

**Original Article**

## ***In Vitro Comparison of Inhibitors of Inducible Nitric Oxide Synthase in a Macrophage Model***

Kyler Hunter, BS; D. Bradford Sanders, BS; Douglas F. Larson, PhD, CCP

Graduate Program in Circulatory Sciences, University of Arizona, Tucson, Arizona

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### **ABSTRACT**

Nitric oxide (NO) has been shown to decrease cardiac performance, induce global hypotension, and generate oxygen free-radicals. Nitric oxide is produced from the conversion of L-arginine to L-citrulline by inducible nitric oxide synthase (iNOS) and is a component of many cellular second messenger systems. It is not clearly understood if NO and iNOS are compensatory mechanisms or pathological processes in heart failure, and this study was designed to understand better inhibition of iNOS in a cell culture model.

Inhibitors of iNOS were compared for in vitro capability of inhibiting the production of NO. Ethanol and *S*-methylisothiurea (MITU) were applied to macrophage populations in 120  $\mu\text{M}$  and 1  $\mu\text{M}$ , 100 and 10 nM for an 8-h incubation. Level of iNOS expression was measured in the ethanol-treated populations using an anti-iNOS primary antibody with a fluorescent labeled secondary antibody. Serum nitrites were measured in both treatment groups by the nonenzymatic Griess method to determine enzyme function.

Our data indicate that ethanol demonstrates a stimulation and simultaneous inhibition of iNOS during an 8-h incubation. No dose-dependent correlation between amount of serum nitrites produced and ethanol treatment was observed. However, MITU demonstrated a clear inhibition of iNOS at 120  $\mu\text{M}$  with a serum nitrite value of  $25.7002 \pm 0.0647$ , with control values of 24.3421  $\mu\text{M}$ . Lower concentrations of MITU also demonstrated no correlation. Although both agents display inhibitory effects upon iNOS, MITU seems to have no apparent simultaneous stimulation and may hold more potential as a post-translational inhibitor of iNOS.

Address correspondence to:  
Douglas F. Larson, PhD, CCP  
Graduate Program in Circulatory Sciences  
University of Arizona  
Arizona Health Science Center  
Tucson, AZ 85724  
dflarson@u.arizona.edu

## INTRODUCTION

Inducible nitric oxide synthase (iNOS) is a nonconstitutive cytoplasmic enzyme expressed by several different cell types, including neurons, kidney tubule cells, macrophages, and neutrophils. It is responsible for the conversion of L-arginine to nitric oxide (NO) and L-citrulline. Different isoforms of nitric oxide synthase (NOS) exist, including neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and iNOS. iNOS is cytoplasmic enzyme expressed in many mammalian cell types and can be produced on demand as a result of stimulation. Macrophages and neutrophils express large amounts of the enzyme for destruction of pathogenic microbes, virus-infected, and necrotic cells. Nitric oxide is a highly reactive species with a half life of approximately 7 sec and can activate many cellular second messengers, such as cyclic adenosine monophosphate (cAMP) and cyclic guanine monophosphate (cGMP). These second messengers can, in turn, affect such ionic gradients as calcium (1–3). Calcium is well understood as important for muscle contraction in skeletal, vascular, and cardiac myocytes, and, consequently, interruption in calcium transients by iNOS through NO can negatively affect cardiac function.

Known inhibitors of iNOS exist, including aminoguanidine, S-methylisothiourea (MITU), L-nitro-amino-methyl-ester, agmatine, andrographolide, and ethanol. Other anti-inflammatory agents, such as dexamethasone and methylprednisolone, decrease the expression of iNOS (4, 5); whereas, the aforementioned agents affect enzyme function. Although in chronic exposures, inhibition of iNOS transcription is possible with ethanol (6, 7).

Cardiopulmonary bypass generates a well-documented inflammatory response that can result in elevated levels of proinflammatory cytokines (8, 9) and monocyte/macrophage populations (10). These cytokines can result in an activation of cellular second messengers in the myocardium that decrease cardiac contractility (11–13). In the postoperative period, this can contribute to delayed patient recovery as well as increased morbidity. Similarly, these same cytokines are elevated in heart failure (14–16) and would also lead to decreased cardiac contractility. Although it is possible that (9, 10, 14) an anti-inflammatory agent would alleviate some of the cytokine-induced myocardial decompensation, this may also contribute to immunosuppression and further complicate recovery. Inhibitors of iNOS may serve a better purpose in these scenarios than anti-inflammatory agents in preventing the production of NO.

To determine the function of iNOS, serum nitrites were analyzed by Griess method. The Griess method involves the spectrophotometric analysis of nitric oxide by conversion into nitrates in the presence of cadmium metal. This method cannot directly determine NO levels because of its high reactivity and conversion to nitrates and nitrites in the presence of oxygen. As a consequence, serum nitrates can only be measured as the stable end products of NO formation through the Griess

method. Quantification of iNOS levels was accomplished through labeling iNOS with an anti-iNOS primary antibody. A fluorescent secondary antibody specific for the primary was then attached, and mean fluorescence was determined by flow cytometry.

## METHODS

### CELL CULTURE

Murine macrophage populations were maintained in Dulbecco's Modified Eagle's Medium F-12 HAM Nutrient Mixture<sup>a</sup> in 10% fetal bovine serum.<sup>b</sup> Liquid media contained penicillin and streptomycin as preventatives against microbial contamination. Cells were allowed to reach confluence in the 25-cm<sup>3</sup> flasks before analysis of iNOS or NO. Cells were treated with 50  $\mu$ l of lipopolysaccharide (LPS) (1  $\mu$ g/mL) and 125  $\mu$ L of murine interferon- $\gamma$  (IFN- $\gamma$ ) (125 ng/mL) to induce iNOS, and ethanol concentrations were added immediately. All cells were allowed an 8-h incubation. Cell culture grade ethanol was utilized in 10 nM, 100 nM, 1  $\mu$ M, and 120  $\mu$ M concentrations diluted with sterile water. Parafilm was used to prevent ethanol evaporation from the canted neck flask by wrapping the cap tightly. Control populations received no LPS, interferon- $\gamma$ , or ethanol, and positive controls received LPS and interferon- $\gamma$  only.

### QUANTIFICATION OF iNOS EXPRESSION

Cells were quantified for iNOS expression using an anti-inducible nitric oxide synthase antibody (Sigma, Inc.) and a FITC secondary antibody (Dako, Inc.) and assayed by flow cytometry. Cells were prepared for the staining procedure by harvesting and suspension in 100  $\mu$ L of Fixation Medium A (CalTag Industries) in a sterile 15-mL Falcon Tube (Beckton Dickinson), and a 45-min incubation at room temperature. Cells were then washed with phosphate buffered saline (PBS) and centrifuged at 4500 rpm for 10 min. Excess PBS was removed by vacuum suction, and the cell pellet was resuspended in 90  $\mu$ L (1:200) of the anti-iNOS antibody and allowed 10 min, and the excess PBS was removed by vacuum suction. One hundred  $\mu$ L (1:20) of the FITC secondary antibody was added in minimal lighting and was allowed to incubate for 60 min in minimal lighting. Following incubation with the secondary antibody, cells were washed in PBS, centrifuged at 4500 rpm, and the excess PBS was removed by vacuum suction. The cell pellet was resuspended in 1.0 mL of PBS, and mean fluorescence was determined within 1 h at the Shared Services Flow Cytometry Lab at the University of Arizona Cancer Center.

### DETERMINATION OF SERUM NITRATES/NITRITES

Serum NO determination was accomplished using the non-

<sup>a</sup> Sigma-Aldrich, St. Louis, MO.

<sup>b</sup> HyClone Laboratories, Logan, UT.

enzymatic Griess Method (Oxis International). Spectrophotometric analysis of serum nitrites was done by conserving the liquid media of each flask of macrophages with their corresponding ethanol concentrations and freezing in  $-80^{\circ}\text{C}$ . Samples were thawed and assayed at convenience. Serum was deproteinized by adding  $75\ \mu\text{L}$  of the media to  $10\ \mu\text{L}$  of  $\text{ZnSO}_4$  and  $115\ \mu\text{L}$  of distilled water. A 15-min incubation at room temperature was allowed, which was followed by centrifugation at 3000 rpm for 10 min to separate protein from supernatant. The supernatant was then removed and added to 0.5–1.0 gram of cadmium beads and placed on a plate rocker overnight at room temperature. The following day,  $100\ \mu\text{L}$  of each sample was pipetted into a microplate with  $50\ \mu\text{L}$  of each color reagent and briefly mixed with a plate rocker for 15 min at room temperature. Raw absorbance was determined at 550 nm, and a standard curve and subsequent equation was generated with known concentrations to extrapolate the concentrations of nitrites.

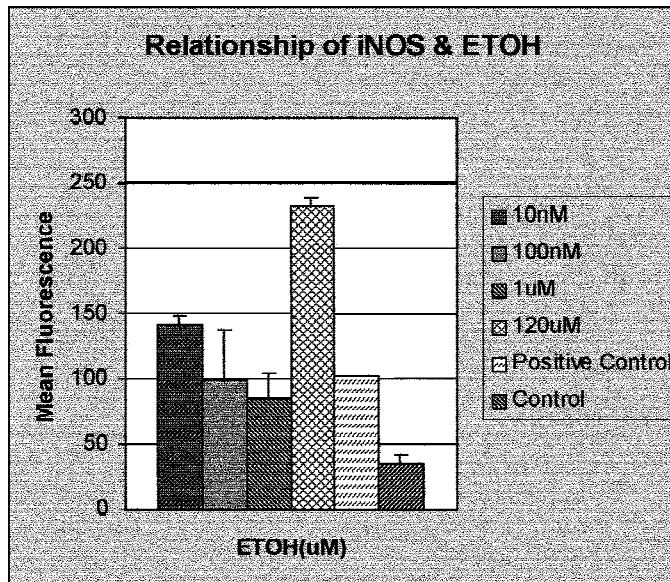
## RESULTS

Values for serum nitrite concentrations and mean fluorescence are shown in Table 1 for ethanol-treated macrophages, and Figure 1 displays the relationship of iNOS expression and ethanol treatment. iNOS expression peaks with  $120\ \mu\text{M}$  ethanol; whereas, lowest expression was observed in  $1\ \mu\text{M}$  concentration. Ethanol concentrations of 10 nM, 100 nM, and  $1\ \mu\text{M}$  demonstrated a decline in serum nitrites, but no overall correlation between treatment of ethanol and serum nitrite was observed. Control values for serum nitrites were  $24.3421\ \mu\text{M}$ , and ambient serum nitrites of media alone were  $10.4018\ \mu\text{M}$ .

Table 2 displays the ratios of serum nitrites and mean fluorescence of ethanol-treated populations with respect to control populations. The ratios of mean serum nitrites differed only slightly from the lowest value of  $3.5037\ \mu\text{M}$  for  $120\ \mu\text{M}$  ethanol treatment and the highest at  $4.3007\ \mu\text{M}$  at 10 nM. These serum nitrite values seem to decrease with increasing ethanol concentrations. The ratio of mean fluorescence has some degree variance as the ethanol concentrations increase. At 10 nM ethanol, the fluorescence is 4.0478 that of control populations, and decreases for 100 nM and  $1\ \mu\text{M}$  concentra-

**Table 1: Mean serum nitrites and fluorescence of ethanol treated macrophages.**

Ethanol concentration	Griess method mean serum nitrites ( $\mu\text{M}$ )	Mean fluorescence
120 $\mu\text{M}$	$85.29 \pm 2.50$	$232.59 \pm 19.64$
1 $\mu\text{M}$	$99.41 \pm 0.95$	$84.60 \pm 40.31$
100 nM	$89.47 \pm 0.3019$	$98.09 \pm 6.45$
10 nM	$104.69 \pm 3.1060$	$142.22 \pm 18.62$
Control	24.34	$35.13 \pm 7.05$
Positive control	$87.82 \pm 4.62$	$88.73 \pm 0.47$
Media only	$10.401 \pm 3.15$	—



**Figure 1: Relationship of iNOS and ETOH.**

tions. At the highest ethanol concentration, mean fluorescence increases to 6.6198 at  $120\ \mu\text{M}$ , a substantial increase in fluorescence from 100 nM and  $1\ \mu\text{M}$  ethanol concentrations.

Table 3 shows the serum nitrite values for MITU treatments.  $120\ \mu\text{M}$  MITU showed the greatest inhibition of iNOS with serum nitrite concentration of  $25.7002\ \mu\text{M}$ , close to that of control values at  $24.3421\ \mu\text{M}$ . 10 nM treatment of MITU resulted in a serum nitrite concentration of  $92.7225\ \mu\text{M}$ , close to that of positive control values of  $102.9\ \mu\text{M}$ . Serum nitrite values for MITU concentrations of 10 nM, 100 nM, and  $1\ \mu\text{M}$  varied and showed no correlation between treatment and nitrite production.

## DISCUSSION

### ETHANOL TREATMENT

Our results seem to have no dose-dependent correlation between ethanol treatment and serum nitrite production. Table 1 demonstrates little change in serum concentration of nitrites as ethanol concentration increases from control to  $120\ \mu\text{M}$  treatment. However, they also seem to indicate that ethanol has a

**Table 2: Ratio of mean serum nitrites and ratio of mean fluorescence of ethanol treated macrophages.**

Ethanol concentration	Ratio of mean serum nitrites to control serum nitrites	Ratio of mean fluorescence to control fluorescence
120 $\mu\text{M}$	3.50	6.62
1 $\mu\text{M}$	4.08	2.40
100 nM	3.67	2.79
10 nM	4.30	4.05
Positive control	3.61	2.525

Table 3:

MITU concentration	Griess method mean serum nitrites ( $\mu\text{M}$ )
120 $\mu\text{M}$	25.70 $\pm$ 0.06
1 $\mu\text{M}$	98.67 $\pm$ 16.97
100 nM	121.63 $\pm$ 16.52
10 nM	92.72 $\pm$ 6.09
Control	24.3421
Positive control	102.9 $\pm$ 0.47
Media only	10.40 $\pm$ 3.15

stimulating and inhibitory affect on level and function of iNOS present in an 8-h time span. The serum nitrite values are similar to positive control values, indicating a stimulation of the production of nitric oxide was occurring despite the presence of ethanol.

However, by comparing the mean fluorescence and serum nitrites of the ethanol-treated populations with control values in Table 2, ethanol seems to retain its inhibitory characteristic of iNOS at higher concentrations. At 120  $\mu\text{M}$ , mean fluorescence is 6.6198 times the control value, indicating a higher iNOS level; whereas, serum nitrite concentrations are 3.5037 times the control values. Ethanol seems to produce a greater stimulation of iNOS production at this concentration, because the fluorescence is approximately seven times that of control values, and serum nitrites have three and one-half times the value of control populations. However, the same observation is not true for 1  $\mu\text{M}$ , which has 2.408 times the mean fluorescence as control with 4.084 times the amount of serum nitrites, indicating a greater stimulation of the production of NO. The stimulatory observation also seems to hold for 100 nM ethanol and seems to have an absolute stimulatory affect at 10 nM, because

the proportional increase in serum nitrites and mean fluorescence is close in value.

Although it is clear our results point to a differential level of iNOS over a wide range of ethanol concentrations, these changes in iNOS resulted in little change in serum nitrites (104.6896  $\mu\text{M}$  for 10 nM; 85.2891  $\mu\text{M}$  for 120  $\mu\text{M}$ ). Our results point to a greater inhibitory affect of ethanol, despite its tremendous stimulatory affect. However, this did not correlate in a dose-dependent manner.

**MITU TREATMENTS**

Our results with MITU seem to demonstrate a total inhibition of iNOS at 120  $\mu\text{M}$ , with serum nitrite values of 25.7002  $\mu\text{M}$  near that of control values at 24.3421  $\mu\text{M}$ . However, lower concentrations of MITU seemed to have a lesser degree of inhibition of iNOS, because the serum nitrite values did not correlate in a dose-dependent manner as expected. Similar to ethanol, lower concentrations of MITU had higher serum nitrite values than that of positive controls. Although it is possible that MITU may be stimulating the expression of iNOS, quantification of iNOS was not determined of MITU-treated populations and have yet to confirm what effects, if any, exist. An  $\text{IC}_{50}$  of 120  $\mu\text{M}$  MITU for inhibition of iNOS has been previously reported (17), but similar to our results, had little effect on serum nitrites for cytokine-induced macrophage populations. However, amino-methyl-isothioureas are reported as highly impermeable to lipid membranes and irreversible inhibitors of NOS (17). It is possible throughout the 8-h incubation that macrophage populations produced more iNOS to compensate for the lack of local NO by a self-feedback mechanism. This may explain the higher levels of serum nitrites observed in the 100 nM MITU concentrations.

It is not clearly understood if NO and iNOS are a compensatory mechanism or a pathological process in heart failure, but

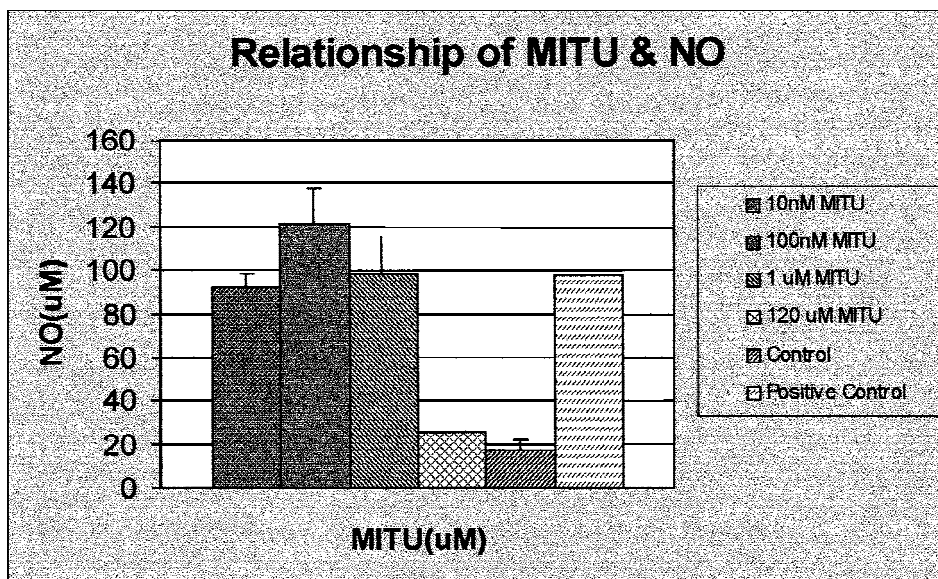


Figure 2: Relationship of S-methylisothiourea (MITU) to nitric oxide (NO).



it is possible that NO is an essential component of normal cardiovascular physiology. However, further investigation of its effects on the myocardium directly could only determine its compensatory or pathological role.

## CONCLUSIONS

Inasmuch as inhibition of iNOS through ethanol is a well-documented phenomenon, our results with ethanol treatment of macrophage populations for 8 h does not indicate a dose-dependent inhibition of iNOS (18–23). It is entirely possible that at shorter time periods of exposure, ethanol maybe a pure inhibitor of iNOS in cytokine-stimulated macrophage populations, in contrast to its stimulatory and inhibitory actions at 8 h of exposure. It is unknown at what time period ethanol may become a stimulant of iNOS in these macrophage populations, and determination of serum nitrites and levels of iNOS at earlier time points needs to be performed to determine this.

MITU seems to be a clear inhibitor of iNOS at higher concentrations (120  $\mu$ m), but presents similar actions as ethanol at lower concentrations. It is difficult to make any solid conclusions on the effect of MITU without the level of iNOS, because further studies must be completed to determine the level of iNOS expression in the MITU macrophage populations. It is possible that sampling of earlier time points could provide greater clarity as to the role of MITU in iNOS inhibition or stimulation.

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