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Rapid Letter

The Specific NOS2 Inhibitor, 1400W, Sensitizes HepG2 Cells to Genotoxic, Oxidative, Xenobiotic, and Endoplasmic Reticulum Stresses

CLAIRE M. PAYNE,[1,3] HARRIS BERNSTEIN,[1,3] CAROL BERNSTEIN,[1]
KATHLEEN KUNKE,[1] and HARINDER GAREWAL,[2–4]

ABSTRACT

We tested the hypothesis that the constitutive activity of the inducible form of nitric oxide synthase (NOS2) serves to protect cells against numerous endogenous stresses. To accomplish this, we treated HepG2 cell lines that were individually transfected with 13 different promoter/response element (RE) chloramphenicol acetyl transferase (CAT) reporter constructs, with a highly selective NOS2 inhibitor, 1400W [*N*-(3-(aminomethyl)benzyl) acetamidine)]. HepG2 cells were incubated for 6 h with 0, 1, 10, 50, 100, and 200 μM 1400W, and the activation of the promoter/RE CAT reporter constructs was simultaneously determined. The highest fold inductions occurred at 200 μM 1400W, a concentration that had no effect on overall cell viability, as determined by the MTT assay. Twelve of the 13 promoter/RE CAT reporter constructs were significantly activated by 200 μM 1400W. These results indicate the extensive protective role of constitutive NOS2 against genotoxic, oxidative, and endoplasmic reticulum stresses. The mechanism of this protection may involve the complexing of iron by nitric oxide (NO) to reduce hydroxyl radical formation, NO inhibition of electron transport and the generation of reactive oxygen species within mitochondria, NO inhibition of cyclooxygenase, lipoxygenase, and cytochrome P450 enzyme activity, and the scavenging of superoxide anions by NO to form peroxynitrite. Antioxid. Redox Signal. 3, 931–936.

INTRODUCTION

THE GENERATION of intracellular nitric oxide (NO) is a double-edged sword (8). On the one hand, physiologic levels of NO are involved in signal-transduction pathways (8) that affect many physiologic processes (2, 7), including neurotransmission, smooth muscle relaxation and vasodilatation, immune function, iron homeostasis, the control of mitochondrial respiration, the inhibition of cellular proliferation, and the inhibition of apoptosis (8, 14, 15). On the other hand, higher levels of NO can contribute to chronic inflammatory disorders and cardiovascular disease and may be cytotoxic to cells (8). The cytotoxicity may be mediated, in part, through the interaction of NO with superoxide anion to form peroxynitrite ($ONOO^-$) and other reactive nitrogen species (RNS) (5). NO is derived through the oxidation of one of the terminal guanidino-nitrogen atoms of L-arginine by the activity of three distinct isoforms of nitric oxide synthase (NOS). Until recently, NOS1 and NOS3 were considered to be constitutive isoforms, whereas NOS2 was considered to be inducible. However, recent evi-

Departments of [1]Microbiology and Immunology and [2]Internal Medicine, College of Medicine, and [3]Arizona Cancer Center, University of Arizona, Tucson, AZ 85724.
[4]Tucson Veteran Affairs Medical Center, Section of Hematology/Oncology, Tucson, AZ 85723.

dence indicates that the constitutive isoforms are also inducible and the inducible isoform can be constitutive, indicating a possible "housekeeping" function for NOS2.

We tested the hypothesis that the constitutive activity of NOS2 serves a major protective role within cells. In the present study, we inhibited NOS2 in HepG2 cells using a highly selective inhibitor, 1400W (4), and determined the activation of 13 different promoter/response element (RE) constructs, each of which responds to a specific type of cellular stress. The findings from these 13 assays indicated an extensive protective role of constitutive NOS2 against genotoxic and other stresses. The possible mechanisms for the protective role of NO are discussed.

MATERIALS AND METHODS

Reporter constructs

Thirteen individual gene promoters or REs have been fused to the chloramphenicol acetyl transferase (CAT) reporter gene to construct pSP-CAT plasmids (13). HepG2 cells were stably transfected with the pSP-CAT plasmids by electroporation using a gene pulser (Bio-Rad Laboratories, Richmond, CA, U.S.A.), as previously described (13).

Treatment of HepG2 cells with 1400W

HepG2 cells containing the reporter constructs were incubated for 6 h with the highly selective NOS2 inhibitor 1400W at the following concentrations: 0, 1.0, 10, 50, 100, and 200 μM. Cells were washed free of 1400W after the 6-h incubation period using fresh media; untreated cells were also washed and replenished with fresh media. All cells were incubated for an additional 44 h and then lysed. The lysates were transferred to eight-well microtiter strips coated with anti-CAT antibodies. The CAT ELISA was performed according to the protocol described in the Xenometrix CAT-TOX assay manual. Microtiter plates were read at an absorbance of 405 nm in a microtiter plate reader (Bio-Tek Instruments, Inc.). Cellular viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Media and MTT solution were aspirated and the formazan salt formed was solubilized with mixing in 200 μl of 100% dimethyl sulfoxide (13). Column 1, containing no cells, was used as a blank against dimethyl sulfoxide and residual MTT. Plates were read at 550 nm on the microtiter plate reader.

Statistical analysis

Xenometrix software was used to collect and analyze data from the CAT and MTT assays (13). Raw optical density (OD) data at 405, 550, and 600 nm were converted into a final fold induction of transcriptional activity and percentage viability (13). Fold induction was calculated using the formula:

$$\frac{OD_{405}(\text{test sample}_N)/OD_{600}(\text{test sample}_N)}{OD_{405}(\text{control}_N)/OD_{600}(\text{control}_N)}$$

Test sample_N represents the specific test well in a row, and control_N represents the control well in column 2 of that row.

Cell viability was determined by using the raw data measured at OD_{550}. The first well of the row was used to blank the spectrophotometer. The second well was the viability control with no test compound, and relative viabilities of exposed cells were compared with this well, recorded arbitrarily as 100% viable. The next five pairs of wells corresponded to the five different concentrations of test compound. The percentage viability of the cells at any particular concentration of test compound was determined by comparing the average OD_{550} reading for the two wells of cells at that dose with the OD_{550} reading for the second well using the following formula:

$$\frac{(OD_{550} \text{ for well N1} + OD_{550} \text{ for well N2})/2}{OD_{550} \text{ for well 2}} \times 100$$

Well N1 is the first well treated at a specific dose, and well N2 is the second well treated at the same specific dose (13). Unequal variance comparison t tests and multiple comparison with a control case (the Dunnett test) were used to determine significance (at the $\alpha = 0.05$ level) of activity at each dose (the corresponding fold

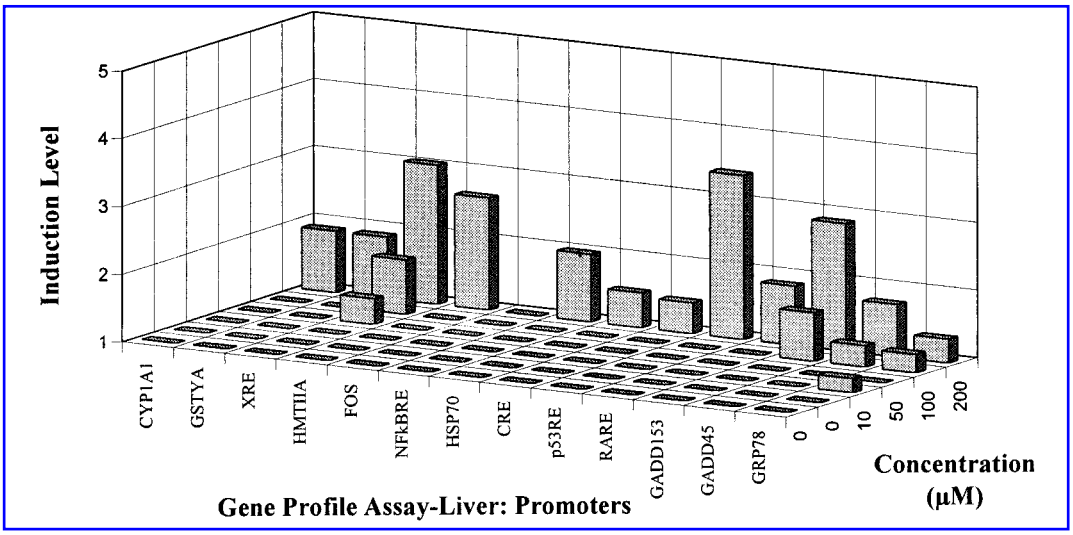

FIG. 1. Profile of CAT induction of the gene promoter/RE CAT response fusion constructs by 1400W. The numbers at the left (*y* axis) indicate fold induction compared with untreated control. The numbers at the lower right (*z* axis) indicate concentrations of 1400W used for treatment of HepG2 cells. Symbols along the bottom (*x* axis) are abbreviations for the respective promoters or RE listed in Table 1. The gene profile assays were performed in triplicate.

increase must be >1.0). The unequal variance *t* test was performed to test the null hypothesis for each concentration of a compound compared with the zero-dose control. All experiments were performed in triplicate to determine the mean fold induction ± SD.

RESULTS

HepG2 cells were treated for 6 h with 1400W, a selective NOS2 inhibitor, at concentrations of 0, 10, 50, 100, and 200 μM, and the fold inductions of 13 different promoter/RE CAT reporter constructs were determined. A CAT reporter plasmid without a gene promoter or RE was used as a control. The statistically significant fold inductions are shown in Fig. 1. The activation of the promoter/RE constructs was subjected to a statistical analysis using unequal comparison *t* tests and multiple comparison with a control case (the Dunnett test; see Materials and Methods for details of analysis). Twelve out of the 13 promoter/RE CAT reporter constructs were significantly activated by inhibiting endogenous NOS2 enzyme activity. The highest fold inductions occurred at the highest concentration of 1400W (200 μM), a concentration that had no effect on overall cell viability, determined using the MTT assay, as previously described (1). The mean fold inductions ±SD of all 13 promoter/RE CAT reporter constructs upon exposure to 200 μM 1400W are listed in Table 1. The p53 RE construct, which contains 10 copies of the p53 RE from the GADD45 gene (16), showed the highest fold induction of 3.42. Although there was a 1.71-fold induction of the *c-fos* promoter construct (Table 1), it was not statistically significant.

TABLE 1. INDUCTION OF PROMOTER/RE CAT REPORTER CONSTRUCTS BY 200 μM 1400W, A HIGHLY SELECTIVE NOS2 INHIBITOR

*Promoter/RE constructs**	*Mean fold induction ± SD*
CYP1A1	1.91 ± 0.37
GSTYA	1.91 ± 0.85
XRE	3.04 ± 0.44
HMTIIA	2.65 ± 1.14
FOS	1.71 ± 0.50
NFκBRE	2.00 ± 0.58
HSP70	1.51 ± 0.28
CRE	1.46 ± 0.08
p53RE	3.42 ± 0.87
RARE	1.87 ± 0.59
GADD153	2.89 ± 0.70
GADD45	1.78 ± 0.31
GRP78	1.35 ± 0.14

*For abbreviations, refer to Abbreviations list.

DISCUSSION

The results from this study, using the highly selective NOS2 inhibitor 1400W (see Table 1, Fig. 1), indicate that constitutive levels of NO (main product of NOS2 activity) protect cells against genotoxic (p53RE, GADD153, GADD45), oxidative (NFκBRE, HMTIIA), xenobiotic (XRE) and endoplasmic reticulum stresses (GRP78), protein malfolding (HSP70), or stresses that activate phase I (CYP1A1) and II (GSTYA) biotransformation enzymes. As most of the promoter/RE CAT reporter constructs used in the present study can be activated by oxidative stress (1, 13), a possible mechanism by which constitutive levels of NOS2 protect cells is through an overall reduction in oxidative stress. Endogenous oxidative stresses come from many sources, such as mitochondrial electron transport (9) and the activity of cyclooxygenases (COX), lipoxygenases (LOX), and members of the cytochrome P450 (CYP450) family of phase I biotransformation enzymes (Fig. 2). Although NO is by definition a free radical, its constitutive presence in cells paradoxically can reduce oxidative stress through four basic mechanisms (Fig. 2): (a) the scavenging of $O_s^{\cdot -}$ with an increase in $ONOO^-$ formation (14); (b) a decrease in mitochondrial respiration through the inhibition of cytochrome oxidase, resulting in an overall decrease in the level of reactive oxygen species (ROS) generated through the electron transport chain ; (c) interaction with hemoproteins that generate O_2^-, including phase I biotransformation enzymes (*e.g.*, CYP450) (10), LOX, and COX, and (d) a reduction in the free iron pool through the formation of nitrosyl–iron complexes, thereby reducing the production of hydroxyl radicals ($^{\cdot}OH$) through the Fenton reaction with a reduction in DNA strand breakage. The activation of promoters and REs reactive to DNA damage (*e.g.*, GADD153, GADD45, p53RE) through the inhibition of NOS2 activity in the present study could be the result of iron-mediated $^{\cdot}OH$ formation in the nucleus. An inhibition of CYP450 activity by NO results in a decrease in CYP transcriptional activity (10). Our results, showing an increase in CYP1A1 promoter activity when NOS2 is inhibited, are consistent with the observations of Stadler *et al.* (10). The increase in GSTYA promoter activity after NOS2 inhibition probably results from an increase in oxidative stress, including organic hydroperoxides and the subsequent activation of redox-sensitive transcription factors (*e.g.*, NF-κB, AP-1, AHR) (1, 3) (Fig. 2). Glutathione *S*-transferases (GSTs) are often associated with chemical detoxification (11). However, GSTs may also serve to protect

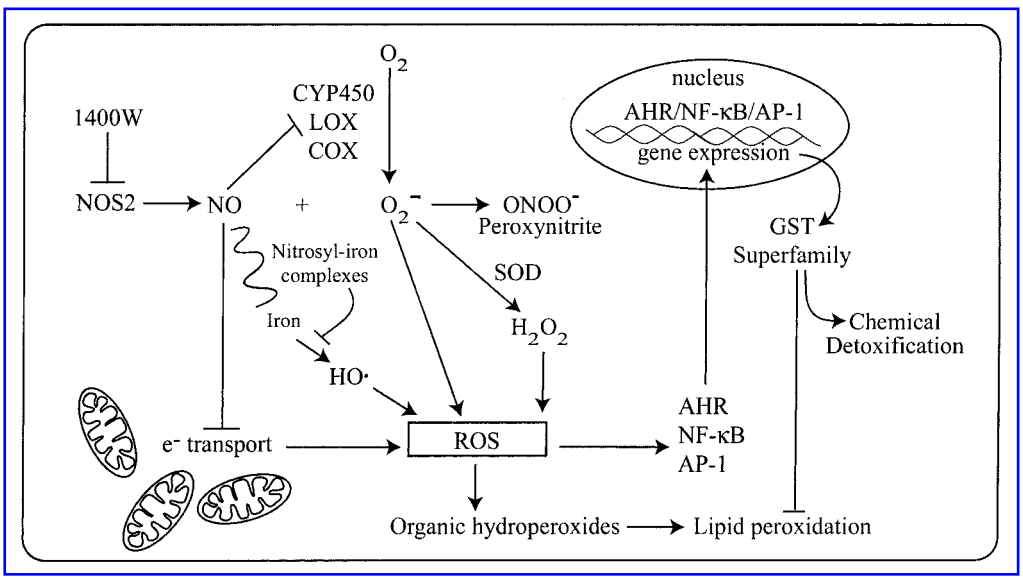

FIG. 2. Schematic showing the possible interactions between NO and other molecules in the cell that serve to reduce endogenous stresses. The cellular perturbations that result from an inhibition of NOS2 by 1400W is explained in the text.

against oxidative stress, including lipid peroxidation (12) (Fig. 2).

Although NOS2 is inducible and is often considered to be associated with inflammation and cytotoxicity when present at high levels, it clearly provides an important housekeeping function when present at lower, constitutive levels. Our results are consistent with the hypothesis that a critical balance of ROS and RNS is important for the redox regulation and control of cell signaling (3), transcription factor activation (6), and gene expression (8).

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ABBREVIATIONS

1400W, *N*-(3-(aminomethyl)benzyl)acetamidine; AHR, aryl hydrocarbon receptor; AP-1, activator protein-1; CAT, chloramphenicol acetyl transferase; COX, cyclooxygenase; CRE, cyclic AMP-response element; CYP1A1, cytochrome P450 1A1; CYP450, cytochrome P450; FOS, c-*fos*; GADD45, growth arrest and DNA damage protein (molecular mass, 45 kDa); GADD153, growth arrest and DNA damage protein (molecular mass, 153 kDa); GRP78, glucose regulated endoplasmic reticulum stress protein (molecular mass, 78 kDa); GST, glutathione *S*-transferase; GSTYa, glutathione *S*-transferase Ya subunit; HMTIIA, metallothionein IIA; HSP70, heat-shock protein 70; LOX, lipoxygenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor κB; NFκBRE, NF-κB response element; NO, nitric oxide; NOS, nitric oxide synthase; NOS2, inducible NOS; $O_2^{\cdot -}$, superoxide anion; OD, optical density, ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite; p53RE, p53 response element; RARE, retinoic acid response element; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; XRE, xenobiotic response element.

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Address all correspondence to:
Dr. Claire M. Payne
Department of Microbiology and Immunology
College of Medicine
University of Arizona
Tucson, AZ 85724

E-mail: cpayne@u.arizona.edu

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