Regulation of iNOS expression and glutathione levels in rat liver by oxygen tension

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Abstract Molecular oxygen (O_2) regulates the expression of a variety of genes. We hypothesized that O_2 tension may regulate iNOS expression in rat liver through the production of reactive oxygen species (ROS) and the reduction of intracellular glutathione (GSH) levels. To investigate this hypothesis, we determined the effects of hyperoxia upon iNOS induction (both at the protein and mRNA level) and the intracellular concentration of GSH in an isolated in vitro perfused rat liver preparation. To study the potential involvement of ROS in the intracellular signaling pathway linking changes in oxygen tension to gene expression, we repeated these determinations in the presence of the thiol antioxidant *N*-acetyl-L-cysteine (NAC). We found that 95% O_2 tension caused a significant induction of the iNOS protein and mRNA levels paralleled by a significant fall in intracellular GSH concentration. The addition of NAC (1 mM) to the perfusate during hyperoxia blocked the induction of iNOS and restored GSH levels. These results indicate that molecular O_2 regulates the expression of iNOS in rat liver at the transcriptional level, most likely through the production of ROS and the reduction of intracellular GSH levels.

Key words: Organ bath; Hyperoxia; Reactive oxygen species; Glutathione; Nitric oxide; Synthase

1. Introduction

Nitric oxide (NO) is an important regulatory molecule in many cell signaling pathways [1]. NO is synthesized by the action of the NO synthases (NOS). To date, three main isoforms of NOS have been identified: type I NOS (also named brain constitutive or bNOS), type II NOS (inducible or iNOS) and type III NOS (endothelial constitutive or ecNOS) [1]. In the liver, ecNOS is expressed constitutively and is highly abundant both at the mRNA [2] and at the protein level [3]. In contrast, the iNOS gene is not expressed constitutively [2,3], but it is upregulated by several pro-inflammatory cytokines [4,5], oxidative stress [6,7] as well as by ischemia/reperfusion [8,9]. This latter condition is characterized by changes in tissue oxygen (O_2) tension, the excessive production of reactive oxygen species (ROS) [10–12] and changes in intracellular reduced glutathione (GSH) levels [13,14].

GSH is a ubiquitous cellular non-protein sulphydryl involved in the detoxification of peroxides, free radicals and electrophilic compounds [15]. It therefore plays an important role in the maintenance of the intracellular redox balance and the regulation of several redox sensitive transcription factors, such as nuclear factor-κB and AP-1 [16]. In endothelial cells, these transcription factors regulate the expression of bNOS, ecNOS and iNOS [5,17–20].

We hypothesized that, in rat liver, O_2 tension may regulate iNOS expression through the production of oxidative stress and the reduction of intracellular GSH levels. To investigate this hypothesis, we determined the effects of hyperoxia upon iNOS induction (both at the protein and mRNA level) and the intracellular concentration of GSH and its oxidized form (GSSG) in an isolated in vitro perfused rat liver preparation. To study the potential involvement of ROS in the intracellular signaling pathway linking changes in oxygen tension to gene expression, we repeated these determinations in the presence of the thiol antioxidant *N*-acetyl-L-cysteine (NAC).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (IFFA-Credo, Barcelona, Spain) (200–300 g) were used in all the experiments. The animals were housed under controlled environmental conditions (22°C, 70% humidity and 12 h light/dark cycle) with free access to food and water. This study was approved by the Research and Ethical Review Board of the Hospital Universitari Son Dureta.

2.2. Isolated rat liver preparation

The isolated rat liver procedures were performed using previously described methodology [21]. Briefly, during intraperitoneal thiobarbital anesthesia (B. Braun Medical SA, Jaen, Spain), rats were subjected to a midline laparotomy. The portal vein and the inferior vena cava were catheterized. Livers were immediately transferred to and perfused in a temperature-controlled (37°C) organ bath (Radnoti, Monrovia, CA, USA) through the portal vein with Krebs–Henseleit buffer (120 mM NaCl, 4.7 mM KCl, 14.5 mM $MgSO_4$, 1.6 mM $CaCl_2$, 25 mM $NaHCO_3$, 11.2 mM glucose; pH 7.4 at 37°C), using a constant flow peristaltic pump (Easy-load, Masterflex, Cole-Parmer Instrument Co., Vernon Hills, USA). Flow was kept constant throughout the experiment. Typically, flow values (measured by timed collection of the effluent flow) ranged between 18 and 25 ml/min. Perfusion pressure was continuously monitored at the entrance of the liver using a pressure transducer (Harvard Apparatus, South Natick, MA, USA). Pressure values ranging from 8 to 13 mm Hg were maintained through the experiment in all animals. The PO_2 in the perfusate was kept constant by bubbling vigorously the Krebs–Henseleit reservoir with 95% O_2, 5% CO_2. The actual PO_2 delivered to the liver was monitored by a polarographic O_2 electrode (Instech, Radnoti, Monrovia, CA, USA). The PO_2 electrode was calibrated in situ, before

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perfusing the liver, by equilibrating the Krebs–Henseleit buffer with calibrated gas mixtures. Typical inflow PO_2 values oscillate around 500–600 mm Hg. At the end of the experiment, after 4 h of perfusion in the organ bath, liver biopsies were taken and frozen immediately in liquid nitrogen.

2.3. Experimental protocol

We studied four experimental groups ($n = 6$, each). Livers were perfused under normoxic (21% O_2) or hyperoxic (95% O_2) conditions, with or without NAC (1 mM) dissolved in the perfusate. Hyperoxic livers were perfused in the organ bath, following the methodology outlined above. By contrast, given that the purpose of the normoxic group was to obtain a baseline value of the different variables studied, normoxic livers were perfused in situ (not in the organ bath) during a brief period of time (typically less than 15 s), until complete blanching of the liver, when a biopsy was immediately taken and frozen in liquid N_2. Frozen liver samples were processed equally in the four groups.

2.4. Immunodetection of iNOS and α-tubulin

Frozen liver samples (300–400 mg) were homogenized (1:10 w/v) in cold 40 mM Tris buffer pH 7.5 containing 1% Triton X-100, 1 mM $MgCl_2$, 5 mM NaCl, 1 mM EDTA and the protease inhibitors phenylmethylsulphonyl fluoride (1 mM) and leupeptin (40 μg/ml). Samples were centrifuged at $12\,000 \times g$ for 10 min at 4°C. 100 μl of the resulting supernatant was mixed with an equal volume of loading buffer (62.5 mM Tris, pH 6.8, 3% sodium dodecyl sulphate (SDS), 20% glycerol, 0.005% bromophenol blue), which was then boiled for 4 min. Proteins were determined by the method of Bradford [22]. 5–20 μl of the resulting suspension was loaded in a 10% polyacrylamide gel and submitted to electrophoresis (SDS–polyacrylamide gel electrophoresis). Proteins were transferred to nitrocellulose membranes (immunoblot, Western blot) that were incubated in phosphate-buffered saline containing 4% non-fat dry milk (blocking solution) for 1 h at room temperature with gentle rocking. Then, membranes were incubated overnight at 4°C in blocking solution containing the primary antibody, anti-iNOS (NOS II) rabbit polyclonal antibody at 1:150 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or anti-α-tubulin mouse monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) at 1:2000 dilution. The specificity of the anti-iNOS antibody was determined by Western blot analysis of a positive control of purified iNOS from mouse macrophages (Transduction Laboratories, San Diego, CA, USA). The secondary antibodies, a horseradish peroxidase-linked sheep anti-rabbit IgG for iNOS and a horseradish peroxidase-linked donkey anti-mouse IgG for α-tubulin (Amersham International, Buckinghamshire, UK), were both incubated at 1:1000 dilution in blocking solution at room temperature for 2 h. Immunoreactivity was detected with a chemiluminescence Western blot detection system (Pierce, Rockford, IL, USA). Films were scanned and densitometric analysis of the immunoreactive bands was performed with the aid of the SigmaGel gel analysis software (Jandel Scientific Corporation, San Rafael, CA, USA).

2.5. RNA extraction

Total RNA was extracted from frozen liver samples by use of a single-step RNA isolation system (TRIzol reagent, Gibco BRL; Life technologies, Grand Island, NY, USA), which is based on the method of Chomczynski and Sacchi [23]. Briefly, 300 mg of rat liver samples was homogenized in 1 ml TRIzol reagent (Gibco BRL) during 30 s using an Ultra-Turrax T25 homogenizer (IKA, Staufen, Germany) and then incubated for 5 min at room temperature. 200 μl of chloroform was added to homogenates, which were then vortexed for 30 s, and incubated at room temperature for 5 min. Samples were centrifuged at $12\,000 \times g$ for 10 min at 4°C. The aqueous phases were separated and mixed with 500 μl of 100% isopropyl alcohol. Mixtures were incubated for 10 min at room temperature and centrifuged at $10\,000 \times g$ for 10 min at 4°C. The RNA precipitates were washed twice with 1 ml of 75% ethanol dissolved in diethyl pyrocarbonate-treated water. RNA was quantitated spectrophotometrically by measuring the absorbance at 260 nm.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

iNOS and tubulin mRNA levels were assessed by RT-PCR using the Access RT-PCR System (Promega, Madison, WI, USA) following the procedure of the manufacturer. Briefly, RNA (2 μg) was mixed with 10 μl of AMV/Tfl 5× reaction buffer, 1 μl Tfl DNA polymerase (5 U/μl), 1 μl AMV reverse transcriptase (5 U/μl), 1 μl dNTP mix (10 mM each dNTP), 2 μl $MgSO_4$ 25 mM and 50 pmol of each primer. The final volume of RT-PCR reaction was 50 μl. Each reaction mixture was overlaid with 50 μl of mineral oil. The RT-PCR was performed with a DNA thermal Cycler (Hybaid Omnigene, UK). The oligonucleotide primers for PCR of iNOS were as follows: iNOS primer 1, 5′-CCACAATAGTACAATACTACTTGG-3′; primer 2, 5′-ACGAGGTGTTCAGCGTGCTCCACG-3′. The PCR product generated using these primers was a 397 bp fragment. The primers for α-tubulin were as follows: primer 1, 5′-CTCCATCCTCAC-CACCCACAC-3′; primer 2, 5′-CAGGGTCACATTTCACCATCT-3′. The PCR product generated using these primers was a 365 bp fragment. The specificity of the primers was assessed by sequence analyses using the basic local alignment search tool program (BLASTN 1.4.10MP) [24] from the National Center for Biotechnology Information and the GenBank nucleic acid sequence database. The cycling program for iNOS was: reverse transcription 45 min at 48°C in one amplification cycle; then denaturation for 1 min at 95°C, annealing for 1 min at 62°C and extension for 2.5 min at 72°C. The cycling program for α-tubulin was: reverse transcription for 45 min at 48°C in one amplification cycle; then denaturation 1 min at 94°C, annealing 1 min at 52°C and extension for 1 min at 72°C. After completion of RT-PCR (25 cycles for iNOS and α-tubulin), 25 μl of the reaction volume was separated on 2% agarose gel electrophoresis and stained with ethidium bromide. Films were scanned and densitometric analysis of the fluorescent bands (integrated optical

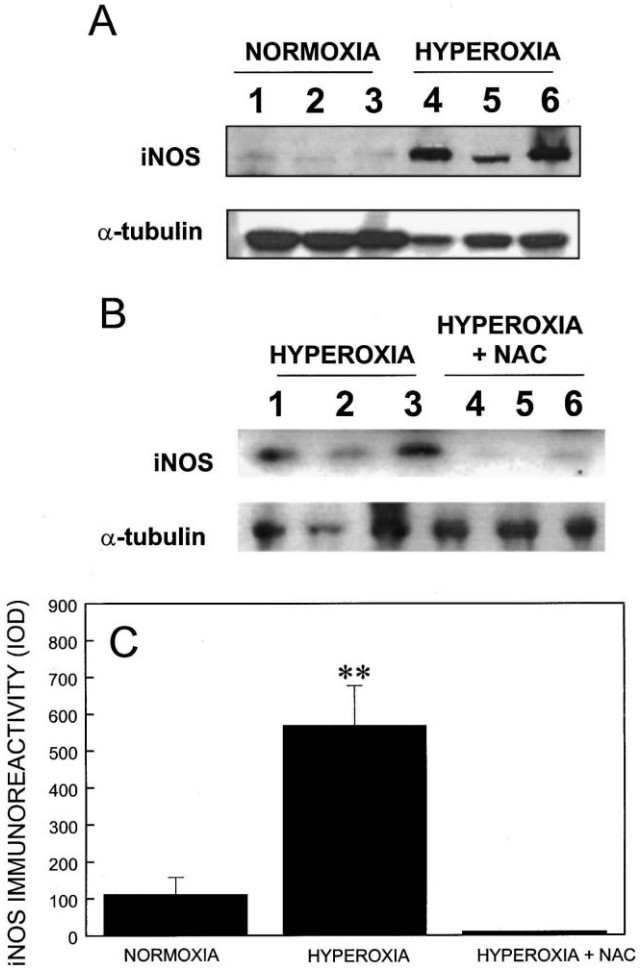

Fig. 1. Representative Western blot showing the iNOS and α-tubulin bands in three normoxic and three hyperoxic livers (A), as well as the effects of NAC (1 mM) during hyperoxia in three other livers (B). C shows the mean (±S.E.M.) values of iNOS immunoreactivity (IOD) in the three experimental groups ($n = 6$, each). $^{**}P < 0.01$, compared with normoxic values.

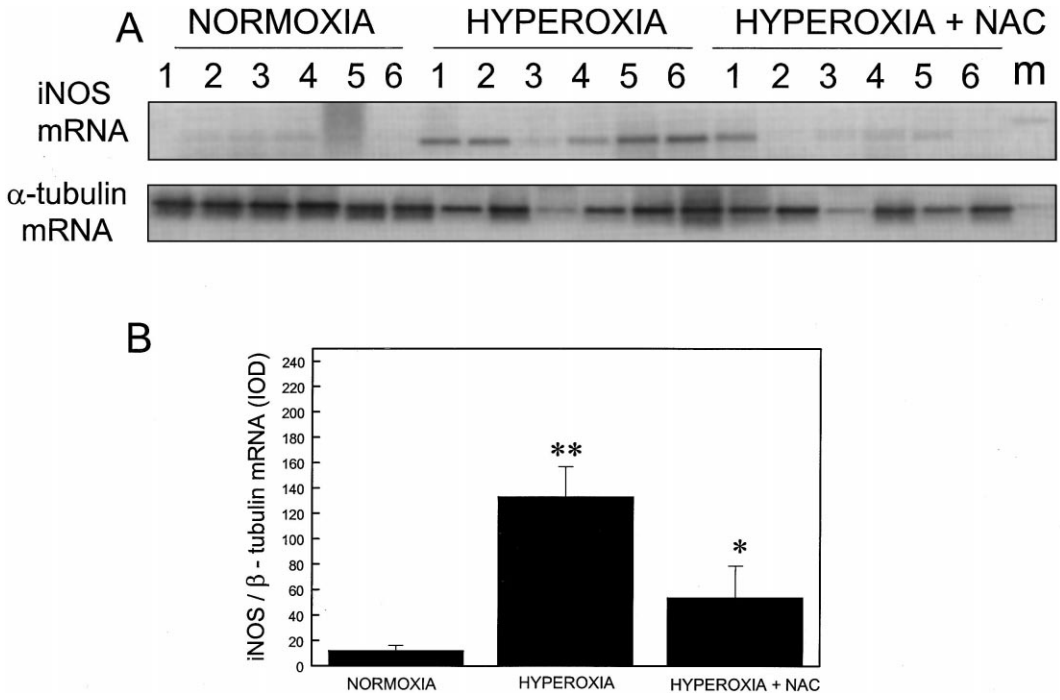

Fig. 2. A: RT-PCR analysis of iNOS and α-tubulin mRNA in all groups ($n=6$, each); m (molecular weight marker) is an internal control that amplifies a 323 bp fragment (Promega, Madison, WI, USA). B: Mean (±S.E.M.) values of iNOS/α-tubulin cDNA ratio in the three experimental groups ($n=6$, each). $^{*}P<0.05$; $^{**}P<0.01$, compared with normoxic values.

density, IOD) was performed with the aid of the SigmaGel analysis software (Jandel Scientific Corporation, San Rafael, CA, USA). iNOS mRNA levels were expressed as the ratio between iNOS and α-tubulin band signal intensities.

2.7. GSH assay

Liver samples (100 mg) were homogenized in 2 ml of cold 0.6% (w/v) 5-sulphsalicylic acid and 0.1% (v/v) Triton X-100 in potassium phosphate buffer (5 mM)–EDTA (1 mM) as described previously [25]. The homogenate was then centrifuged at 4000×*g* for 10 min at 4°C. The supernatant was used immediately in the soluble GSH assay by the 5,5′-dithiobis-(2-nitrobenzoic acid)–GSSG reductase recycling method described by Tietze [26]. To determine GSSG, supernatant was treated with 2-vinylpyridine and triethanolamine as previously described [27], and thereafter was used in the assay for GSH as described above.

2.8. Statistical analysis

Data are presented as mean ± S.E.M. The statistical significance of differences between groups was assessed using the Mann–Whitney test. A P value lower than 0.05 was considered significant.

3. Results

3.1. iNOS expression

In all experimental groups, the relative molecular mass of iNOS (~130 kDa) detected by Western blot was in the range described for this protein [28]. As shown in Fig. 1, during normoxia, iNOS bands were barely detectable (109.78 ± 48.07 IOD). In contrast, the immunoreactivity of the iNOS band was strongly increased during hyperoxia (566.93 ± 109.57 IOD, $P<0.005$) (Fig. 1A,C). When NAC was added to the perfusion buffer, hyperoxia did not upregulate iNOS immunoreactivity (Fig. 1B,C). NAC did not influence iNOS immunoreactivity during normoxia (130.33 ± 16.82 vs. 141.00 ± 28.00 IOD, P = ns).

Fig. 2A,B shows the results of the amplification of specific cDNAs fragments for iNOS (395 bp) and α-tubulin (365 bp), as a RNA loading control. During normoxia, the iNOS cDNA product was very low (iNOS/α-tubulin: 12.13 ± 3.98 IOD) (Fig. 2A,B). Hyperoxia increased the iNOS message very significantly (iNOS/α-tubulin: 133.26 ± 23.42 IOD, $P<0.005$) (Fig. 2A,B). The presence of NAC in the perfusion buffer completely prevented the upregulation of iNOS mRNA levels during hyperoxia (iNOS/α-tubulin: 53.83 ± 24.79 IOD, $P<0.05$) (Fig. 2A,B).

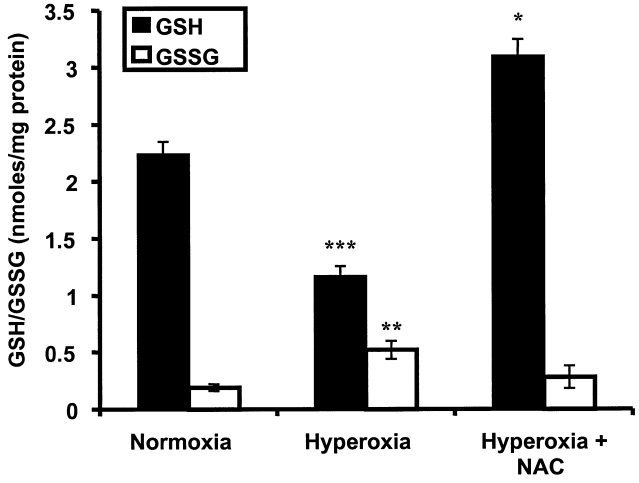

Fig. 3. Effect of normoxia and hyperoxia in the absence or presence of NAC (1 mM) on GSH and GSSG levels in rat liver. Each histogram represents the mean (±S.E.M.) of three experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, compared with normoxic values.

3.2. GSH levels

Compared to control normoxic livers (2.24 ± 0.19), hyperoxia exposure significantly decreased GSH (1.17 ± 0.15, $P < 0.001$) and increased GSSG levels (hyperoxia: 0.52 ± 0.13 vs. controls: 0.19 ± 0.05) (Fig. 3). As a result, hyperoxia produced a 5-fold decrease in the GSH/GSSG ratio, as compared to control normoxic values (11.8 ± 6.40 vs. 2.3 ± 1.9, $P < 0.001$). The presence of NAC during hyperoxia prevented the fall of the GSH/GSSG ratio (11.1 ± 1.9, $P =$ ns), which was close to control normoxic values (3.1 ± 0.25, $P < 0.01$ compared to controls) (GSSG: 0.28 ± 0.17, $P =$ ns compared to controls) (Fig. 3).

4. Discussion

This study shows that O_2 tension modulates the expression of the iNOS gene in rat liver. Given that parallel changes occurred in GSH and GSH/GSSG levels, and that all these effects are prevented by NAC, our results suggest that ROS are likely to be key factors in the signal transduction pathway linking changes in O_2 tension and iNOS expression in rat liver.

The iNOS isoform is expressed in a variety of cell types and tissues, including human and rat hepatocytes, by the action of cytokines, or lipopolysacharide [29–33]. Whether or not molecular O_2 regulates iNOS expression in the liver has not been investigated before. Our results show that control livers (perfused in situ with 21% O_2) express very low levels of iNOS immunoreactivity and iNOS mRNA (Figs. 1 and 2). It is normally assumed that iNOS is not constitutively expressed in the liver [2,3]. Probably, the very low levels of iNOS expression detected in our normoxic samples can be explained by the mechanical stress suffered by the organ during the experimental procedure [34,35]. We observed that hyperoxia strongly induced both the iNOS protein (Fig. 1) and iNOS mRNA (Fig. 2). A similar induction of iNOS by hyperoxia has been demonstrated in human lung [6] and, in a very recent report, in rat liver by ischemia and reperfusion [9]. This latter condition involves changes in tissue oxygenation. However, at variance with our study, it also involves changes in blood flow and substrate delivery. Therefore, our study confirms and extends previous studies by showing that molecular oxygen by itself can trigger the expression of iNOS in isolated, perfused rat livers (Fig. 1), and that this induction appears to be regulated at the transcriptional level (Fig. 2).

Molecular oxygen can regulate iNOS transcription through several potential mechanisms. In this study, we investigated if ROS (produced during hyperoxia [20]) were capable of modulating intracellular redox GSH levels and thus trigger signaling pathways that link changes in O_2 tension to gene expression [20]. During hyperoxia (and compared to normoxia), we observed that GSH levels fell and GSSG concentration increased, with a corresponding decrease in the GSH/GSSG ratio (Fig. 3). These observations already suggest a role of ROS in the hyperoxia-mediated induction of the iNOS gene. However, to further investigate this possibility, we repeated the same experiments discussed above in the presence of thiol antioxidant NAC (1 mM). NAC is known to buffer cysteine/GSH levels in the cell and to scavenge ROS, both in vitro and in vivo [10,36]. Our results show that during hyperoxia, the presence of NAC in the perfusate inhibited the upregulation of the iNOS gene, both at the protein (Fig. 1) and mRNA levels (Fig. 2), and reversed the GSH/GSSG ratio close to normoxic values (Fig. 3). Our results are in coincidence with other recent studies showing an increase of GSH levels by NAC [39,40]. Therefore, our results suggest that ROS are involved in the induction of the iNOS gene in rat liver. This would be in keeping with previous reports in RAW264.7 cells [37], rat glomeruli [38], endothelial cells [14], rat pleural mesothelial cells [7] and lung epithelial cells [13]. Nonetheless, apart from a direct effect of ROS upon gene expression [20], other potential mechanisms by which changes in O_2 tension can theoretically modulate the expression of the iNOS gene include the effects of O_2 on the expression of several pro-inflammatory cytokines, such as IL-1β and TNF-α [41,42]. By their own, these cytokines are potent inducers of iNOS expression in many cell types and tissues, including human and rat hepatocytes [29–33,43]. Therefore, the possibility of a direct involvement of these pro-inflammatory mediators in the upregulation of iNOS gene in this model cannot be ruled out. In any case, however, the decrease in GSH/GSSG ratio caused by O_2 and the fact that NAC fully prevented the induction of the iNOS gene under these circumstances indicate that, even if operative, these alternative mechanisms should also be mediated by ROS.

In summary, the results of this study indicate that molecular oxygen modulates the expression of the iNOS gene in rat liver. Given that this effect is paralleled by changes in GSH/GSSG ratio and it is inhibited by the thiol antioxidant NAC, we suggest that ROS are likely to be involved in the transcriptional induction of the iNOS gene by molecular oxygen.

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