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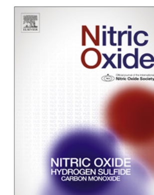

Nitric oxide sets off an antioxidant response in adrenal cells: Involvement of sGC and Nrf2 in HO-1 induction

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ABSTRACT

Induction of microsomal heme oxygenase 1 (HO-1) activity is considered a cytoprotective mechanism in different cell types. In adrenal cells, HO-1 induction by ACTH exerts a modulatory effect on steroid production as well. As nitric oxide (NO) has been also regarded as an autocrine/paracrine modulator of adrenal steroidogenesis we sought to study the effects of NO on the induction of HO-1 and the mechanism involved. We hereby analyzed the time and dose-dependent effect of a NO-donor (DETA/NO) on HO-1 induction in a murine adrenocortical cell line. We showed that this effect is mainly exerted at a transcriptional level as it is inhibited by actinomycin D and HO-1 mRNA degradation rates were not affected by DETA/NO treatment. HO-1 induction by NO does not appear to involve the generation of oxidative stress as it was not affected by antioxidant treatment. We also demonstrated that NO-treatment results in the nuclear translocation of the nuclear factor-erythroid 2-related factor (Nrf2), an effect that is attenuated by transfecting the cells with a dominant negative isoform of Nrf2. We finally show that the effects of the NO-donor are reproduced by a permeable analog of cGMP and that a soluble guanylate cyclase specific inhibitor blocked both the induction of HO-1 by NO and the nuclear translocation of Nrf2.

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Introduction

Heme oxygenases (HO) catalyze the rate controlling step on heme degradation [1] and its activity has been classically involved in the catabolism of heme proteins, mainly hemoglobin but also cytochromes, nitric oxide synthases, guanylate cyclase and cyclooxygenases. In the presence of NADPH-cytochrome P450 reductase and NADPH, heme catabolism by HO generates carbon monoxide (CO), free iron, and biliverdin that is subsequently transformed to bilirubin by the activity of biliverdin reductase [2]. Unlike HO-2, that is mainly constitutively expressed, HO-1 is induced by a wide variety of cellular stressors including cytokines, endotoxins, heavy metals and heme [3]. Based on its antioxidant and anti-inflammatory effects this activity has been considered a cellular defensive mechanism [4]. In addition to this, we have previously demonstrated that HO-1 activity modulates steroid production in adrenocortical cells in an autocrine/paracrine way [5,6].

In a previous study we showed that adrenal HO-1 induction triggered by LPS involved the stimulation of nitric oxide (NO) generation [6]. In this sense, the induction of HO-1 has been associated with cytoprotective effects triggered by NO in different cell types [7–9]. These effects may involve the activation of the NF-E2 related factor 2 (Nrf2) transcription factor and the induction of antioxidant and cytoprotective genes such as HO-1, glutathione cysteine ligase (GCL) and NADPH quinone oxydoreductase 1 (NQO), among others [7].

Activation of the transcription factor Nrf2 occurs as a consequence of an unbalance in the redox state of the cell. In basal conditions, Nrf2 is maintained in the cytoplasm by interacting with Klech-like ECH-associated protein 1 (Keap1) and is targeted to proteosomal degradation by ubiquitin modification [10]. Previous studies suggested that various stimuli could induce the dissociation of Nrf2 from Keap1 in the cytosol and/or promote its nuclear translocation. In particular, different mechanisms could be involved in Nrf2 activation under NO stimulation. Among them NO-mediated thiol modification of specific cysteine residues in Keap1 (S-nitrosylation) [7] and serine phosphorylation of Nrf2 by PKC or other kinases have been suggested [9,11,12].

Stimulation of soluble guanylate cyclase (sGC) by endogenously generated NO has been demonstrated in different cells types including adrenal cells [13,14]. Its product, cGMP could in turn, activate several intracellular proteins including cGMP-dependent protein kinase (PKG), cGMP-gated channels, cGMP-regulated phosphodiesterases (PDEs) [15] and, depending on its levels, even cAMP-dependent protein kinase (PKA) [16]. Recently, an 8-nitro-cGMP caused modification of Keap1 (*S*-guanylation) that results in Nrf2 release, translocation to the nucleus and consequent

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activation of ARE containing promoters has been also detected in NO-treated cells [7,17].

In the present study we demonstrate that treatment of murine adrenocortical cells with a NO-donor result in the induction of HO-1. We also show that this effect does not appear to entail the generation of ROS and supply evidence for the involvement of a cGMP signal transduction pathway and transcription factors like Nrf2 in this mechanism.

Materials and methods

Chemicals

8Br-cGMP and diethylenetriamine/nitric oxide adduct (DETA/NO) were purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies raised against HO-1, were acquired from StressGen Biotechnologies Corp. (Victoria, Canada). Those against Actin and Nrf2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); peroxidase-conjugated goat anti rabbit-IgG was purchased from Bio-Rad (Hercules, CA, USA). Fetal calf serum, penicillin, streptomycin, MMVL reverse transcriptase, DNAse I and Taq polymerase were from Invitrogen (Life Technologies, Buenos Aires, Argentina). All other chemicals were of the highest quality available.

Cell culture and treatments

Y1 cells (ATCC, CCL-79) are an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor cell line isolated by Yasumura et al. [18]. Cells were grown in monolayers in Ham's F10 medium containing heat-inactivated fetal bovine (2.5%) and horse (12.5%) serum, 200 U/ml penicillin G and 270 μg/ml streptomycin sulfate, in a humidified atmosphere of 5% CO2 in air at 37 °C [19]. Diethylenetriamine/nitric oxide adduct (DETA/NO) was used as a NO-donor due to its long half-life at 37 °C, between 7 and 8 h in culture media [20]. As DETA/NO releases NO with a first order kinetics Cortese-Krott et al. [21] calculated that at concentrations similar to those used in this paper (1 mM) this donor generates 3.3 nM/min of NO. These levels are within the range of NO concentrations produced endogenously in different experimental conditions [22,23].

No differences in cell viability, assessed by the trypan blue dye exclusion test, resulted from any of the treatments.

Determination of intracellular ROS generation

At the end of the treatments the cells were washed twice in 1× PBS and further incubated in 1× PBS containing 10 μmol/l of the fluorophore 2′,7′-dichlorodihydrofluorescein diacetate for 1 h at 37 °C. After that period, cells were rapidly washed twice with 1× PBS. Fluorescence (excitation 485 nm, emission 535 nm) was determined in culture wells using Multi-mode microplate reader, FLUOstar Omega (BMG Labtech, Germany). ROS levels are expressed as Fluorescence Relative Units (FRU) normalized to total protein content.

Measurement of reduced glutathione (GSH)

Cells were homogenized in 0.2 ml of 50 mmol/l pH 7.4 phosphate buffer containing 1% Triton-X. Protein and cellular debris were precipitated by the addition of 50 μl of 50% trichloroacetic acid and 1 mmol/l EDTA and dithionitrobenzene (0.25 mg/ml in 0.5 mol/l phosphate buffer) to the supernatant. The mixtures were incubated at 37 °C for 15 min. Absorbance was measured at 412 nm in a JENWAY 6405 spectrophotometer (Bibby Scientific Limited, Staffordshire, UK). GSH levels were determined as nanomoles of GSH per milligram of protein and expressed as percentage (%) of control. A standard GSH solution was used for the calibration curve.

Measurement of lipid peroxides

Lipoperoxide levels, as thiobarbituric acid-reactive substances (TBARS), were determined as previously described [24]. Briefly, cells were suspended in 1 ml of 1× PBS with 0.075% SDS. Then, 0.5 ml of 10 mg/ml 2-thiobarbituric acid in 10% sodium acetate buffer pH 3.5 was added. Mixtures were heated in a boiling water bath at 100 °C for 60 min and fluorescence was measured (515 nm excitation and 555 nm emission) using Multi-mode microplate reader, FLUOstar Omega (BMG Labtech, Germany). Values were expressed as μmol/l of nanomoles of malondialdehyde (MDA) per milligram of protein using MDA as a standard prepared from 1,1,3,3-tetramethoxypropane.

RNA isolation and RT real time PCR

Total RNA was extracted from Y1 cells using TriZol® reagent (Invitrogen). RNA (2 μg) was pre-treated with RNAse-free DNAse I, heated at 70 °C for 10 min, placed on ice for 1 min, and then incubated with a mixture containing 0.5 mmol/l dNTPs mix, 25 ng/μl random primers, 1× first-strand buffer, 25 units of RNAse inhibitor, 200 units of MMLV reverse transcriptase and water in a final volume of 25 μl, for 1 h at 42 °C. The reaction was stopped by heating at 90 °C for 5 min and the mixture was brought to 100 μl with diethylpyrocarbonate-treated water and stored at −70 °C. In selected tubes reverse transcriptase was omitted as a genomic contamination control. Amplifications by real-time PCR were carried out in a Rotor-GeneTM 6000© Corbett Life Science Real Time Thermal Cycler (Corbett Research, Sidney, Australia) and were performed using 0.5 μl cDNA in a final volume of 25 μl in the following reaction mixture: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM of each specific oligonucleotide primer, 0.625 U GoTaq® polymerase and 0.96 × EvaGreen. The sequence for the oligonucleotide primers were as follows: for HO-1: forward: 5′-CAACAGTGGCAGTGGGAATTT-3′ reverse: 5′-CCAGGCAAGATTCTCCCTTAC-3′; for GLCL: forward: 5′-AGTGGCC-CAGAAGCGAGATGC-3′ reverse: 5′-ACCACAGCGTTGCCGCCTTT-3′; for GLCM: forward: 5′-GCATCCCCGAGGTGCGAGTG-3′ reverse: 5′-AGTGGTTCCTGTGAGCGCGC-3′; for NQO1: forward: 5′-CATGGC GGCGAGAAGAGCCC-3′ reverse: 5′-CAGCAGCCTCCTTCATGGCGT-3′ and for 18SrRNA: forward: 5′-ACGGAAGGGCACCACCAGGA-3′ reverse: 5′-CACCACCACCCACGGAATCG-3′. The expression levels of 18S rRNA were used as an internal control. We previously determined that the stability of the 18S rRNA was not affected by DETA/NO treatment in our experimental conditions.

The cycling conditions were as follows: denaturation at 95 °C for 3 min, 40 cycles of 20 s at 95 °C and 20 s at 52 °C and finally 30 s at 72 °C. Real-time PCR results were analyzed according the $2^{-\Delta\Delta Ct}$ method [25] using Rotor Gene 6000 Series Software (version 1.7 Build 40) with 18S expression as internal control.

Transfection of Y1 cells – luciferase assay

Transient transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA). Briefly, one day before transfection, 10^5 cells/well were seeded in 24-well plates. 0.8 μg of total plasmid DNA (a mixture of Nrf2 reporter plasmid and pcDNA3-βgal or DN-Nrf2 or pcDNA3-βgal) and 2 μl of lipofectamine were mixed in optiMEM (Invitrogen, Buenos Aires, Argentina) and added to cells. Three hours later, complete medium was added and the cells incubated for 24 h before initiating the experiments.

Nrf2 reporter assays were carried out using a 96-well luminometer with the Steady-Glo® Luciferase Assay System (Promega, USA). Relative luciferase activity was normalized to the internal control β-galactosidase activity.

Preparation of nuclear/cytoplasm protein

After treatments, cells were washed with 1× PBS and scrapped in ice-cold hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and 0.1% NP40). After incubation in an ice bath for 10 min, cells were centrifuged at maximum speed in a tabletop centrifuge for 30 s. Supernatants were used to extract proteins from the cytoplasmic fraction. After being washed twice in hypotonic buffer, proteins from the nuclear fraction were extracted.

Immunoblot analysis

Y1 cells were washed twice in 1× PBS and lysed in 20 mmol/l Tris–HCl pH 7.4, 250 mmol/l NaCl, 1% Triton X-100 and 1× of protease inhibitor cocktail. Samples were boiled for 5 min in loading buffer with 0.1 mol/l dithiothreitol and electrophoresed on 10% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidenefluoride (PVDF) membranes for 1 h at 15 V in a Bio-Rad Trans-Blot SD system with 25 mmol/l Tris–HCl pH 9.2, 192 mmol/l glycine and 20% methanol. PVDF membranes were blocked in TBS-Tween (50 mmol/l Tris–HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20) with 5% skimmed milk for 60 min at room temperature and then incubated overnight with the following antisera: HO-1 (1:5000), Nrf2 (1:500) or β-actin (1:10,000) at 4 °C. Membranes were washed with TBS-Tween and then incubated for 1 h with a 1:5000 dilution of a goat anti-rabbit immunoglobulin G antibody–horseradish peroxidase conjugate. Membranes were washed and the bands were visualized by chemiluminescence (Enhanced chemiluminescence reagent, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) in an ImageQuant Imaging System (GE Healthcare) and quantified with the Alpha Ease Fluorchem software (V. 4.1.0, Alpha Innotech Corporation).

Immunocytochemistry

Y1 cells grown on poly-L-lysine treated glass cover slips were washed once with 1× PBS and then fixed at room temperature with 4% (w/v) paraformaldehyde in 1× PBS for 10 min. Cover slips were then rinsed in 1× PBS and incubated with blocking solution (2% fetal clef serum, 0.3% Triton-X100, 1× PBS) for 1 h at RT and then with rabbit polyclonal antibodies raised against Nrf2 (1:500) or with vehicle (control of specificity) in a humidified chamber for 24 h at 4 °C. Alexa 488-conjugated goat anti-rabbit IgG (© 2012 Life Technologies Corporation, Argentina) was used as a secondary antibody. After immunostaining, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and fields were examined using a fluorescence microscope (BX50; Olympus, Tokyo, Japan) connected to a video camera (3CCD; Sony, Tokyo, Japan) and attached to a computer running image analysis software (Image-Pro Plus; Media Cybernetics Inc., Bethesda, MD).

Statistical analysis

All data is expressed as means ± SEM of n replicates. Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test or by Student's *t* test as considered necessary, using

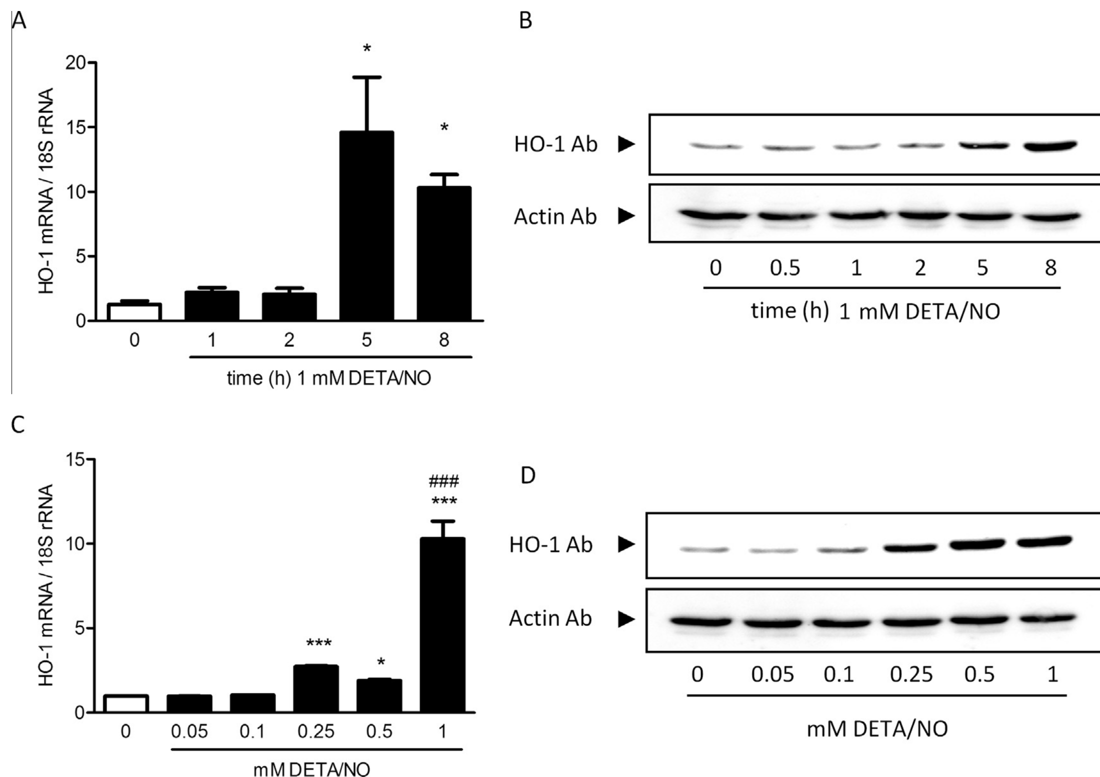

Fig. 1. Effect of DETA/NO on HO-1 expression in Y1 cells. HO-1 mRNA and protein levels were measured in Y1 cells treated with DETA/NO for different times and doses. mRNA levels of HO-1 and 18S rRNA were determined by real-time qRT-PCR performed upon RNA extracted from lysates of cells exposed to 1 mM DETA/NO for different time intervals (A) or lysates of cells exposed to increasing concentrations of DETA/NO for 5 h (C). Protein levels of HO-1 were analyzed by immunoblotting using anti-HO-1 antibody. Actin protein levels were tested using anti-actin antibody as an internal control. Cells were exposed to 1 mM DETA/NO for different intervals of times (B) or to increasing concentrations of DETA/NO for 5 h (D). Representative immunoblots out of 3 independent experiments are shown. Data are presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$, $^{*}p < 0.05$ *vs.* 0 by Tukey's test.

GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego CA). A p value <0.05 was considered significant.

Results

DETA/NO increases HO-1 mRNA and protein levels in Y1 cells

As a first attempt to study the regulatory mechanisms that control HO-1 expression triggered by nitric oxide we decided to test our cell system of choice. Incubation of murine adrenocortical Y1 cells in the presence of a NO-donor resulted in increased levels of HO-1 mRNA and proteins. Treatment of Y1 cells with 1 mM DETA/NO, significantly induced HO-1 mRNA after 5 h of incubation (Fig. 1A). Minimal DETA/NO concentration required to detect a significant induction of mRNA levels was 0.25 mM (Fig. 1C). Protein expression patterns correlated with mRNA expression levels (Fig. 1B and D). In contrast, incubation of Y1 cells in the presence of the exhausted NO-donor had no effect (data not shown).

HO-1 induction by DETA/NO depends on gene transcription and does not involve mRNA stabilization

Increased levels of mRNA usually result from a balance between transcription and mRNA degradation. To test the effect of DETA/NO upon mRNA expression, Y1 cells were incubated with actinomycin D in order to completely prevent RNA synthesis, and its effect at both mRNA and protein levels was measured. As depicted, addition of actinomycin D abolished any increases in HO-1 expression nduced by DETA/NO (Fig. 2A and B). HO-1 mRNA stability was assessed following mRNA decay over a time course after the addition of actinomycin D to Y1 cells previously incubated with DETA/NO for 5 h. Data was plotted and tendency curves were drawn showing no significant differences in the degradation rates of HO-1 mRNA in cells treated with DETA/NO as compared to control cells (Fig. 2C). Altogether these experiments show that the effects of DETA/NO upon HO-1 mRNA expression are exerted mainly on transcription and not on mRNA stabilization.

Induction of HO-1 by NO does not involve generation of oxidative stress

The generation of oxidative stress has proven to play an important role in the induction of HO-1. In order to test a link between the roles of oxidative stress and DETA/NO in HO-1 induction we tried alternative approaches. On one hand, DETA/NO treatment of Y1 cells resulted in a significant decrease in reduced GSH, ROS and lipid peroxide levels (Fig. 3A–C). In addition, treatment of Y1 cells with antioxidants (α-tocopherol, VE; bilirubin, Bi or butylated hydroxytoluene, BHT) did not prevent the increase in HO-1 protein levels induced by the NO-donor (Fig. 3D).

NO induces Nrf2 dependent phase II enzymes

Gene expression activation induced by DETA/NO is not restricted to HO-1. As our qRT-PCR analysis demonstrate, DETA/NO also triggered the up-regulation of detoxifying and antioxidant

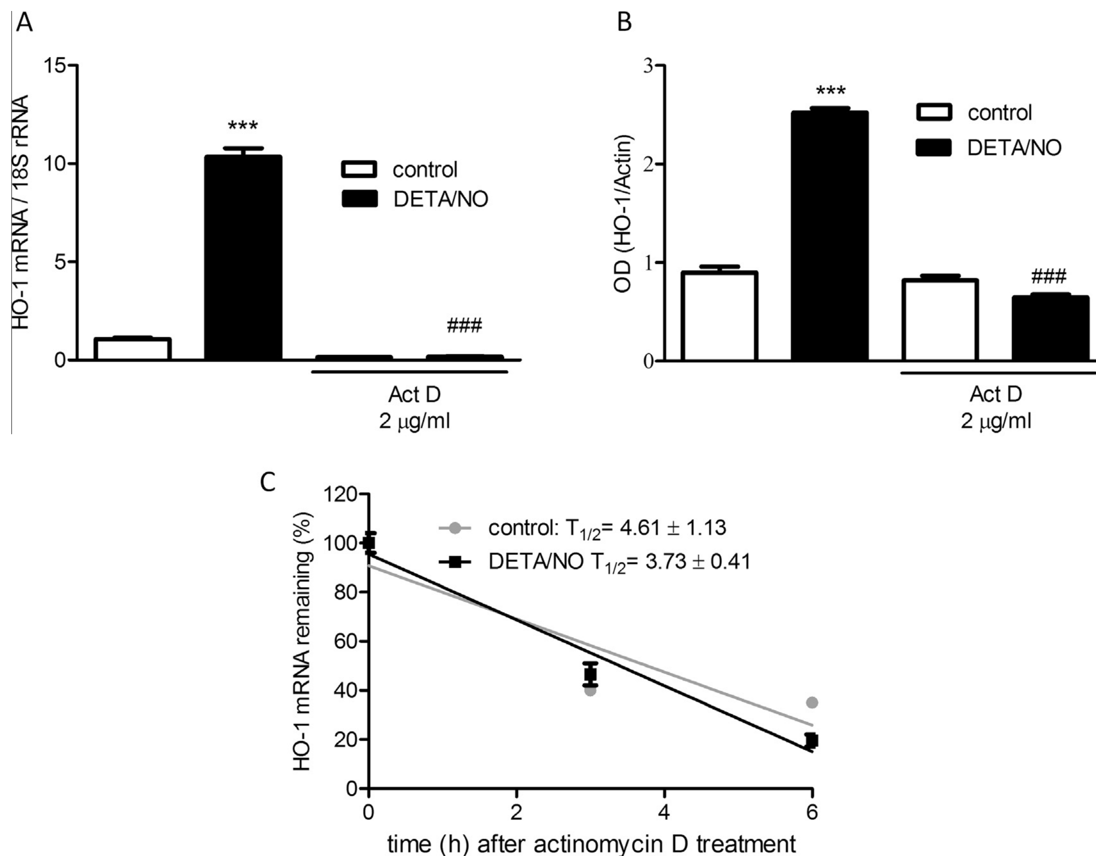

Fig. 2. Effect of actinomycin D on HO-1 induction by DETA/NO. Y1 cells were incubated for 30 min with or without 2 μg/ml actinomycin D (Act D). The incubation was continued for 5 h in the presence or absence of 1 mM DETA/NO. (A) mRNA levels of HO-1 and 18S rRNA were measured by real-time qRT-PCR. (B) Quantification of three independent immunoblots for HO-1 and Actin protein expression, average data is shown. (C) Cells were incubated in the absence (grey) or in the presence (black) of 1 mM DETA/NO for 5 h. After that, incubation was continued with actinomycin D (2 μg/ml) for different times in order to analyze mRNA degradation. Total RNA was isolated at the times indicated, and levels of HO-1 mRNA were measured by real-time qRT-PCR. Data in A and B are presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$ *vs.* control, $^{###}p < 0.001$ *vs.* DETA/NO by Tukey's test. Data in C are presented by a tendency plot of normalized individual points from three independent experiments. Half-life calculation is depicted as an insert. No significant differences were observed by ANOVA ($p = 0.22$).

genes. Among them, substantial increases in glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase regulatory subunit (GCLM) and NAD(P)H Dehydrogenase Quinone 1 (NQO1) gene transcripts were observed after 5 h of exposure to 1 mM DETA/NO (Fig. 4). Interestingly, all these genes, and HO-1, have been previously described as targets for the activation by the transcription factor Nrf2, suggesting a relationship for DETA/NO and Nrf2 in the mechanism of HO-1 induction.

Activation of Nrf2 is involved in HO-1 induction by DETA/NO

The transcription factor Nrf2 is continuously being degraded in the cytosol in resting cells. Our data shows that incubations in the presence of DETA/NO resulted in higher protein levels of Nrf2 in whole cell extracts (Fig. 5A). Cell lysate fractionation show that while treatment with DETA/NO stabilizes cytosolic protein levels, a significant increase is observed in the nuclear fraction of Y1 cells (Fig. 5B). In addition, an increase in the Nrf2 signal in the nucleus of these cells was also observed by immunofluorescence analysis (Fig. 5C). All these data is consistent with the activation of Nrf2 in Y1 cells upon treatment with the NO donor.

In order to test if the Nrf2 activation has any effects on HO-1 expression we employed a dominant negative approach. Transfection of Y1 cells with a plasmid that expresses a dominant negative isoform of Nrf2 significantly reduced the induction of HO-1 by DETA/NO to 25% (Fig. 6).

8Br-cGMP induces HO-1 expression by activating Nrf2

A classical target of NO activation widely acknowledged in the literature is the soluble guanylate cyclase (sCG), enzyme that catalyzes the conversion of GTP into cGMP. Therefore, the involvement of the cGMP pathway on the activation of Nrf2 was analyzed. In this sense, we previously demonstrated NO-dependent cGMP production in adrenal cells [26]. These observations, added to the results from Fig. 5, support a potential role for cGMP in Nrf2 activation and ultimately HO-1 increased expression.

Present results showed that treatment of Y1 cells with a permeable analog of cGMP (8Br-cGMP) induces an increase in both mRNA and protein levels of HO-1 (Fig. 7A–B). In addition, incubation of Y1 cells with 8Br-cGMP resulted in a significant increase in the activity of a luciferase reporter plasmid for Nrf2 activity (Fig. 7C) and in the nuclear accumulation of Nrf2 as determined by immunocytochemical analysis (Fig. 7D).

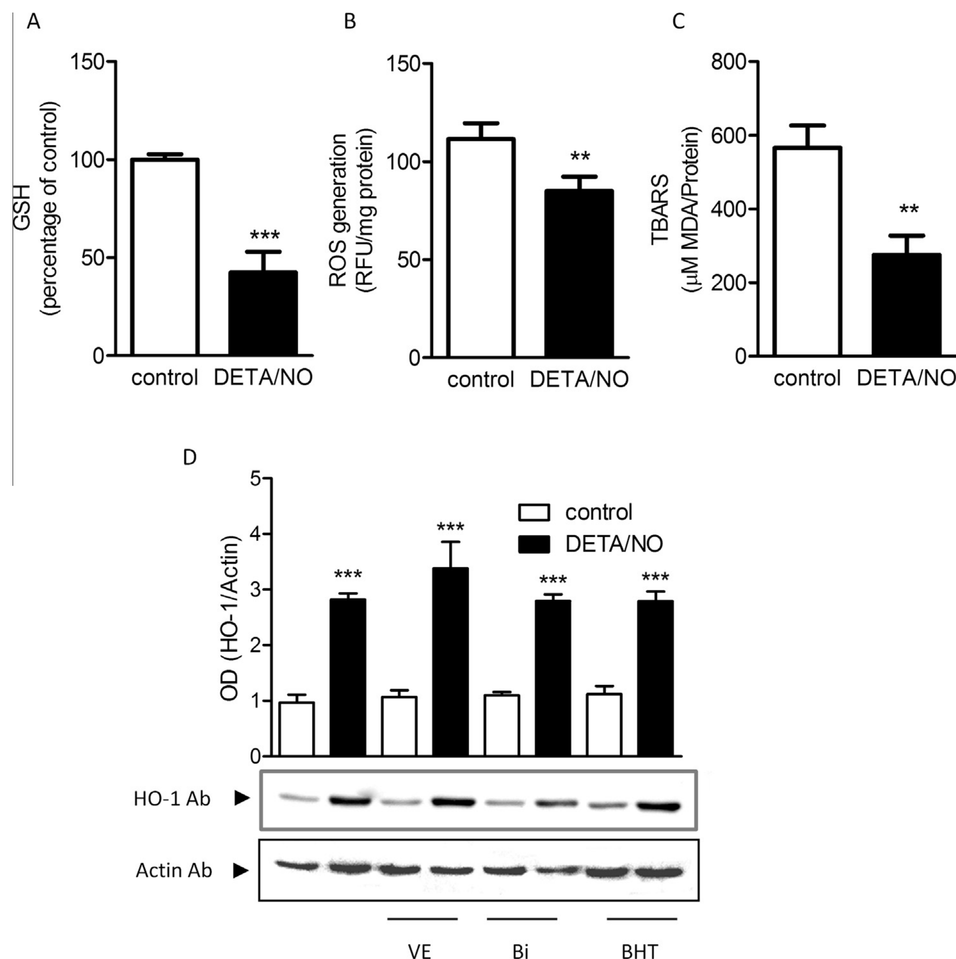

Fig. 3. Role of oxidative stress on HO-1 induction by DETA/NO. Y1 cells were treated during 5 h with 1 mM DETA/NO as described in Materials and methods. Reduced glutathione concentration (B), Reactive oxygen species (ROS) (B) and lipoperoxide levels (TBARS) (C) were tested. Data are presented as mean ± SEM, $N = 4$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control by Student's *t* test. Y1 cells were pre-incubated with 100 μM vitamin E (VE), 1 μM Bilirrubin (Bi) or 25 μM Butylated hydroxytoluene (BHT) and then treated with DETA/NO for 5 h. Protein levels of HO-1 and Actin were analyzed by immunoblotting using anti-HO-1 antibody and anti-Actin antibody as a loading control (D). Data is depicted as columns (D, upper panel) that result from the quantification of 3 independent experiments and a representative western blot (D, lower panel). Data is presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$ *vs.* control by Tukey's test.

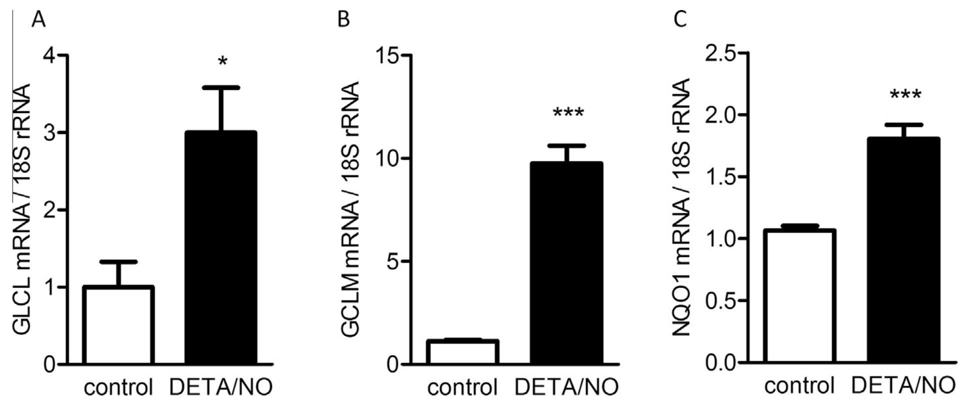

Fig. 4. DETA/NO induces expression of antioxidant phase II enzyme genes in Y1 cells. After 5 h of incubation with 1 mM DETA/NO mRNA levels of glutamate-cysteine ligase catalytic subunit, GCLC (A), glutamate-cysteine ligase regulatory subunit, GCLM (B) and NAD(P)H Dehydrogenase Quinone 1, NQO1 (C) were analyzed by real time qRT-PCR. Data are presented as mean ± SEM, $N = 3$, $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* control by Student's *t* test.

Fig. 5. DETA/NO activates Nrf2 pathway in Y1 cells. Y1 cells were incubated for 0, 1, 3, 5 h with 1 mM DETA/NO. Nrf2 protein levels were determined in complete cell lysates by immunoblot using specific antibodies (A). Cytoplasmatic/nuclear cell fractions were prepared and analyzed for Nrf2 subcellular distribution (B). Actin or Lamin B protein levels were tested as internal controls. A representative western blot is shown for each cell fraction. (A–C) Quantification of different experiments is shown above each picture. Data is presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$ and $^{*}p < 0.05$ *vs.* control by Tukey's test. (C) Immunofluorescence staining was performed using the same Nrf2 antibody in Y1 cells incubated for 3 h in the absence or presence of 1 mM DETA/NO. Nuclei are denoted by staining with 4′,6-diamidino-2-phenylindole (DAPI). Representative fields are shown for each case. Magnification 400×.

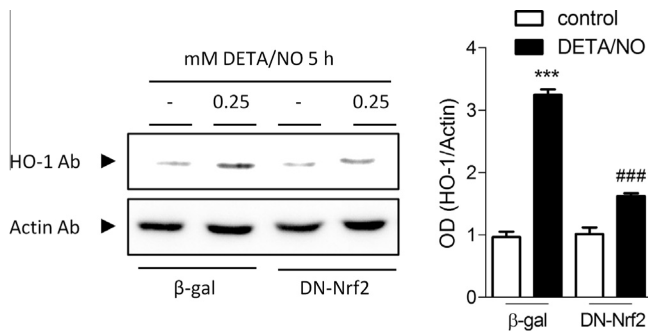

Fig. 6. HO-1 induction by DETA/NO involves Nrf2. Y1 cells were transfected with expression plasmids containing β-galactosidase as an internal control of transfection efficiency (β-gal) or a dominant negative construct for Nrf2 (DN-Nrf2). 24 h later, 0.25 mM DETA/NO was added and cells were incubated for an extra 5 h. Protein levels of HO-1 and Actin were analyzed by immunoblotting using anti-HO-1 antibody and anti-actin antibody as an internal control. Representative western blots are shown (left panel) and a quantification of 3 independent experiments is presented (right panel). Data is presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$ *vs.* control (β-gal), $^{\#\#\#}p < 0.001$ *vs.* DETA/NO (β-gal) by Tukey's test.

Differential effect of sGC and protein kinases inhibitors upon Nrf2 and HO-1 activation

In order to test the involvement of the cGMP pathway on Nrf2 and HO-1 expression we decided to use a pharmacological inhibitor of sGC and, in addition, different protein kinases inhibitors. Treatment of Y1 cells with a specific inhibitor for sGC (ODQ) prevented the increase on HO-1 protein levels triggered by DETA/NO (Fig. 8A). On this regard, when we measured nuclear levels of Nrf2 using this inhibitor, ODQ prevented the Nrf2 translocation (Fig. 8B). In another set of experiments, specific inhibitors for PKC, PKG, MAPKs and PI3K were analyzed. Results led us to discard a significant participation of these kinases in the mechanism involved in HO-1 induction by DETA/NO as only a slight inhibitory effect on HO-1 induction was obtained with the PKC and the PKG inhibitors (data not shown).

Discussion

Signals from outside the cell or generated within them are transduced by intricate molecular mechanisms that affect metabolism and other biochemical processes. Among them, changes in the levels of second messengers and activation of preexisting proteins ultimately end up in arranging a signaling mechanism that impinging upon discrete promoter elements defines a new pattern of gene expression. Identifying signaling components and its target genes in healthy cells contributes to assemble a picture of the response mechanism that, if deregulated, contributes to pathology.

In this study, we show that treatment of Y1 adrenal cells with a NO-donor to mimic endogenous NO production results in the induction of the HO-1 gene. Our evaluation of signal transduction pathways triggered by NO in Y1 cells demonstrates the involvement of sGC activity and the activation of the transcription factor Nrf2, which has been shown to play a major role at the HO-1 promoter [10]. To our knowledge, this is the first study that links those molecular components to the mechanism of HO-1 induction by NO in adrenal cells.

Nitric oxide exerts dual effects on cell physiology. While high concentrations of NO will eventually generate cytotoxicity, relatively lower levels of NO, as those used in our study, promote cell survival and cytoprotection [27]. Accordingly, our results showed that NO treatment of murine adrenocortical cells induces a rapid increase in mRNA and protein levels of HO-1 and that this effect was completely prevented by actinomycin D treatment. Similar results were obtained in HeLa cells [28], and in rat glioma cells among other cell types [8].

Current knowledge of the biology of mRNA considers that promoter activity and mRNA stability contribute equivalently to regulate gene expression. In this regard, an increase in HO-1 mRNA levels by NO has been attributed to decreased turnover rates [29]. Léautaud and Demple reported the induction of HO-1 in NIH3T3 cells through the stabilization of its mRNA by inhibition of its deadenylation [30]. Alternatively the participation of HuR riboprotein in this mechanism has been postulated in human and mouse fibroblasts. Analysis of nitric oxide-stabilized mRNAs in human fibroblasts reveals HuR-dependent HO-1upregulation [31]. As the stability of the HO-1 mRNA was not affected by DETA/NO treatment, in our experimental conditions, we conclude that in murine adrenocortical Y1 cells NO induces HO-1 gene expression acting mainly through transcriptional activation.

Our results obtained using a permeable analog of cGMP and a specific inhibitor of soluble guanylate cyclase support the notion that HO-1 induction by NO treatment of adrenal cells also involves the stimulation of sGC and the generation of cGMP. Similar results were obtained by Kurauchi et al. in dopaminergic neurons [32], Polte et al. in bovine lung endothelial cells [33] and Immenschuh et al. in hepatocytes [34].

HO-1 induction has been associated with higher rates of ROS generation. We analyzed oxidative stress in NO-treated Y1 cells. Notably, our results showed that, in parallel to HO-1 induction, both ROS generation and lipoperoxide levels were significantly decreased in Y1 cells treated with DETA/NO. These results could be explained by an increase in the activity of SOD and glutathione peroxidase activities in NO-treated adrenal cells as reported by Dovinová et al. in rat myocardium [35]. In a similar way, attenuation of lipoperoxidation by NO generators has been observed in endothelial cells [36], and in a renal injury model [37]. However, many studies report increases in oxidative stress parameters by NO treatment in several cellular systems suggesting that this effect could depends on molecular parameters of the cell type under study [38,39]. This point deserves deeper attention and is currently the subject of further examination.

In spite of the high levels of glutathione detected in the adrenal cortex [40] and the increase in the expression of the key regulatory enzyme in glutathione synthesis (GLCM and GLCL), we observed a decrease in GSH levels in Y1 cells treated with a NO-donor. This could be the result of higher GSH oxidation rates and/or the generation of S-nitrosoglutathione (GSNO) as was reported by Meloche et al. in rat liver cells treated with a NO donor [41]. In addition, SOD induction by GSNO treatment has been also demonstrated in rat glomerular mesangial cells and kidneys of endotoxemic rats [42]. Based on our results we suggest that NO treatment caused a redox imbalance that favored the removal of reactive oxygen species through a variety of mechanisms. However, antioxidant treatment did not prevent the increase in HO-1 protein levels induced by NO suggesting that, in our model, ROS generation appears not to be responsible for HO-1 induction triggered by DETA/NO.

The involvement of the transcription factor Nrf2 in the induction of phase II antioxidant enzyme genes including glutathione S-transferase (GST), NADP (H): quinone oxidoreductase (NQO1), gamma-glutamylcysteine synthetase (γGCS) and HO-1 has been reported elsewhere [10,27,43,44]. Our results demonstrate that NO release in Y1 cells raised mRNA levels of genes like NQO1, GLML and GLCL. It also increased Nrf2 protein levels, promoting its relocalization to the nucleus. The role of Nrf2 activation in HO-1 induction has already been documented in different cellular settings [45–48]. Involvement of Nrf2 in NO-dependent induction of HO-1 was also confirmed in Y1 cells by using a dominant negative isoform of Nrf2. In sum, present results depict a NO-dependent

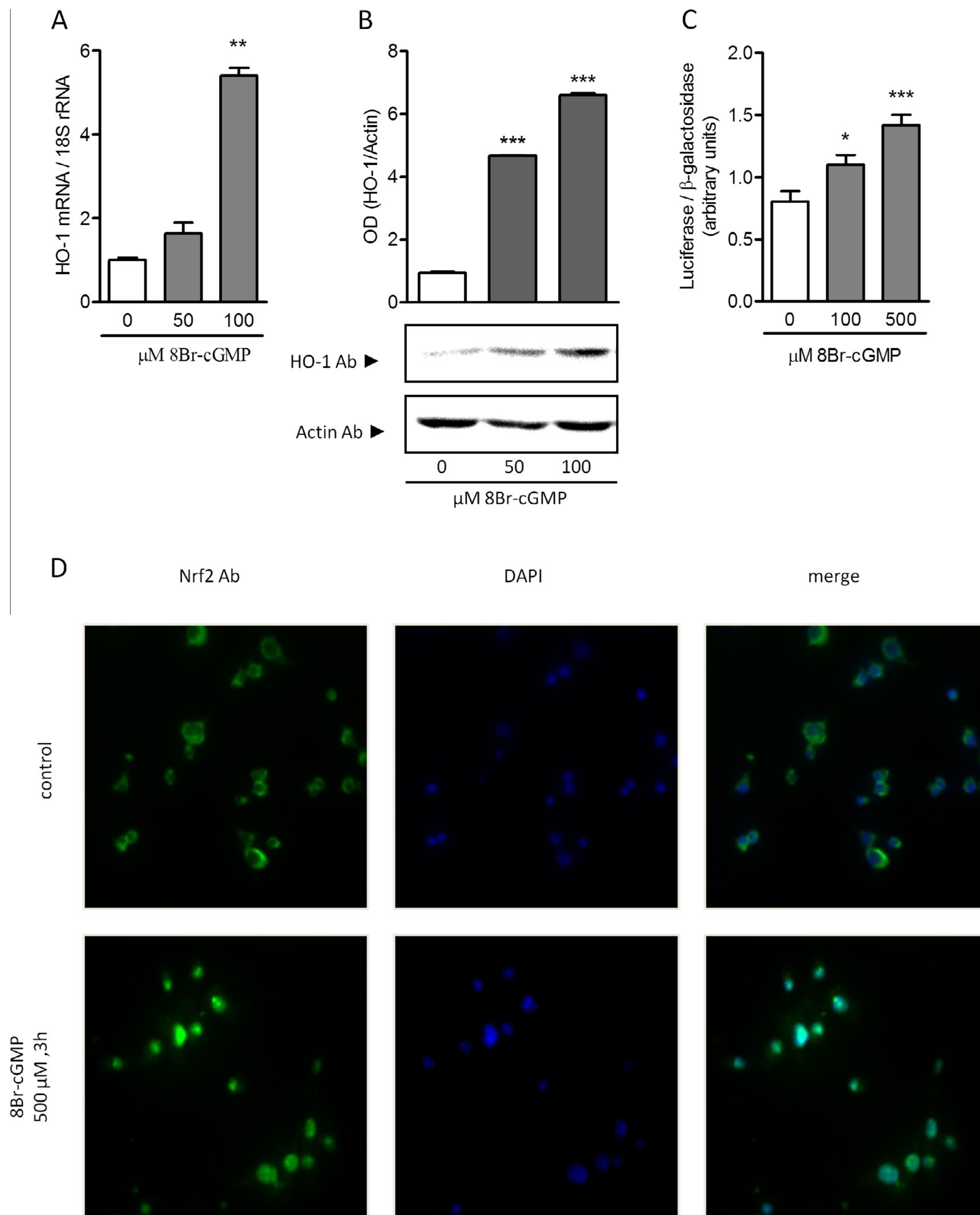

Fig. 7. 8Br-cGMP induces HO-1 expression and activates Nrf2. Y1 cells were incubated with 0, 50 and 100 μM 8Br-cGMP for 5 h. (A) mRNA levels of HO-1 and 18S rRNA were analyzed by qRT-PCR. (B) Protein levels of HO-1 and Actin were detected by immunoblotting using anti-HO-1 antibody and anti-Actin antibody as an internal control. Quantification of 3 independent experiments is shown above the immunoblot. Data is presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$ *vs.* control by Tukey's test. (C) Y1 Cells were transfected with a reporter plasmid for Nrf2 activity. 24 h later, 100 or 500 μM 8Br-cGMPC was added and cells were incubated for another 24 h. Then, luciferase activity was assessed as described in Materials and methods. Data are presented as mean ± SEM, $N = 3$, $^{**}p < 0.01$ by Tukey's test. (D) Immunostaining for Nrf2 in Y1 cells incubated for 3 h in the absence or presence of 500 μM 8Br-cGMP. Representative fields are shown for each case. Magnification 400×.

mechanism for the activation of Nrf2 and its consequent involvement in HO-1 induction in adrenal cells.

Activation of the transcription factor Nrf2 has been associated with the generation of oxidative stress [24,49]. As ROS generation has been ruled out as responsible for the Nrf2-dependent induction of HO-1, alternative mechanisms for the activation of Nrf2 by NO in adrenal cells could be suggested: (1) Critical cysteine residues of the repressor protein Keap1 could be subjected to S-nitrosylation. This modification may inhibit the degradation of Nrf2, favoring its accumulation in the cytoplasm and its subsequent mobilization to the nucleus [45]. In concordance, this mechanism has been characterized in the induction of HO-1 in PC-12 cells [45]. (2) S-guanylation of Keap1 has been suggested as an alternative mechanism for the activation of Nrf2 as NO can react with cGMP to form the guanine nucleotide derivative, 8-Nitro-cGMP, which could transfer the guanylyl group to cysteine residues of proteins [50], (3) Finally, Nrf2 activation could also be triggered by phosphorylation by PKC, PKCK2, MAPKs or other kinases [51–53]. However, our results suggest that in our experimental system the contribution of phosphorylation mechanisms might not be significant (data not shown).

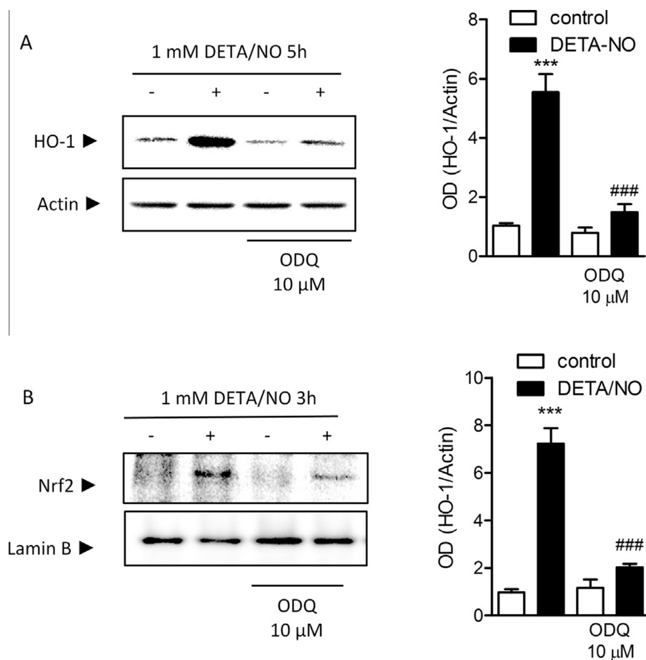

Fig. 8. Role of sGC/cGMP pathways on HO-1 induction by DETA/NO. Y1 cells were pre-incubated for 30 min with 10 µM ODQ and then treated for 5 h with 1 mM DETA/NO. Total cell homogenates were used to analyze HO-1 protein levels (A) and nuclear accumulation of Nrf2 by immunoblotting (B). Actin or Lamin B protein levels were used as internal controls. Representative western blots taken out of 3 independent experiments are shown. Quantification of data from 3 independent experiments is shown beside each immunoblot. Data is presented as mean ± SEM, $N = 3$, $^{***}p < 0.001$ *vs.* control and $^{\#\#\#}p < 0.001$ by Tukey's test.

Present results confirm the participation of cGMP in the activation of Nrf2, but do not allow us to determine the precise mechanism involved. We then speculate that the Nrf2-dependent induction of HO-1 by NO could entail the activation of one or more pathways, thus providing adrenal cells with redundant mechanisms of activation of Nrf2 that would ensure induction of HO-1 under these conditions.

In conclusion, results presented here indicate that NO induces the expression of HO-1 in Y1 adrenal cells by an effect exerted mainly at the transcriptional level, ruling out any effect of NO on mRNA stability. While oxidative stress does not appear to be involved in the inductive effect of NO in our experimental setting, the signal transduction pathway leading to the induction of HO-1 in adrenal cells involves the activation of the redox-dependent transcription factor Nrf2 by a cGMP-dependent effect.

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