# Dexamethasone on Inducible Nitric Oxide Synthase and Nitrite/Nitrate Production in Myocardial Infarction (44326)

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*Abstract.* **The effect of dexamethasone (DEX) on the activation of the inducible form of nitric oxide synthase (iNOS) and its relation to the formation of oxidation products of nitric oxide ($NO_{2-}$ + $NO_{3-}$, $NO_X$) in infarcted heart muscle of rabbits was investigated. Myocardial infarction was produced by ligation of the first anterior branch of the left circumflex coronary artery. $NO_X$ was determined by chemiluminescence and iNOS by conversion of L-[$^{14}$C]-arginine to L-[$^{14}$C]-citrulline. The rise in myocardial iNOS production, which followed onset of myocardial ischemia, was suppressed by DEX. In addition, following coronary artery ligation, coronary arterial venous differences of $NO_X$ increased markedly; this effect was also partially abolished by DEX. Cardiac origin of $NO_X$ was confirmed by the linear relationship between coronary A-V $NO_X$ difference and myocardial formation of iNOS. This relationship was preserved after administration of DEX. Therefore glucocorticoids interfere with the myocardial NO production and thus diminish the concentration of its oxidative products in plasma.**

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The free radical molecule nitric oxide (NO) exhibits diverse biological functions in the vasculature such as vasodilation, inhibition of platelet aggregation (1) and of vascular smooth muscle cell growth (2). Basal levels of nitric oxide are generated by the constitutive isoform of nitric oxide synthase (cNOS). In endothelial cells, cNOS mediates endothelial dependent relaxation in adjacent vascular smooth muscle cells, thereby regulating coronary circulation (2, 3). Under pathological conditions such as in heart failure (4), dilated cardiomyopathy (5) and cardiac allograft rejection (6), the inducible isoform of nitric oxide synthase (iNOS) is activated by various cytokines (7–10) and bacterial endotoxins such as lipopolysaccharide (11). This occurs in many cell types found in the myocardium such as endothelial cells, vascular smooth muscle cells, monocytes, neutrophils, and macrophages (12, 13). Once iNOS is activated, it remains active for up to several days and produces a relatively large amount of NO (12, 13).

In infarcted rabbit heart muscle, following ligation of a coronary artery, iNOS activity increased on the first postoperative day, peaked at Day 3 following ligation, and remained elevated for at least 14 days (14, 15). Macrophages were identified by immunohistochemistry as the major source of increased iNOS activity (16). The levels of oxidation products of NO (nitrite ($NO_{2-}$) + nitrate ($NO_{3-}$), $NO_X$) were increased in plasma of animals with myocardial infarction (15). This was attributed to elevated production of NO in the infarcted myocardium (15). Glucocorticoids such as dexamethasone (DEX), which inhibit the activity of macrophages, also interfered with iNOS expression at multiple levels (17). Several studies have shown that DEX inhibits iNOS expression at the level of transcription (18–20) or, as shown in rat insulinoma cell cultures, by post-transcriptional mechanisms (21).

Although the effect of DEX on iNOS activity has been well documented, its relationship to NO formation has been controversial (22). It was the purpose of this study to examine the effect of dexamethasone on the activation of the inducible form of nitric oxide synthase and the relationship

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of iNOS to the formation of the oxidation products of nitric oxide in infarcted heart muscle.

## Materials and Methods

The investigation conforms with the Declaration of Helsinki and the *Guiding Principles in the Care and Use of Animals.* The experimental protocol was approved by the institutional Animal Care and Use Committee. As previously described, healthy male New Zealand rabbits (3.2–4.2 kg) were anesthetized with pentobarbital (10 mg/kg iv), ketamine (40 mg/kg im), and xylazine (10 mg/kg im) (15). The animals received atropine (0.5 mg im), were anticoagulated with heparin (500 IU/kg iv), intubated with an endotracheal tube (3.0, cuffed), and mechanically ventilated with 95% oxygen (rate 40 strokes/min, tidal volume 25 ml; Harvard Rodent Ventilator, Model 683, Harvard Apparatus, South Natick, MA). The rabbits received ciprofloxacin (5 mg/kg iv) before surgery as an antibiotic prophylaxis.

After administration of anesthesia but prior to surgery, blood (0.95 ml) was drawn from the peripheral ear vein (EV) using a heparinized syringe (0.05 ml heparin). A left thoracotomy was performed in the fourth intercostal space under sterile conditions. Left ventricular hemodynamics were measured by inserting a 22-gauge × 1-inch catheter connected to a DIGI-MED Heart Performance Analyzer 100 (Micro-Med, Louisville, KY) directly into the left ventricle through the apex of the heart. Blood (0.95 ml) was drawn from the coronary sinus (CS), left ventricle (LV), and right ventricle (RV) using heparinized syringes (0.05 ml heparin).

The first anterolateral branch of the left circumflex coronary artery was exposed and ligated at 50% apex to base of the heart using a 4-0 Prolene suture. The changing color and bulging of the left ventricle documented coronary occlusion. The chest was then closed in layers, and the animals were allowed to recover from surgery; they were fed *ad libitum.* All animals were observed frequently, and buprenorphine (0.03 mg/kg sc) was given as an analgesic twice daily following surgery until sacrifice. Dexamethasone (DEX, $n = 7$) was administered intravenously into a peripheral ear vein (2 mg/kg) at 1, 12, 24, and 36 hr following coronary artery ligation. Rabbits not receiving DEX served as controls ($n = 7$).

Forty-eight hours after coronary ligation, the animals were anesthetized and intubated, and EV blood was drawn as described above. They were then mechanically ventilated as described above. The heart was exposed with a median sternotomy and pericardiectomy. CS, LV, and RV blood samples were drawn, and hemodynamics were measured as described above. All blood samples were prepared as described below. Several samples of CS, LV, and RV blood taken before and after coronary artery ligation were analyzed for $O_2$ saturation using an AVL Omni-3 Blood Gas Analyzer (AVL Medical Instruments, Roswell, GA), to confirm the source of the blood.

After the blood was drawn, the heart was rapidly excised, weighed, and prepared for analysis.

**Measurement of Plasma $NO_X$ ($NO_2^-$ + $NO_3^-$).** Blood was drawn in heparinized tubes for $NO_X$ determination. It was centrifuged at 1200*g* for 10 min to remove formed elements. Deproteinization of the plasma samples was achieved by placing them in an ultrafiltration device (MPS Micropartition, Millipore Corporation, Bedford, MA) and centrifuging at 2500*g* for 90 min. The deproteinized plasma samples were stored at −20°C until analysis. Samples treated with the enzyme aspergillus nitrate reductase (NR) measure both $NO_2^-$ and $NO_3^-$. The $NO_3^-$ content of samples was reduced to $NO_2^-$ with NR (23). For analysis of plasma $NO_X$ ($NO_2^-$ + $NO_3^-$), 50 µl of NR (0.1 U, reconstituted in phosphate buffered saline (PBS) as 2 U/ml) was added to 5 µl of flavin adenine dinucleotide (FAD, 5 m*M*), 5 ml NADPH (6 m*M*), 40 µl PBS (10 m*M*, pH = 7.4) and 50 µl deproteinized plasma for a final volume of 150 µl. All samples were incubated at 37°C for 1 hr. After incubation, 30 µl samples were injected into a reaction vessel. All samples were analyzed in duplicate.

For chemiluminescence, a reducing system consisting of a ferrocene derivative dissolved in acetonitrile containing 1% perchloric acid was used (24). In this solution, NO is generated from $NO_2^-$ by a one-electron reduction pathway. This reaction is very fast, and the dilution with water does not influence the speed of the reaction. In the reaction vessel, helium at a rate of 35 ml/min is used as the carrier gas of NO to the NO-analyzer (270 B, Sievers Res. Inc., Boulder, CO). Reducing solutions (75 mg of 1,1-dimethylferrocene in 7.5 ml of acetonitrile, acidified with 150 µl of 70% perchloric acid) (all reagents from Sigma Chemical Co., St. Louis, MO) were freshly prepared and used within 2 hr. Under this condition, $NO_2^-$ in the injected samples (30 µl) was reduced to NO and detected by chemiluminescence with a NO-analyzer. Data were recorded automatically to a chart recorder (Soltec Corporation, Sun Valley, CA) as previously described (25).

**$NO_X$ Standard Curve.** The standard curve for $NO_X$ was obtained by adding 50-µl aliquots of sodium nitrate ($NaNO_3$, dissolved in degassed distilled water, $NO_3^-$ concentrations of 0–120 µ*M*) to 5.0 µl of flavin adenine dinucleotide (FAD, 5 m*M*), 5.0 µl of NADPH (6 m*M*), 40 µl of PBS, and 50 µl of NR (0.1 U) for a final volume of 150 µl. All samples were incubated a 37°C for 1 hr. The chemiluminescence signal (mV) was related to the concentration of $NO_2^-$ and $NO_3^-$ (µmol/l) and was fitted to a straight line (linear regression) (15). Correlation coefficients of all standard curves were $r > 0.98$.

**Calculation of $NO_X$ Production Equivalent.** To obtain information on $NO_X$ production by the heart, myocardial flow and cardiac output must be known. In an identical experimental model, myocardial blood flow in the rabbit has been previously determined (26). Accordingly, mean values of 1.18 and 0.9 ml/g/min were used for myocardial

blood flow in normal and ischemic preparations, respectively. With a coronary blood flow estimated at 5% of cardiac output, and a heart weight of 10 g, a cardiac output of 236 and 180 ml/min was calculated in normal and ischemic myocardium, respectively. These values agree with previously published data of Wolf and associates who found a mean cardiac output in the normal rabbit of 220 ml/min (27). Input into the heart (delivery) of $NO_X$ is the product of coronary flow times $NO_X$ concentration in the left ventricle; output from the heart is the product of coronary flow times $NO_X$ concentration in the coronary sinus. The myocardial production equivalent of $NO_X$ is then calculated as:

Production equivalent of $NO_X$ (%) = [($NO_X$ Output − Input)/Input] × 100.

As previously described, the term "production equivalent" was used in preference to "production," because coronary flow was not determined in these experiments (15). The values for coronary flow were obtained from published data in similar experimental conditions (28).

**Assay of iNOS.** iNOS activity was measured by the conversion of L-[$^{14}$C]-arginine to L-[$^{14}$C]-citrulline (29). Thirty to sixty mg of tissue were prepared from each of the right ventricle, left ventricle remote, and left ventricle infarcted regions. Tissue designated as "left ventricle remote" originated from the noninfarcted region above the coronary artery ligation. Tissue designated as "left ventricle infarcted" originated from the region immediately below the ligation and was easily identifiable by its whitish color and thinning wall. The heart was homogenized in 1 ml of cold Tris-HCl buffer (0.05 *M*, pH = 7.4) containing: DL-dithiothreitol (1.0 m*M*), leupeptin (10.0 m*M*), phosphoramidon (25.0 μg/ml) and aprotinin (100.0 μg/ml) (all from Sigma Chemical Co., St. Louis, MO), and sonicated for 15 sec. The homogenate which, depending on the location of the specimens, contained myocytes, polymorphonuclear leukocytes, macrophages, and some fibroblasts, was centrifuged (1200*g*, 5 min.) and EDTA (0.5 m*M*) and NADPH (1 m*M*) were added to 65–70 μl of supernatant. L-[$^{14}$C] arginine (200,000 cf/min., Amersham Life Science, Buckinghamshire, UK) was added, and the samples were incubated for 30 min in a 37°C water bath. The reaction was stopped by adding ice-cold Tris-HCl buffer (pH = 5.5). Radiolabeled citrulline was separated from arginine by cation exchange chromatography (Dowex 50-WX8, 200–400 mesh, Na-form, Bio-Rad Laboratories, Hercules, CA). The eluate (approx. 3.5 ml) was mixed in 10 ml of scintillation fluid and counted in duplicate in a Beckman LS 100SC scintillation counter. All values were corrected for protein content of the samples (determined by the modified Lowry Assay), and calculated in pmol/mg protein/min.

**Statistical Analysis.** Data are presented as the mean ± SEM. The data from the same animals, which were obtained before and after coronary artery ligation, were compared by the paired Student's *t* test. The data from different animals were compared by nonpaired Student's *t* test. The correlation coefficient was determined by simple regression analysis. Values of $p < 0.05$ were considered statistically significant.

## Results

**Plasma $NO_X$ Concentration.** In general, dexamethasone (DEX) had a significant effect on $NO_X$ production. Figure 1 illustrates that in the control group (DEX not administered), myocardial ischemia significantly increased $NO_X$ production in plasma obtained from a coronary sinus (before ligation (bef.) = 54.6 ± 1.8; after ligation (aft.) = 90.5 ± 12.4 μmol/l, $p < 0.05$), left ventricle (bef. = 53.3 ± 1.7; aft. = 82.1 ± 10.9 μmol/l, $p < 0.05$), right ventricle (bef. = 56.4 ± 1.4; aft. = 89.0 ± 10.6 μmol/l, $p < 0.05$) and a peripheral vein (ear vein) (bef. = 55.6 ± 2.2; aft. = 82.9 ± 10.1 μmol/l, $p < 0.05$). DEX administration suppressed this rise. Following coronary artery ligation, the differences in $NO_X$ concentrations between coronary venous and arterial plasma (obtained from left ventricular blood) were significant (1.3 ± 0.5 as compared to 8.4 ± 2.1 μmol/l plasma, $p < 0.05$) whereas differences in $NO_X$ concentration between right ventricular and left ventricular plasma and between peripheral venous (ear vein) and left ventricular plasma were not significant (Fig. 2). However, after ligation, DEX significantly diminished the coronary arterial-venous (A-V) difference as compared to its control (Fig. 2).

**$NO_X$ Production Equivalent.** The production of $NO_X$ (production equivalent) by the heart rose significantly following the onset of ischemia (2.4 ± 1.0 as compared to 10.2 ± 2.4%, $p < 0.05$). With DEX administration, the production equivalent in the myocardium failed to reach significance (1.6 ± 0.6 as compared to 0.7 ± 1.1%).

**iNOS Activity.** The myocardial iNOS activity in the infarcted region was significantly greater than in the non-

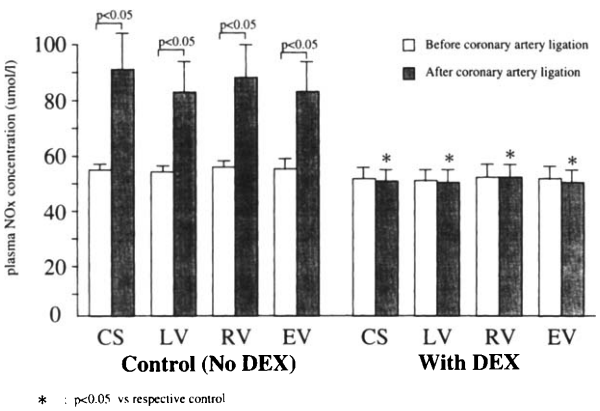


**Figure 1.** Effect of DEX on plasma $NO_X$. In control experiments, a significant increase in plasma $NO_X$ concentration collected from different locations is noted ($p < 0.05$) (left panel). The right panel shows a significant decrease in $NO_X$ concentration following dexamethasone ($p < 0.05$). CS = Coronary sinus; LV = Left ventricle; RV = Right ventricle; EV = Ear vein. (Control: $n = 7$; DEX: $n = 7$).

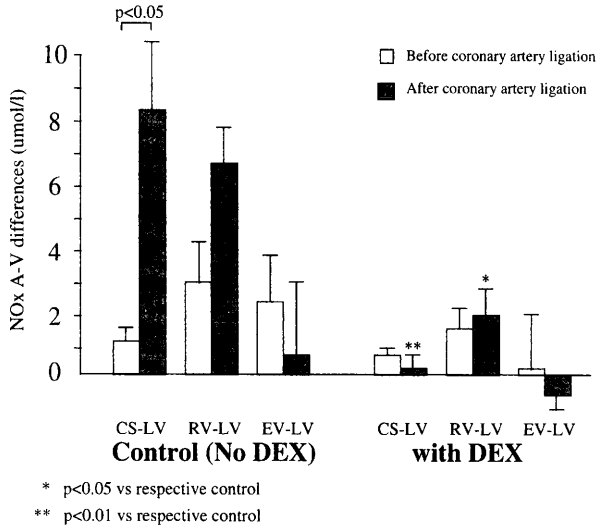


**Figure 2.** Effect of DEX on $NO_X$ coronary A-V differences. Left panel shows a significant increase of coronary A-V difference of $NO_X$ concentration following ligation of a coronary artery. Differences in plasma $NO_X$ concentration between RV and LV and between EV and LV are not significant. Following administration of dexamethasone, the difference between CS and LV plasma $NO_X$ concentration was abolished. Following dexamethasone, there were also significant differences between RV and LV $NO_X$ concentrations. (*n* as in Fig. 1)

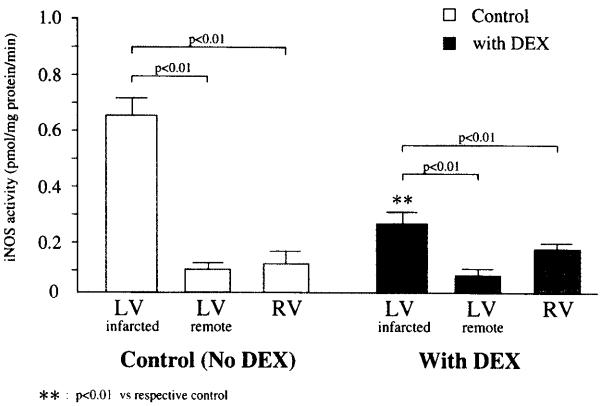


**Figure 3.** Effect of DEX on iNOS activity. Myocardial activity of iNOS. Left panel shows a significant increase in activity in infarcted portion (LV infarcted) compared to activity in remote portion of LV (LV remote) and RV portion (RV). Right panel illustrates a significant reduction in iNOS activity in infarcted LV as compared to its control. The difference between infarcted LV, LV remote, and RV remains significant. (*n* as in Fig. 1)

infarcted regions (left ventricle remote and right ventricle) ($p < 0.01$, Fig. 3). In addition, with DEX administration, the iNOS activity in the infarcted region was significantly lower as compared to its control (0.63 ± 0.08 as compared to 0.27 ± 0.03 pmol/mg protein/min, $p < 0.01$, Fig. 3).

## Discussion

A rise in concentrations of the oxidation products of nitric oxide ($NO_2^-$ + $NO_3^-$, $NO_X$) in plasma following myocardial ischemia has been previously reported and ascribed to increased production of NO by activated macrophages in the infarcted heart muscle (14, 15). In addition to myocar-

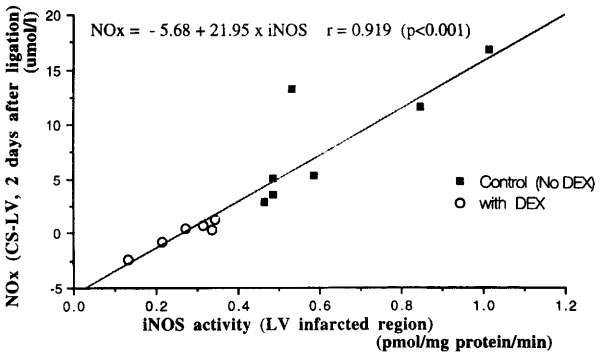


**Figure 4.** $NO_X$ coronary A-V differences (Y axis) versus iNOS activity (X axis). A linear relationship is seen between iNOS activity in the infarcted portion of the left ventricle (pmol/mg protein/min) and the coronary sinus-LV $NO_X$ differences (μmol/L) ($r = 0.919$, $p < 0.001$). (*n* as in Fig. 1)

dial infarction, $NO_X$ levels are also elevated following infusion of interleukin 2 (30). Citterio *et al.* found elevations of plasma nitrite and nitrate levels during and after administration of this cytokine for treatment of malignant melanoma and renal cell carcinoma (30). This elevation results from increased NO formation. Citterio *et al.* established a dose- and course-dependent pattern for plasma nitrite and nitrate changes in these patients (30). In our experiment, cardiac origin of $NO_X$ is illustrated by the linear relationship between coronary A-V $NO_X$ difference and myocardial formation of iNOS (Fig. 4) and between cardiac production equivalent and iNOS ($r = 0.911$, data not shown). Figure 4 shows that this linear relationship is preserved after administration of DEX.

Glucocorticoids repress the expression of a variety of genes, mediated by binding the hormone to the glucocorticoid receptor to block transcription. Additionally the inhibition of DEX on iNOS protein synthesis and decrease in iNOS protein stability play important roles. These mechanisms could be responsible for prevention of iNOS activity and cGMP formation by dexamethasone in infarcted heart muscle (22). Dudek also found that DEX inhibits *de novo* iNOS formation *in vivo* (14).

Previous reports have stressed the effect of glucocorticoids on NO production in a variety of organs. For example, glucocorticoids inhibit NO synthesis in peritoneal neutrophils (31) and prevent induction of NO synthesis by endotoxin in the lung, liver, and aorta of rats (32). In kidney and vascular endothelium, anti-inflammatory steroids inhibit cytokine induction of NO synthase in mesangial and endothelial cells (20, 33). The inhibitory effects of glucocorticoids on NOS in macrophages are also well documented. Di Rosa *et al.* describe inhibition of NO synthase by glucocorticoids in a macrophage cell line after *in vivo* treatment with LPS (17).

Pathophysiological effects of NO are diverse. NO in infarcted heart muscle increases myocardial perfusion and collateral circulation (16) and, because of c-GMP production, results in dephosphorylation of myosin light chains, which are involved in myocardial contractility (34). NO

may lead to the formation of peroxynitrite and other damaging superoxide anions (35). The hemodynamic effects of NO have been well documented by Paulus *et al.* who described that infusion of a NO donor in the coronary artery bed resulted in reduction in left ventricular peak and end-systolic pressures, early onset of left ventricular isovolumic relaxation, and increased left ventricular diastolic distensibility (36). An important part of the evolution of myocardial infarction is the initiation of the inflammatory phase by cytokine-activated macrophages with NO formation and the termination of this phase by apoptosis of macrophages, the NO producing cells (37): this terminates the inflammatory phase and eliminates the source of NO production. Glucocorticoids interrupt these processes with both potentially beneficial and harmful effects.

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