GR is inhibited by a linkage of the
dinitrosyl-iron group to the active site cysteine residues.

We next assessed the formation of a S-nitroso group in DNIC-

inhibited GR by the Saville reaction. No S-nitrosation was
detectable, although 1 mol of the DNIC-inhibited enzyme con-
tained about 0.8 mol/subunit Griss-positive \( \text{NO} \) released from the
enzyme by 0.25 M HCl, but not by 0.1 M HCl. Similar acid
lability of the NO moiety was observed with \( \text{N-nitrosopropyri-}

derine}, a nitrosamine of a cyclic secondary amine, whereas the
C-nitroso bond of 1-nitroso-2-hydroxynaphthalene-3,6-disulfo-
nic acid was acid-resistant. This result indicates that DNIC-
inhibited GR bears a \( \text{N-nitroso moiety} \).

A similar conclusion was derived from the comparison of the
guanylyl cyclase-stimulating activity of DNIC-inhibited GR,
S-nitrosoalbumin, N-nitrosoppyrrolidine, and 1-nitroso-2-hydroxynaphthalene-3,6-disulfonic acid. Only S-nitrosoalbumin was able to enhance guanylyl cyclase activity.

Since in vitro a nitrosation of cyclic secondary amines by S-nitrosothiols has been described (31), and a histidine residue (His-467) located in the vicinity of Cys-58 participates in the catalytic reduction of GSSG, we considered N-nitrosation of this residue as a likely mechanism of GR inhibition by DNIC. In fact, GR activity was also lost after N-carboxylation of histidine residues by DEPC. This finding implies that blocking of an imidazole nitrogen could account for inhibition of GR activity by DNIC.

Our results are consistent with the following hypothetical mechanism of GR inhibition by DNIC (Fig. 6). In a rapid pre-equilibrium DNIC interacts reversibly with active site thiols. DNIC then S-nitrosates Cys-58, which is characterized by a higher electronegativity than Cys-63 (32) and is located at the entrance of the active site. This reaction is immediately followed by an intramolecular trans-nitrosation yielding a N-nitrosamine, presumably at the imidazole nitrogen of His-467. In this context it should be noted that the covalent modification achieved by bichloroethylnitrosourea is stabilized by a hydrogen bond between the carbamoyl oxygen and the His-467 (29). This implies that a trans-nitrosation between Cys-58 and His-467 should be sterically feasible. However, the involvement of His-467 in the inhibitory mechanism and its N-nitrosation by DNIC has to be proven by x-ray diffraction analysis. Since minor species differences in the protein structure of GR have a great influence on GR’s susceptibility to different kinds of inhibitors, the scheme proposed here may be valid only for GR from bovine intestinal mucosa.

In conclusion, we have shown that low mass DNIC irreversibly inactivate GR and exhibit a much higher inhibitory potency than other known inhibitors of GR. From our study two biochemical principles might emerge. First, N-nitrosation is a novel mechanism by which proteins may be post-translationally modified and which broadens the spectrum of NO-mediated signal transduction. Adjacent cysteine and histidine residues would be expected to be susceptible to this modification. As a consequence of its size and charge distribution, DNIC should nitrosate proteins more selectively than NO2/N2O3, as accessibility to DNIC is determined by protein three-dimensional structure in the immediate vicinity of the target thiol. Second, we have shown that the thiol ligand structure influences the interaction of DNIC with macromolecular targets. A similar effect of the NO carrier structure on the interaction of NO with proteins has been reported. For instance, human glutathione peroxidase specifically liberates NO from S-nitrosglutathione, but not from S-nitroso-l-cysteine (33) or di-

**References**

1. Meyer, D. J., Kramer, H., Ozer, N., Coles, B. & Ketterer, B. (1994) FEBS Lett. 345, 177–180
2. Boese, M., Mordvintsev, P. I., Vanin, A. F., Busse, R. & Mulsch, A. (1995) J. Biol. Chem. 270, 29244–29249
3. Stamler, J. S. (1994) Cell 78, 931–936
4. Jia, L., Bonaventura, C., Bonaventura, J. & Stamler, J. S. (1995) Nature 376, 221–226
5. Stamler, J. S., Jaraki, O., Osborne, J., Simon, D. I., Keaney, J., Vita, J., Singel, D., Valeri, C. R. & Loscalzo, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7674–7677
6. Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J., Loscalzo, J. & Stamler, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10957–10961
7. Hegg, N., Singh, R. J. & Kalyanaraman, B. (1996) FEBS Lett. 382, 223–228
8. Mohr, S., Stamler, J. S. & Brune, B. (1996) J. Biol. Chem. 271, 4209–4214

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**Fig. 6. Scheme of the putative mechanism of GR inhibition by DNIC-GSH.** The reduced form of GR (1) bearing the charge-transfer complex between the flavin and Cys-63 reacts with a nitrosyl moiety of DNIC (2) to form an intermediate S-nitrosothiol at Cys-58. Trans-nitrosation from Cys-58 to an imidazole nitrogen of His-467 (3) yields a stable N-nitroso bond at His-467 (4) and accounts for irreversible inhibition of GR (35).

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2 M. Boese, M. A. Keese, K. Becker, R. Busse, and A. Mulsch, unpublished results.
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9. Caselli, A., Camici, G., Manao, G., Moneti, G., Pazzagli, L., Cappugi, G. & Ramponi, G. (1994) J. Biol. Chem. 269, 24878–24882
10. DeMaster, E. G., Quast, B. J., Redfern, B. & Nagasawa, T. (1995) Biochemistry 34, 11494–11499
11. McDonald, B., Reep, B., Lapetina, E. G. & Molina y Vedia, L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11122–11126
12. Stamler, J. S., Singel, D. J. & Loscalzo, J. (1992) Science 258, 1898–1902
13. Travis, M. D. & Lewis, S. J. (1997) Am. J. Physiol. in press
14. Chamulitrat, W., Jordan, S. J., Mason, R. P., Litton, A. L., Wilson, J. G., Wood, E. E., Wolberg, G. & Molina y Vedia, L. (1995) Arch. Biochem. Biophys. 316, 30–37
15. Williams, C. H. Jr. (1992) Chemistry and Biochemistry of Flavoenzymes (Müller, F., ed) Vol. III, pp. 121–211, CRC Press, Boca Raton, FL
16. Becker, K., Gui, M. & Schirmer, R. H. (1995) Eur. J. Biochem. 234, 472–478
17. Stamler, J. S., Osborne, J. A., Jaraki, O., Rabbani, L. E., Mullins, M., Singel, D. J. & Loscalzo, J. (1995) J. Clin. Invest. 91, 308–318
18. Saville, B. (1995) Anal. Chem. 63, 670–672
19. Misko, T. P., Schilling, R. I., Salvermini, D., Moore, W. M. & Currie, M. G. (1993) Anal. Biochem. 214, 11–16
20. Lee, M., Arosio, P., Cozzi, A. & Chasteen, N. D. (1994) Biochemistry 33, 3679–3687
21. Worthington, D. J. & Rosemeyer, M. A. (1976) Eur. J. Biochem. 67, 231–238
22. Kitz, R. & Wilson, I. B. (1962) J. Biol. Chem. 237, 3225–3249
23. Musch, A. & Gerzer, R. (1991) Methods Enzymol. 195, 377–385
24. Cleland, W. W. (1964) Biochemistry 3, 480–486
25. Pietraforte, D., Mallorzi, C., Scorza, G. & Minetti, M. (1995) Biochemistry 34, 7177–7185
26. Musch, A., Mordvinov, P. I., Vanis, A. F. & Busse, R. (1991) FEBS Lett. 294, 252–256
27. Pui, E. F. & Schulz, G. E. (1983) J. Biol. Chem. 258, 1752–1757
28. Schallreuter, K. U., Gleason, F. K. & Wood, J. M. (1990) Biochem. Biophys. Acta 1054, 14–20
29. Jochers-Scherübl, M. C., Schirmer, R. H. & Krauth-Siegel, R. L. (1989) Eur. J. Biochem. 180, 267–272
30. Vanin, A. P., Kiladze, S. V. & Kubrina, L. N. (1975) Biofizika 20, 1068–1073
31. Oae, S., Kim, Y. H., Fukushima, D. & Takata, T. (1977) Chem. Lett. 893, 65–69
32. Karplus, P. A., Krauth-Siegel, R. L., Schirmer, R. H. & Schulz, G. E. (1988) Eur. J. Biochem. 171, 193–198
33. Freedman, J. E., Frei, B., Welch, G. N. & Loscalzo, J. (1995) J. Clin. Invest. 96, 394–400
34. DeGroote, M. A., Granger, D., Xu, Y., Campbell, G., Prince, R. & Fang, F. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6399–6403
35. Schirmer, R. H., Müller, J. G. & Krauth-Siegel, L. (1995) Angew. Chem. Int. Ed. Engl. 34, 141–154