

Modulation of the Inflammatory Response in the Cardiomyocyte and Macrophage

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Abstract: Numerous cardiac disease processes have been linked to the overproduction of nitric oxide (NO) via inducible nitric oxide synthase (iNOS) in the cardiovascular system. Chronic and acute exposure to hyperphysiologic levels of NO has been suggested as an agent in chronic transplant rejection, various cardiomyopathies, reperfusion injury, and the inflammatory state following cardiopulmonary bypass. Proinflammatory cytokines and inflammatory cell types, such as macrophage and neutrophils, have also been implicated in the pathophysiology associated with the previously mentioned syndromes. Previous work by this group has shown that lipopolysaccharide (LPS) in combination with tumor necrosis factor- α (TNF- α) can increase iNOS expression and the production of NO in macrophage. With this in mind, we hypothesized that increased iNOS expression and NO production generated by LPS and TNF- α in the macrophage could be mimicked in the cardiomyocyte and potentially account for some aspect of the cardiac dysfunction attributed to NO. Furthermore, this increased expression of iNOS and NO production could be returned to control using the glucocorticoid, dexamethasone, a known iNOS transcription blocker. Using fetal rat cardiomyocytes in primary culture cell line and a murine macrophage cell line, RAW 264.7, the expression of iNOS was quantified with specific FITC-conjugated antibodies using fluorescence activated cell sorter (FACS) and NO production with a Bioxytech nitric oxide spectrophotometric assay. The myocytes and macrophage were separated into three groups, Control, TNF + LPS, and (+) Dexamethasone. The control groups received no TNF or LPS or dexamethasone, TNF + LPS groups received

TNF- α and LPS for 8 hours with no dexamethasone, and the (+) Dexamethasone groups were pretreated with dexamethasone for 8 hours and stimulated with TNF- α and LPS along with a second 8-hour treatment of dexamethasone. The macrophage cell groups treated with TNF- α and LPS showed a 335% increase over control in iNOS expression, and NO production was increased 494% from control. Macrophage treated with dexamethasone experienced an attenuation of iNOS expression of 200% toward control from stimulated levels and 202% decrease in NO production from stimulated levels toward control. Cardiomyocytes exhibited no statistically significant change in the expression of iNOS or NO production with stimulation or dexamethasone treatments. In conclusion, iNOS and NO were elevated in macrophage, which can be blunted in the presence of dexamethasone in the macrophage. Curiously, iNOS could not be stimulated in the cardiomyocyte, suggesting inflammatory cells may be largely responsible for the elevated iNOS and NO experienced in some cardiovascular diseases. The clinical relevance of this study is the introduction of specific iNOS inhibitors into the cardiopulmonary bypass circuit could serve as a potential mechanism for modulating the inflammatory response surrounding cardiopulmonary bypass. Likewise, therapeutic glucocorticoid administration could improve outcomes for patients with inflammatory cardiovascular disease states related to elevated NO production. **Keywords:** nitric oxide, inducible nitric oxide synthase, tumor necrosis factor- α , lipopolysaccharide, cardiac myocyte, macrophage, dexamethasone. *JECT. 2001;33:167-174*

The American Heart Association has identified cardiovascular disease (CVD) as the leading cause of morbidity and mortality in the United States. Furthermore, approximately 60,000,000 Americans have some form of CVD.

More than 953,000 deaths or 41.2% of all mortality in 1997 was attributed to this group of diseases. Interestingly, if CVD were eliminated, the average life expectancy would increase 7 years. As research progresses and molecular techniques improve, the biological mediators have begun to emerge in this syndrome of disease. Inflammatory cells, such as macrophage, neutrophils, and so forth, are potent reservoirs for proinflammatory cytokines. These cells are activated in response in pathophysiologic conditions and

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are the likely causal agents in the pathology of cardiac dysfunction via cytokines and other cytotoxic substances, such as NO, myeloperoxidase, and so forth. Abnormal serum levels of proinflammatory cytokines have been suggested or directly indicated in many cardiovascular diseases. The elevated secretion of proinflammatory cytokines during cardiopulmonary bypass has been definitively established (1–12). These proinflammatory cytokines include: TNF- α , Interleukin-1 β (IL-1 β), Interferon- γ (IFN- γ), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), and Interleukin-8 (IL-8). Numerous other pathologic states, including: dilated and ischemic cardiomyopathy (13, 14), chronic allograft rejection (15–18), accelerated graft atherosclerosis (19,20), reperfusion injury (21, 22), and senescent cardiovascular disease (23–25) have been associated with these same cytokines. Cytokines bind extracellular receptors and transduce signals intracellularly, ultimately affecting transcription and translation within the nucleus of the target cell. Figure 1 demonstrates this mechanism of intracellular signal transduction.

The target gene of interest in this study, iNOS, has garnered much attention within the realm of inflammatory and sepsis research. The iNOS protein is member of a family of nitric oxide synthases, which catalyze the bio-conversion of L-arginine to NO. NO has numerous physiologic functions; however, when NO levels become elevated, the pathophysiology can become quite perverse. Similar to proinflammatory cytokines, increased iNOS expression and NO production has also been linked to a

variety of inflammatory states and cardiovascular diseases such as: dilated cardiomyopathy (26, 27), cardiac allograft rejection (28–31), accelerated graft atherosclerosis (32), congestive heart failure (33, 34), reperfusion injury (35–38), as well as myocardial infarction and stunning (39, 40), and cardiopulmonary bypass (6, 41–43).

Proinflammatory cytokines have been shown to be capable of upregulating the expression of iNOS, in addition to increasing the production of NO in the macrophage (44). Although macrophage and neutrophils have been shown to contribute to cardiac dysfunction, the participation of the cardiomyocyte has not been definitively established. Regulation of iNOS and its product during these pathologic states would intuitively suggest a more favorable outcome for the patient. With this in mind, the hypothesis of this study was to determine whether iNOS expression and NO production could be stimulated with TNF- α and LPS and modulated using the corticosteroid, dexamethasone, a pretranscriptional blocker of iNOS in the macrophage and cardiomyocyte.

METHODS AND MATERIALS

Cell Culture

A fetal, rat cardiomyocyte and a murine macrophage, RAW 264.7 (ATCC, Bethesda, MD) were utilized for this research. Fetal cardiomyocytes have been established as a cell culture standard, because no immortalized cardiac cell lines exist, so this type of cell was employed in this study. These cardiomyocytes were prepared from 17-day-old fetal Sprague–Dawley rat embryos. The tissue was dissected into 1- and 2-mm segments and predigested with pancreatin (Life Technologies, USA) to remove red blood cells. The cardiac tissue was again digested with 0.12% pancreatin. The dissociated cells were collected by centrifugation and were resuspended in Ham's F-12 medium (Life Technologies, USA) with 1.0% bovine serum albumin (Sigma, St. Louis, MO), 0.025% Fetuin (Sigma, St. Louis, MO), 0.1mM ascorbate (Sigma, St. Louis, MO), 100 units/mL of penicillin (Sigma, St. Louis, MO), and 100 units/mL of streptomycin (Sigma, St. Louis, MO). The dissociated cells were placed in uncoated 100-mL Petri dishes and incubated at 37°C in a 5% CO₂ incubator for at least 1 hour, but not more than 3 hours. This procedure, differential plating, results in predominately fibroblasts attaching to the dishes; whereas, most cardiomyocytes remain in solution, unattached. The enriched population of cardiomyocytes separated by differential plating are then collected and counted. These cells were then aliquoted into flasks and grown to confluency in tissue culture flasks in a humidified incubator (Sheldon Laboratories, Cornelius, OR) in room air and 5% carbon dioxide at 37°C. The cardiomyocytes were grown in Dulbecco's modified Eagle's medium (DME/F12) (Sigma, St. Louis, MO), with the addition of 1 gm of NaHCO₃, 1.19 gm of HEPES into 475 mL

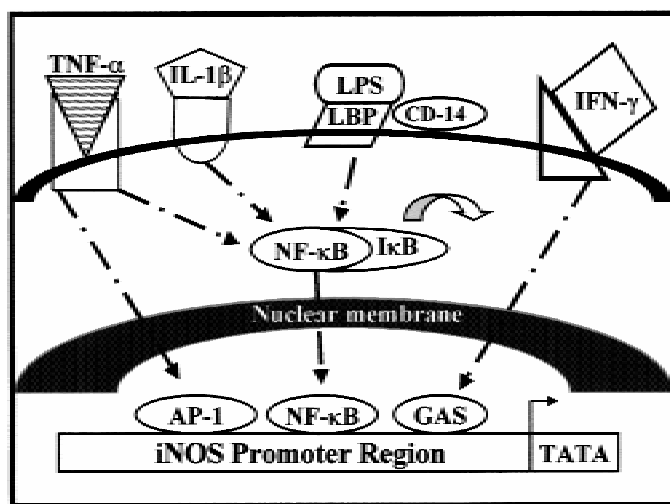


Figure 1. Cytokine modulation of the iNOS promoter. IL-1 β , and LPS converge at the nuclear factor κ B (NF- κ B) binding site on the iNOS promoter to activate transcription. IFN- γ binds the gamma-activating sequence (GAS), and TNF- α can diverge either to a pro-apoptotic pathway with activator protein (AP-1) or as a pro-inflammatory mediator and merge with NF- κ B to enhance iNOS transcription.

of double distilled H₂O (ddH₂O). The medium was then enriched with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), endothelial growth factor (Sigma, St. Louis, MO), and penicillin/streptomycin (Sigma, St. Louis, MO) (1 mL/500 ml).

The macrophage were incubated in a humidified incubator with room air and 5% carbon dioxide at 37°C. The murine macrophage were grown to confluency to ensure a homogenous cell population in the aforementioned medium utilized for cardiomyocytes.

Stimulation Treatment

Dose–response curves, not shown, were generated using macrophage with TNF- α and LPS stimulation to determine what concentration of dexamethasone would attenuate iNOS expression and NO production in the macrophage. This dexamethasone concentration of 50 μ g/mL of culture medium was used in both cell types. Following confluent growth, the macrophage ($N = 6$) and myocytes ($N = 3$) in the (+) dexamethasone treatment group were incubated with dexamethasone (Sigma, St. Louis, MO) (50 μ g/mL) for 8 hours before stimulation. Cells were then, again incubated with dexamethasone (50 μ g/mL), as well as, the stimulation cocktail, TNF- α (Calbiochem, San Diego, CA) (10 ng/mL) and LPS (Sigma, St. Louis, MO) (10 μ g/mL) for another 8 hours. This dexamethasone concentration of 50 μ g/mL of culture medium was used in both cells types. Cells in the stimulated groups received TNF- α and LPS only, where cells in the (+) dexamethasone received dexamethasone and TNF- α and LPS. Control groups received neither stimulation nor dexamethasone. At this time, cells were then rinsed with phosphate-buffered saline (PBS), lifted from the flask with gentle scraping, and transferred into tubes. A 10% dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) was made fresh, and cells were frozen in this solution at -70°C until the time of assay.

Fluorescence Staining Protocol

The myocytes and macrophage were thawed and rinsed in PBS solution and centrifuged at 500 g . Cells were permeabilized, and stained with a primary antibody (Sigma, St. Louis, MO) specific to iNOS and incubated for an hour in darkness. Following a rinse, cells were incubated with secondary antibody to the primary antibody and prepared for flow cytometry. A more specific fluorescence staining protocol is available in the publication by (44).

Spectrophotometric NO Analysis

Spectrophotometric NO analysis kit (Bioxytech, Oxis International, Portland, OR) was utilized to conduct this examination. This kit is designed to reduce nitric oxide radicals, such NO⁻, NO[•], and NO₃⁻ to nitrite, which is detected by differences in absorbance of light. These radi-

cals are collectively referred to as NO_x. The culture media from each flask of cells was stored and frozen for determination of the concentration of NO_x. The enclosed instructions were followed with a few exceptions, which can be followed in (44).

Statistical Method

The sample data were compared using the Student's t -test statistical analysis. The FACS data were expressed as peak fluorescence. The percentage change was calculated with the standard percentage change formula, the difference between the origin and the endpoint divided by the origin. Nonlinear regression analysis and correlation testing were conducted to test the relationship between iNOS expression and the generation of NO production in macrophage. The sample data were analyzed using Student Minitab 4.0 (Minitab Corporation, USA) and Microsoft Excel 7.0 (Microsoft Corporation, USA). P -values less than .05 were accepted as statistically significant.

RESULTS

Induction of iNOS Expression in Cardiomyocyte and Macrophage Cultures

The FACS data are summarized in Table 1 for both cell types. The column labeled, "Control" represents the basal expression of iNOS without stimulation with TNF- α and LPS. The adjacent column labeled, "TNF + LPS" denotes iNOS expression following a challenge with TNF- α and LPS for 8 hours. The third column classified, "(+) Dexamethasone" indicates the expression of iNOS following an 8-hour pretreatment and 8-hour coinubation of dexamethasone and the stimuli, TNF- α and LPS. The myocyte control group peak fluorescence was 199.0 ± 11.5 , standard deviation of the mean. Peak fluorescence in the TNF + LPS group was 153.6 ± 68.2 , the standard deviation of the mean. The (+) dexamethasone group was found to have a peak fluorescence of 205.8 ± 18.5 , the standard deviation of the mean. Among the cardiomyocytes, no statistically significant increase among the three groups was demonstrated in iNOS expression. Figure 2 represents the peak fluorescence among the cardiomyocytes.

The FACS data for the macrophage demonstrate an elevated response with stimulation as compared to control. The control column again indicates the basal expres-

Table 1. Peak fluorescence for cardiomyocytes and macrophage treatment groups.

Peak Fluorescence	Control	TNF + LPS	(+) Dexamethasone
Cardiomyocytes	199.0 ± 11.5	153.6 ± 68.2	205.8 ± 18.5
Macrophage	$53.5 \pm 2.2^*$	$233.1 \pm 4.9^*$	$111.43 \pm 9.0^*$

*Denotes $p < .01$.

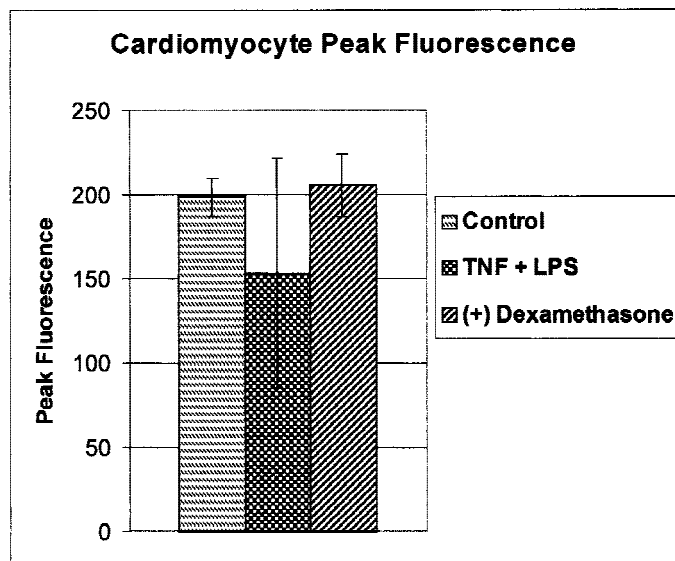


Figure 2. Peak fluorescence among cardiomyocyte treatments.

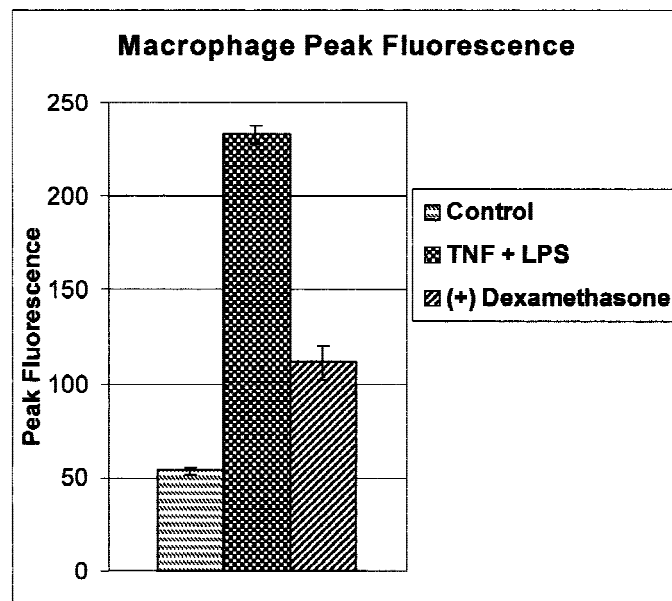


Figure 3. Peak fluorescence among macrophage treatments.

sion of iNOS without stimulation. Likewise, the TNF + LPS column in Table 1 illustrates the iNOS induction following exposure to TNF- α and LPS for 8 hours. The last column represents the effect of dexamethasone on iNOS expression. The stimulated macrophage exhibit a 335% increase over control ($p < .0001$). The macrophage pretreated with dexamethasone demonstrated a distinct reduction of iNOS expression 199% from the level of the stimulated groups ($p < .0003$). The difference between control and (+) dexamethasone in the macrophage was statistically significant ($p < .008$). Figure 3 represents the peak fluorescence among the macrophage.

Spectrophotometric Analysis of NO Production

The NO_x concentration data for both cell types are summarized in Table 2. NO_x concentration, for the purposes of this discussion is synonymous with NO concentration or NO production. The media that cardiomyocytes were grown in was analyzed for the presence of NO_x. Table 2 illustrates the mean and standard deviation of the mean for NO production in the macrophage and myocytes. The control column corresponds to the control column in the Table 1; likewise, the TNF + LPS and (+) dexamethasone columns also represent the corresponding NO concentrations from the iNOS expression in Table 1. The control group of cardiomyocytes generated a NO concentration of 13.2 $\mu\text{M} \pm 4.7$ the standard deviation of the mean. The stimulated myocyte group produced 5.6 $\mu\text{M} \pm 1.8$, the standard deviation of the mean. The (+) dexamethasone treatment displayed a NO concentration 21.4 $\mu\text{M} \pm 1.5$, the standard deviation of the mean. The differences among the myocyte NO concentration groups were found

Table 2. NO concentration for cardiomyocyte and macrophage treatment groups.

	Control	Stimulation	Dexamethasone
Cardiomyocytes	13.2 \pm 4.7	5.6 \pm 1.8	21.4 \pm 1.5
Macrophage	12.9 \pm 6.5*	76.3 \pm 4.9*	25.3 \pm 1.6

*Denotes $p < .01$.

to be not statistically significant. Figure 4 represents the NO concentration among the three cardiomyocyte groups.

The media from the macrophage group was analyzed for NO concentration. The data can be found in Table 2. Similar to the myocytes, the macrophage NO concentration data correspond to the iNOS expression data found in Table 1. The macrophage control group generated a NO concentration of 12.8 $\mu\text{M} \pm 6.5$, the standard deviation of the mean. The group stimulated with TNF- α and LPS demonstrated a concentration of 76.3 $\mu\text{M} \pm 4.9$, the standard deviation of the mean. This difference corresponds to an increase of 498% over control. The elevation is statistically significant ($p < .0005$). The stimulated macrophage treated with dexamethasone demonstrated a 25.3 $\mu\text{M} \pm 1.6$, the standard deviation of the mean. This concentration of NO, represents a decrease of 202% from the stimulated NO concentration. This decrease in NO concentration from TNF + LPS to (+) dexamethasone is statistically significant ($p < .002$). The difference between control and (+) dexamethasone was found to be not significant. Figure 5 demonstrates the NO concentration among the three macrophage groups.

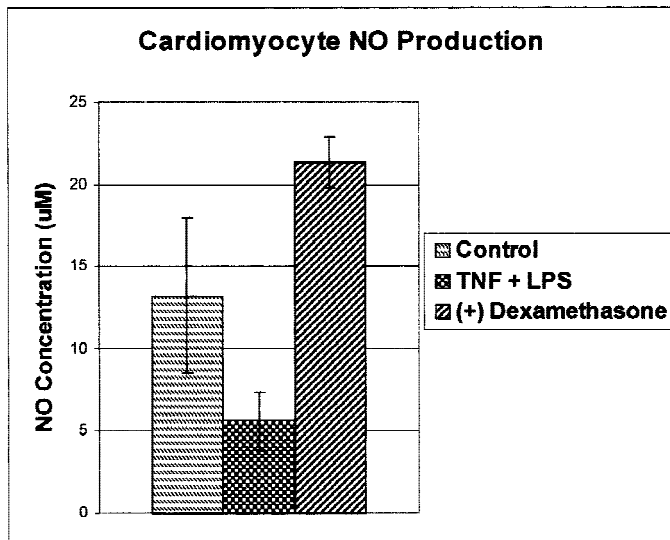


Figure 4. NO production among cardiomyocytes.

Regression and correlation analysis was examined in the macrophage groups to ascertain whether a relationship between expression of iNOS and the concentration of NO exists. The correlation value was found to be 0.939 ($p < .0001$), which indicated a strong relationship between iNOS peak fluorescence and NO concentration. The regression analysis confirmed a linear relationship with an R^2 -value = 0.881. Figure 6 demonstrates the linear regression analysis for the peak fluorescence and NO production.

DISCUSSION

The findings from this in vitro experiment indicate that in the presence of TNF- α and LPS, iNOS is stimulated to produce large quantities of NO in the macrophage. The macrophage cell groups stimulated with TNF- α and LPS show a 335% increase from control. The NO production is concomitantly increased (498%) in these same stimulated groups. The stimulated groups of macrophage pretreated and treated with dexamethasone reveal a 199% decrease in iNOS expression toward basal level and a 202% decrease in NO production. This research suggests a potential prophylaxis in dexamethasone and other glucocorticoids for the prevention of cytokine-induced NO production and other inflammatory processes generated by macrophage. The cardiomyocyte groups treated with TNF- α and LPS and dexamethasone exhibit no statistically significant change in iNOS expression or NO production. The fetal nature of these cells or the small number of repetitions ($N = 3$) could be likely contributors to this response. The fetal nature of cells is perhaps an explanation for the lack of response caused by to some as-

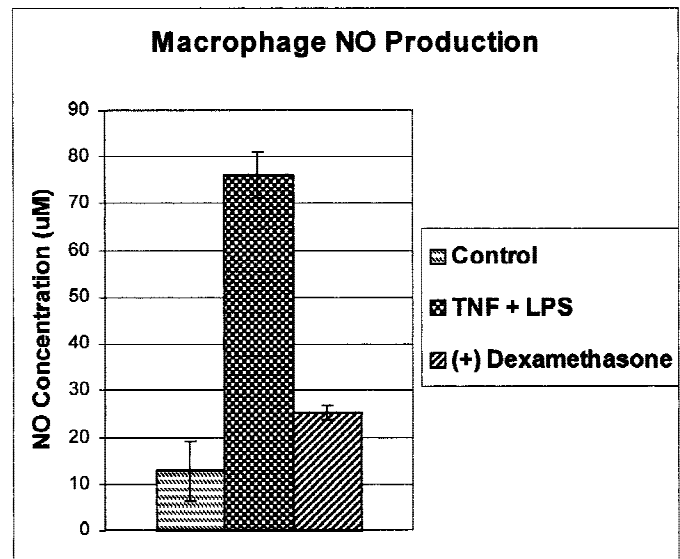


Figure 5. NO production among macrophage. The macrophage groups demonstrated a statistically significant difference at a $p < .01$ among the two groups, Control vs. TNF + LPS, and TNF + LPS versus (+) Dexamethasone; however, Control versus (+) Dexamethasone was not statistically significant.

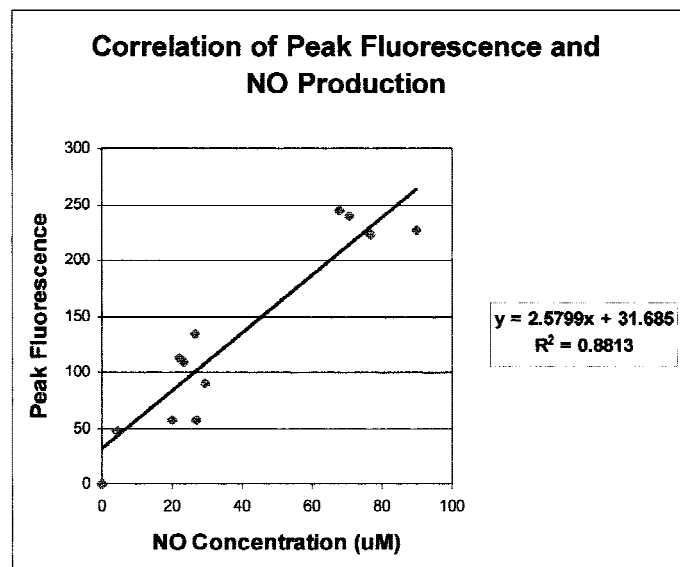


Figure 6. Regression analysis of iNOS peak fluorescence and NO concentration.

pect of the produced iNOS enzyme; that is, enzyme kinetics, lack of an inducible enzyme, receptor discrepancy, or differences in signal transduction cascade between fetal cells and immortalized cells. The small number of repetitions may contribute in a statistical manner; however, this is unlikely.

The cytokines, IFN- γ , TNF- α , and IL-1 β , and LPS mediate their activity through the binding of their respective

response element on the promoter of the iNOS gene. Figure 1 illustrates how the iNOS promoter is activated by each of the cytokines. The TATA box is a region on the iNOS promoter where RNA polymerase recognizes and begins to synthesize mRNA. LPS is bound extracellularly by lipopolysaccharide binding protein (LBP) and CD-14, which transduces an intracellular signal via cascade of cytoplasmic mediators (45). As a result, nuclear factor- κ B (NF- κ B) is dephosphorylated, releasing inhibitory- κ B (I- κ B), it then traverses the nuclear membrane and binds the κ B response element on the promoter of iNOS to activate gene transcription (46).

Similarly, IL-1 β binds to its dimerized, extracellular receptor and initiates a cascade, resulting in NF- κ B-mediated gene transcription augmentation (47). Conversely, IFN- γ , binds its extracellular receptor complex, and initiates an intracellular cascade that activates the gamma-activating sequence (GAS) on the iNOS promoter, resulting in increased gene transcription (48).

Finally, TNF- α demonstrates a dual pathway of iNOS activation. TNF- α can operate via NF- κ B activation (49), initiating a pro-inflammatory cascade, or it can activate a different pathway causing the dimerization and translocation of *c-fos* and *c-jun* to the nucleus. The dimer then binds activating protein-1 (AP-1) on the iNOS promoter generating an increase in gene transcription (50). Activation of the AP-1 cascade is generally considered proapoptotic and outside the context of this discussion. As shown in previous research in this lab, IFN- γ , TNF- α , and IL-1 β have little or no effect in vitro. However, when these cytokines are co-incubated with LPS or one another, the activation of iNOS protein expression is appreciated.

The mechanism of action of dexamethasone-induced attenuation of iNOS expression seems to be mediated through blockade of normal NF- κ B translocation to the nucleus (51). More specifically, the inhibitory action of dexamethasone with respect to proinflammatory cytokine-induced iNOS expression and NO production is propagated at different levels. The deactivation of NF- κ B results from decreased dephosphorylation, degradation, and decreased transcription of I- κ - α , liberating more NF- κ B for nuclear translocation (52). Furthermore, the data from Katsuyama et al. indicate that via protein-protein interaction, glucocorticoid receptor activation prevents the binding of NF- κ B to the iNOS promoter, hence blocking induction of iNOS transcription (52).

The introduction of glucocorticoids into the treatment regimen should not be limited to treatment of cytokine-induced NO production alone. Glucocorticoids have proved efficacious in the reduction of elevated proinflammatory cytokines themselves. Specifically, glucocorticoids with anti-inflammatory potential, such as dexamethasone, have been shown to repress interleukin-6 gene expression in a NF- κ B-dependent mechanism (53). Other research

has established similar results with regard to TNF- α , IL-1 β , IL-8, and IL-16 (54–61). These same cytokines have been indicated in the depression of cardiac efficiency via an NO-dependent pathway (62). So, dexamethasone and other glucocorticoids are not only a potential prophylaxis for iNOS-induced NO production, these compounds can be of therapeutic value in the reduction of certain proinflammatory cytokine elevations or the downstream effects thereof.

Although studies have demonstrated the anti-inflammatory effects of glucocorticoids during cardiopulmonary bypass (63–65), the exact mechanism of this benefit remains to be completely discerned. This research suggests that glucocorticoids may mediate their anti-inflammatory effects via attenuation of NF- κ B-dependent mechanisms in the macrophage or neutrophils; thereby, blocking the effects of proinflammatory cytokines and NO production.

CONCLUSIONS

Proinflammatory cytokines and NO can have serious systemic effects, such as multiple organ dysfunction when not treated. This presented data, as well as the comprehensive review of the literature demonstrate clearly a potential prophylactic role for dexamethasone or other glucocorticoids in the conduct of cardiopulmonary bypass, in addition to other inflammatory states that potentiate cytokine expression and cytokine-induced NO production. These pharmaceutical products are readily available and a staple of most hospital formularies.

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