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LIDOCAINE METABOLISM IN ENDOTOXIN INDUCED SEPSIS

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LIDOCAINE METABOLISM IN ENDOTOXIN INDUCED SEPSIS

BY

JUDAH BOULET

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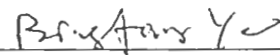UNIVERSITY OF RHODE ISLAND
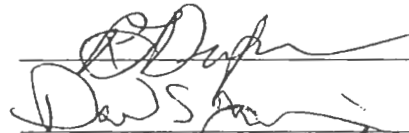
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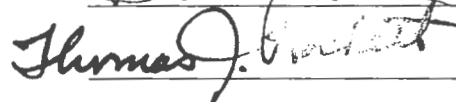
MASTER OF SCIENCE THESIS

OF

JUDAH A. BOULET

APPROVED:

Thesis Committee

Major Professor _____

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DEAN OF THE GRADUATE SCHOOL

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Abstract

Septic shock is the body's inflammatory response to an infection, and is usually associated with gram negative bacteria. It is characterized by hypotension and hypoperfusion, despite adequate fluid resuscitation, and can lead to multiple organ failure. At these advanced stages there is a high morbidity and mortality rate associated with it. One mediator of this inflammatory response is a lipopolysaccharide (LPS), or endotoxin, found in the gram negative bacterial cell wall.

Besides having effects on the body as a whole, there are many cellular processes affected by endotoxin. In the body, there is a family of enzymes, the cytochrome P450 enzymes, which are the body's drug metabolizing enzymes. Although found in all tissue types, the P450 enzymes are mainly located in the liver. There are many families and isoforms of these enzymes, of which several are inhibited by endotoxin. The regulation of the P450 enzymes is very complex. During the systemic inflammatory response to endotoxin, many cytokine mediators are released into the bloodstream. These mediators, specifically tumor necrosis factor-α, interleukin-1, and interleukin-6, all are capable of regulating the activity, and production of the P450 enzyme system.

Lidocaine is a widely used antiarrhythmic drug, which is metabolized by cytochrome P450 enzymes, to its major metabolite monoethylglycinexylidide (MEGX). Lidocaine is metabolized specifically by P450 3A2, 2C11, and 2B1 in the rat. The conversion of lidocaine to MEGX has been looked at as a method of evaluating liver function or dysfunction in a variety of liver related diseases, including viral hepatitis, cirrhosis, liver transplantation, and hypovolemic shock.

This present study was undertaken to determine whether the conversion of lidocaine to MEGX was inhibited *in vivo*, in rats given a lethal dose of endotoxin. It was hoped that the degree of drug metabolizing inhibition could be determined using this whole animal model, since many of the systemic effects of septic shock, i.e. hypotension, also factor into the clearance of a high extraction compound, like lidocaine. Results from this study could potentially be helpful in determining drug metabolizing alterations in humans experiencing septic shock.

Acknowledgements

I would like to thank Dr. Clinton Chichester for serving as my major professor and providing me with the guidance, support, resources and encouragement necessary for completion of my thesis. I would also like to thank Dr. David McKindley, for playing a large role in guidance, support and resources. I thank Dr. Bingfang Yan and Dr. Robert Dufresne for being on my committee and offering any help they could. I also would like to thank Dean Joanne Lausier for accepting the responsibility to serve as the chairperson at my defense.

I thank the following graduate students for their support: Brian Felice, Tom Daniels, Swapnil Raut, Selma Sadovic, Weifang Tang, Uma Krishnamoorthy, Maowen Hu, and Guangzu Wang. I would also like to thank Dr. Shaikh for sharing his knowledge with me in both conversation and in the classroom. I thank Dr. Babson, and Dr. Rodgers for their wisdom in class and in discussion on matters of both science and life.

I dedicate this thesis to my parents who have always inspired and encouraged me to be successful, and have also provided me with any help, both financial and emotional throughout the years. I also dedicate this to Mrs. Jeraldine Ferry, for it was through her inspiration that led me to choose a career in science.

Preface

This thesis was written in accordance with the manuscript format.

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Abstract

Gram-negative bacterial endotoxin causes a systemic inflammatory response, known as sepsis. High exposure to endotoxin can lead to septic shock, which is characterized by hypoperfusion and hypotension, which can ultimately lead to multiple organ failure, and death. One of the organs highly effected by endotoxin is the liver. The liver has a family of enzymes, the cytochrome P450 enzymes, which handle the biotransformation of many xenobiotics, to excretable compounds. Endotoxin inhibits this biotransformation by inhibiting and down regulating many P450 isoforms. This paper describes a study into the metabolism of lidocaine during a endotoxin induced shock. Lidocaine is metabolized via P450 enzymes, to MEGX, the major metabolite. Serum concentrations of lidocaine and MEGX were analyzed at various time points. Blood pressure measurements were also taken to determine if hypotension played any role in reduced lidocaine clearance.

A lethal dose of endotoxin caused severe hypotension eight hours after administration (mean±SEM; 51.7±50.7) when compared to control animals (mean±SEM; 108±9.5). Endotoxin significantly inhibited the clearance of lidocaine *in vivo* at 120 minutes (mean±SEM: 1.16±0.52) when compared to control animals (mean±SEM; 0.08±0.067) ($p < 0.05$). The degree of inhibition was greater than cimetidine treated animals (mean±SEM; 0.15±0.073) though, which is a positive control for P450 inhibition, suggesting that the reduction of blood pressure could possibly be a factor in the clearance ($p < 0.05$). The serum level MEGX was elevated in the endotoxin treated animals (mean±SEM; 626.8±123.2) compared to control (mean±SEM; 137.5±44.2) and cimetidine animals (mean±161.1±71.1) at 120 minutes ($p < 0.05$). It is possible that the

elevated concentration of metabolite in the serum of septic animals is the result of

reduced clearance of metabolites secondary to blood flow in the kidneys, and/or reduced

bile flow. Since the serum MEGX did not appear to reflect the decreased lidocaine

clearance, ratios of lidocaine:MEGX were calculated. A significant difference between

the endotoxin treated and cimetidine treated groups was seen at 15 minutes was seen

(p<0.05). The ratio of lidocaine:MEGX appears to be a potential measure of inhibition of

drug metabolism, but further exploration in this model is needed.

Introduction

Lidocaine, is the most widely used intravenous anti-arrhythmic agent for the management of acute ventricular arrhythmias. (Zito, et. al., 1978) Lidocaine is totally and rapidly metabolized in the liver, (Bennet, et. al., 1982; Keenaghamp, et. al., 1972) with one of its major metabolites produced by oxidative N-deethylation to monoethylglycinexylidide (MEGX). (Nyberg, et. al., 1977) It has been postulated that the conversion of lidocaine to MEGX be used as a marker of liver function and drug metabolism during different disease states. It has been shown that after administration of lidocaine, serum MEGX levels at 15 minutes is a sensitive and specific indicator of hepatic damage, (Berdelski, et. al., 1987; Oellerich, et. al., 1987) compared to indocyanine green and galactose. (Burdelski, et. al., 1988) Lidocaine and its metabolism has been suggested as a way to monitor pre-transplant liver function (Oellerich, et. al., 1987; Schroeder, et. al., 1989) and as an early predictor of rejection in transplant recipients. (Schroeder, et. al., 1989) MEGX formation can also be used for the rapid assessment of liver function in cirrhosis, (Oellerich, et. al., 1990) and after hypovolemic shock. (Chandel, et. al., 1995)

Sepsis is the developed condition of the body's systemic inflammatory response to the presence of an infection in the body by a microorganism, most frequently derived from gram-negative bacteria. When sepsis further deteriorates, septic shock can result. Shock is frequently associated with poor circulation, hypotension and hypoperfusion, and can lead to multiple organ failure. A number of studies have shown that there is severe liver dysfunction following sepsis. (Wang, et. al., 1995; Wang, et. al., 1991) Hepatic function and effective hepatic blood flow, as measured by clearance of indocyanine

green, was decreased in studies using the cecal ligation and puncture septic animal model. (Wang, et. al., 1991; Wang, et. al., 1995) The activity of the cytochrome P450 drug metabolizing enzyme system also changes in sepsis. It has been shown in rats and mice, that after endotoxin administration, P450 2B1 (Stanley, et. al., 1988), 2C11 (Morgan, 1989; Sewer, et. al., 1996; Morgan, 1993), 2C12 (Morgan, 1993), 2C6 (Morgan, 1993), 2C7 (Morgan, 1993), 3A2 (Sewer et. al., 1996) and 2E1(Morgan, 1993; Sewer et. al., 1996) are suppressed.(Ishikawa, et. al., 1991)   Also, it has been shown that there is a significant decrease in drug metabolism in human subjects exposed to significantly smaller dose of endotoxin than would be expected in a clinical case of septic shock. (Shedlofsky, et. al., 1996)

Systemically, endotoxin has profound effects on the heart, other organs and the vasculature, with a major cause of morbidity associated with a hypotensive state, which persists despite adequate fluid resuscitation. (Parillo, et. al., 1993) Endotoxin also inhibits P450 activity, including the enzymes that metabolize lidocaine, P4502B1, P4502C11, and P4503A2, which have the highest MEGX formation turnover in the rat. (Oda, et. al., 1989; Imaoka, et. al., 1990; Nakamoto, et. al., 1997) Although it has been shown that endotoxin inhibits these P450 isoforms, and that these isoforms are responsible for the metabolism of lidocaine to MEGX, lidocaine metabolism during septic shock has not been studied. Since MEGX formation has been considered as a model for liver dysfunction in cirrhosis (Oellerich et. al., 1990), hypovolemic shock (Chandel, et. al., 1995) liver transplantation (Oellerich et. al., 1989; Schroeder et. al. 1989), ischemic liver injury (LeClercq, et. al, 1997) and chronic viral hepatitis (Elin, et. al., 1997), we examined if MEGX formation was a good marker for the metabolic

changes induced by endotoxin. It is thus needed to examine the metabolism of lidocaine to MEGX in the *in vivo* animal model, where the blood pressure, and subsequent liver blood flow are dramatically different between septic and control animals.

Materials and Methods

Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Rhode Island. All reagents were purchased from Sigma Chemical, with the exception of the lipopolysaccharide, purchased from Difco Laboratories, and the TDx analyzer kits, which were graciously supplied by Abbott Laboratories.

Male Sprague Dawley rats, ranging from 250-550g, were administered endotoxin or cimetidine. Endotoxin (E. Coli 026:B6 LPS, lot #91608), 45.6 mg/kg, was injected IP approximately 5 hours before surgery. Cimetidine, 80mg/kg, was injected IP daily for three days prior to surgery. This group served as a positive control for inhibition of cytochrome P450 (Levine, et. al., 1998; Bruck, et. al., 1990; Fruncillo, et. al., 1983) Initial blood pressure readings and lidocaine administration occurred at six hours after endotoxin injection, which was 24 hours after the third cimetidine injection.

In Vivo Study of Blood Pressure

Rats, weighing 250-550 g, were anaesthetized with pentobarbital (50 mg/kg). A mid-line incision from the chin to the upper part of the thorax was made so that both the trachea and left carotid artery could be cannulated. A short cannula was secured in the trachea, while a cannula, filled with a 10 units/ml heparin/saline solution, attached to a 1 mL syringe, was secured into the artery. An infusion of 0.4 mL of the heparin/saline solution was made, and the rat was allowed to stabilize for 30 minutes. After the rat had stabilized, blood pressure recordings were taken at 0, 60, 90, and 120 minutes using a Statham P-23A transducer, and recorded on a Grass Model 7 Polygraph.

In Vivo study of Lidocaine Metabolism

Lidocaine (2mg/kg) was administered via the cannula at time zero. The catheter was then flushed with 0.3 mL of saline. Blood, 0.4 mL, was drawn via the cannula at times 15, 25, 45, 60, 90, and 120 minutes, and centrifuged at 3,000 x g for 3-5 minutes to collect the serum. After each blood withdrawal, the animal was infused with the same amount of saline to make up for fluid loss. Serum samples were analyzed for lidocaine and its major metabolite monoethylglycinexylidide (MEGX) using a fluorescence immunoassay (TDx). (Abbott Diagnostics, Abbott Park, Illinois) The standard curve was linear between 0 and 10 µg/mL for lidocaine, and 0-250 ng/mL for MEGX. Coefficients of variation for each of these assays were 10% at all control concentrations.

Statistical Analysis

Repeated measures ANOVA was used to compare blood pressure data. After a significant overall difference was found, a Student-Newman-Keuls method was used as the pairwise multiple comparison procedure. All lidocaine and MEGX data was tested with a one-way ANOVA, and after a significant difference was found, a Tukey's multi-comparison post hoc test was used. Significance was determined by a p value of <0.05.

Results

Endotoxin had a detrimental effect on the blood pressure. (Figure 1) There was a significant decrease in blood pressure between the endotoxin and the control groups at 90 and 120 minutes ($p < 0.05$) and between the endotoxin and the cimetidine groups at 90 and 120 minutes ($p < 0.05$). The average blood pressure of the endotoxin group dropped from 105 ± 23.5 mmHg, at time zero, to 51.7 ± 50.7 mmHg two hours later, while the blood pressures of the untreated and cimetidine group stayed constant throughout the experiment. The average blood pressure at eight hours after endotoxin administration decreased by approximately 50% of the untreated and cimetidine groups. The blood pressures of the endotoxin group at 60, 90, and 120 minutes were significantly lower than the blood pressures at time zero ($p < 0.05$). After examination of blood pressure data of the endotoxin group, two sub-populations of endotoxin treated rats were distinguished; those that showed a drop in blood pressure (n=4), and those that were unaffected (n=3). (Figure 2) These two groups were significantly different at time points 90, and 120 minutes ($p < 0.05$). The animals whose blood pressure dropped were significantly different versus the control group at 90 and 120 minutes ($p < 0.05$).

Lidocaine elimination was significantly lower than the untreated and cimetidine groups at 120 minutes after administration ($p < 0.05$). (Figure 3) At 120 minutes after lidocaine administration, the endotoxin group had a 14.5 fold and 7.5 fold higher concentration of lidocaine than the untreated and cimetidine groups, respectively. The cimetidine group had a 1.9 fold greater lidocaine concentration than the untreated group.

MEGX concentrations were determined at the same time points as lidocaine. (Figure 4) MEGX serum levels increased during the first 60 minutes after lidocaine

administration and then began to plateau for the last 60 minutes of the experiment in the control groups, while in the endotoxin group, MEGX concentrations continued to increase throughout the experiment. The concentrations in the endotoxin group were significantly higher than control and cimetidine groups at all time points except 15 minutes ($p<0.05$). The endotoxin group had a 3.4 fold greater concentration of MEGX than the untreated group, and a 2.9 fold higher level than the cimetidine, at 120 minutes after lidocaine administration. The cimetidine group had a slightly higher serum level of MEGX than the untreated group, but the average difference in concentration between the two groups was approximately 24 ng/mL, and was found insignificant.

Lidocaine:MEGX ratios were derived to normalize the effects of endotoxin on the changes of lidocaine concentration on MEGX formation. (Figure 5) At 15 minutes there was a significant difference between the lidocaine:MEGX ratio for the endotoxin group and the control group($p<0.05$). Significant differences were also shown at 15 minutes between the endotoxin and cimetidine groups ($p<0.05$), and the cimetidine and control groups ($p<0.05$). At later time points, the lidocaine:MEGX ratio of the endotoxin group at 120 minutes was 5.6 fold higher than the untreated group, and twice that of the cimetidine group. The cimetidine group had a lidocaine:MEGX ratio that was 2.5 fold higher than the untreated at the same time point.

In order to examine the effect of blood pressure on the concentration of lidocaine and MEGX, we divided the endotoxin group into those that had a significant blood pressure decrease (shock), and those that showed no effect on blood pressure (non-shock). The two groups were examined for changes in MEGX and lidocaine concentrations. Lidocaine concentrations for the group whose blood pressures dropped

had a 1.5 fold larger lidocaine serum level at 120 minutes after lidocaine administration,
compared to the group whose blood pressures did not change, but the changes were not
significant at the $p<0.05$ level. (Figure 6) MEGX levels were significantly higher for
both the shock and non-shock at 45, 60, 90, and 120 minutes compared to control animals
($p<0.05$). The non-shock group and the control group were significantly different at 25
minutes ($p<0.05$). At 120 minutes, average MEGX concentrations were 128% higher for
those that were not in shock compared to those in shock at 120 minutes. (Figure 7) The
ratio of lidocaine:MEGX was approximately twice as great for the shocked compared to
non-shocked animals. (Figure 8) At 15 minutes there was a significant difference
between the shock and non-shock sub-groups ($p<0.05$) and between the shock and
control group ($p<0.05$). These data suggest that blood pressure may have an effect on the
clearance of lidocaine and MEGX.

Figure 1. Blood pressures (mmHg) of untreated (n=5), cimetidine (n=5, 80 mg/kg, for three days), and endotoxin (n=7, 6 hours pre-treatment with endotoxin, LD_{100}) treated animals, over two hours. The blood pressures were measured on a Stratham polygraph in mmHg (* p<0.05 LPS vs. Control at 90 and 120 minutes) (+ p<0.05 LPS vs. Cimetidine at 90 and 120 minutes) (- p<0.05 LPS vs. Time 0 at 90 and 120 minutes). Means ± SEM are reported.
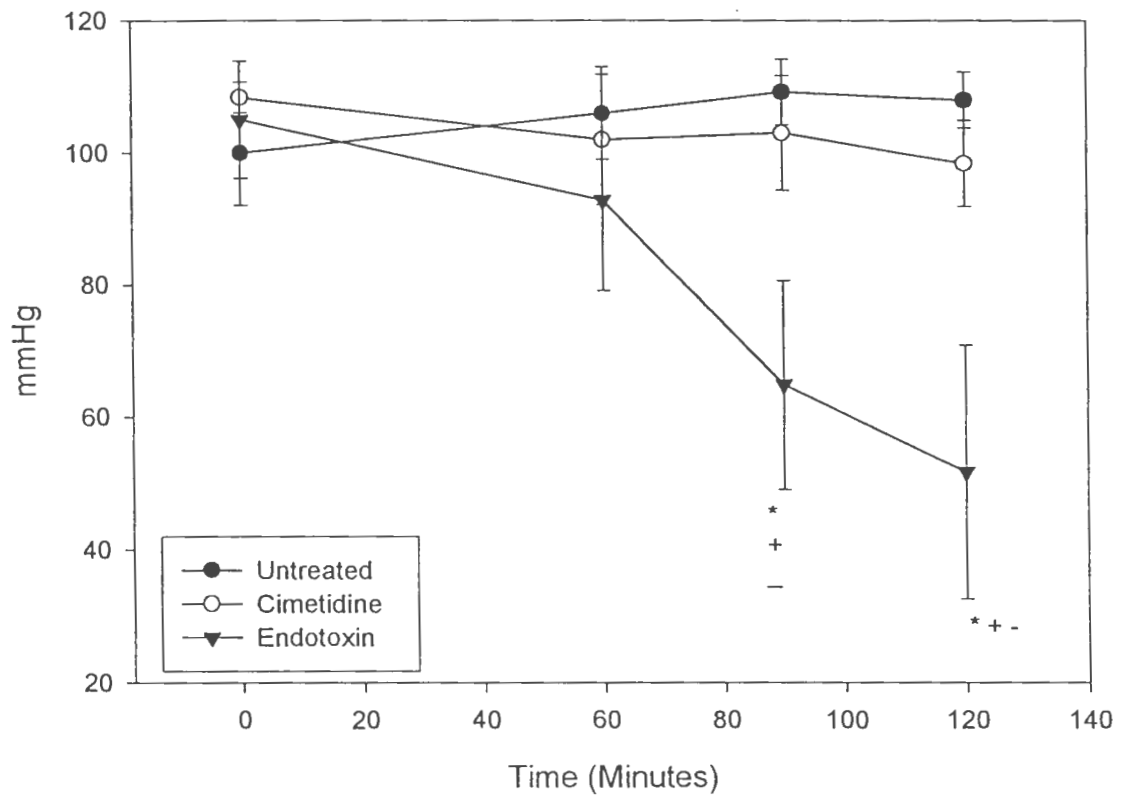
Figure 2. Blood Pressures (mmHg) of the two sub-populations of endotoxin treated rats (six hour pre-treatment, LD_{100}), shock (n=4), and non-shock(n=3), over the course of two hours. Blood pressures are measured in mmHg (* $p<0.05$ shock vs. non-shock at 90 and 120 minutes) (+ $p<0.05$ Shock vs. Control at 90 and 120 minutes) (- $p<0.05$ Shock vs Time 0 at 60, 90, and 120 minutes). Means $\pm$ SEM are reported.

Figure 3. Lidocaine concentrations of untreated (n=5), cimetidine (n=5, 80 mg/kg, for three days) and endotoxin (n=7, six hour pre-treatment, LD_{100}) groups over a course of two hours. Lidocaine (2mg/kg) was administered and serum samples were taken at 15, 25, 45, 60, 90, and 120 minutes after dosage (* $p<0.05$ LPS vs. Control at 25, 45,60,90, and 120 minutes) (+ $p<0.05$ LPS vs. cimetidine at 45, 60, 90, and 120 minutes). Means $\pm$ SEM are reported.

Figure 4. MEGX concentrations of untreated (n=5), cimetidine (n=5, 80 mg/kg. For three days) and endotoxin (n=7, six hour pre-treatment, LD_{100}) groups over a course of two hours. Lidocaine (2mg/kg) was administered and serum samples were taken at 15, 25, 45, 60, 90, and 120 minutes after dosage. Serum samples were assessed for monoethylglycinexylidide (MEGX), the major metabolite of lidocaine (* $p<0.05$ LPS vs. Control at 15, 25,45, 60, 90, and 120 minutes) (+ $p<0.05$ LPS vs. Cimetidine at 15, 25, 45, 60, 90, 120 minutes). Means ± SEM are reported.

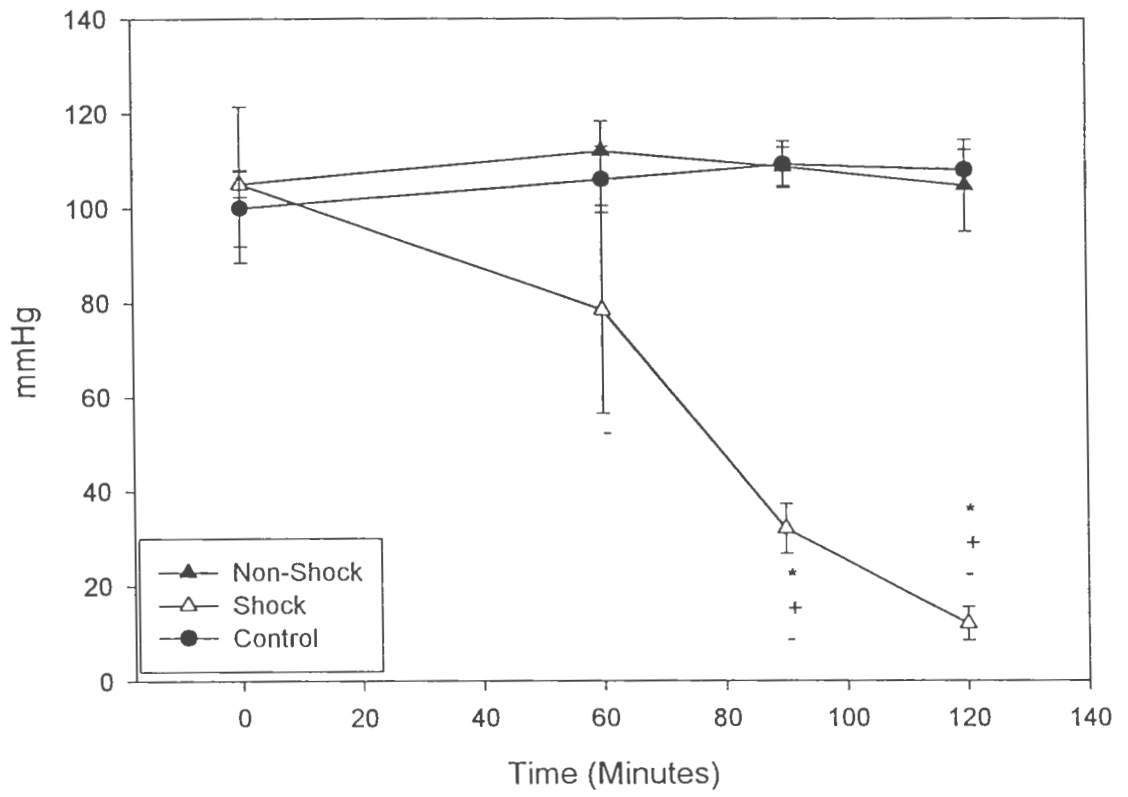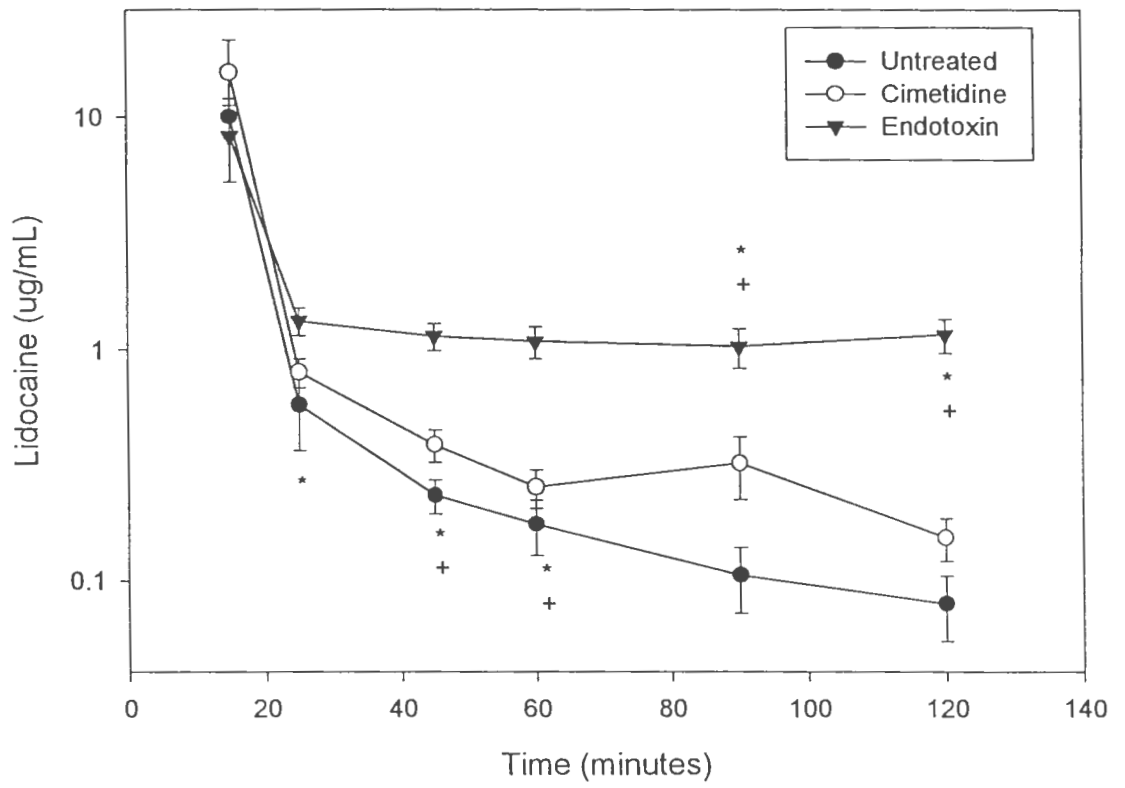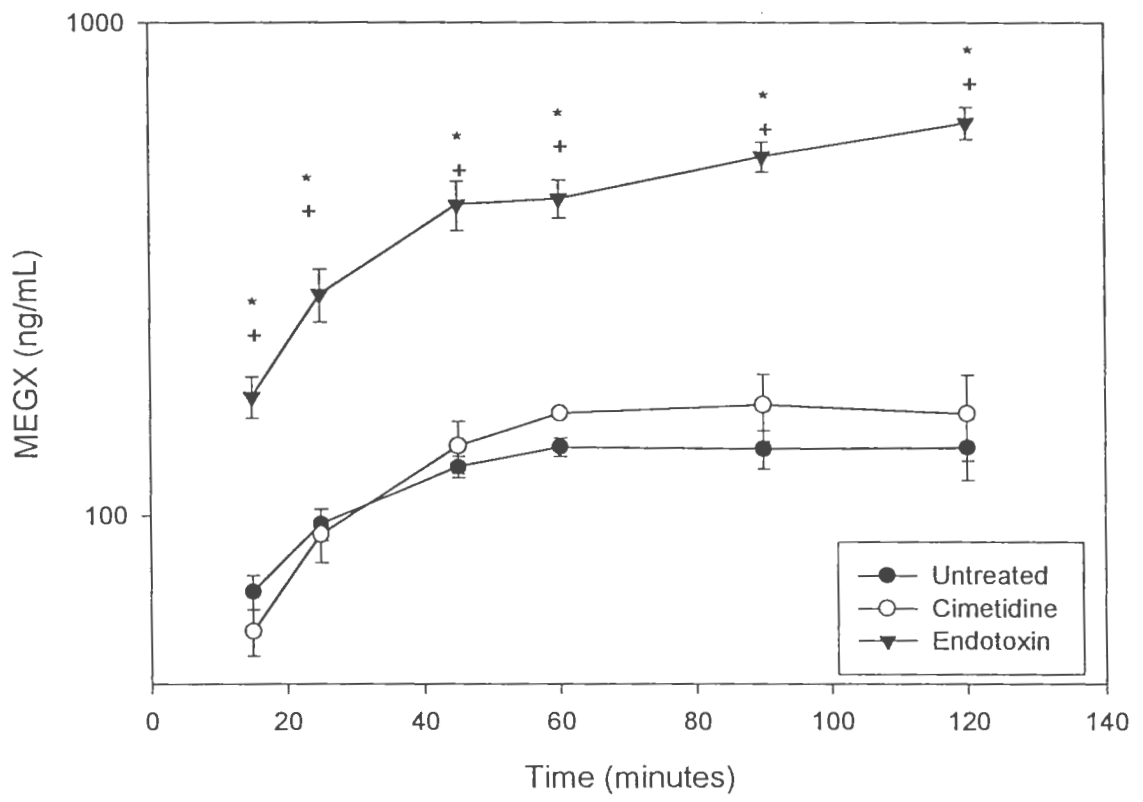**Figure 5.** Ratio of Lidocaine:MEGX of untreated (n=5), cimetidine (n=5, 80 mg/kg, for three days) and endotoxin (n=7, six hour pre-treatment, LD_{100}) groups over a course of two hours. Lidocaine (2mg/kg) was administered and serum samples were taken at 15, 25, 45, 60, 90, and 120 minutes after dosage (* $p < 0.05$ LPS vs. Cimetidine at 15 minutes). Means ± SEM are reported.
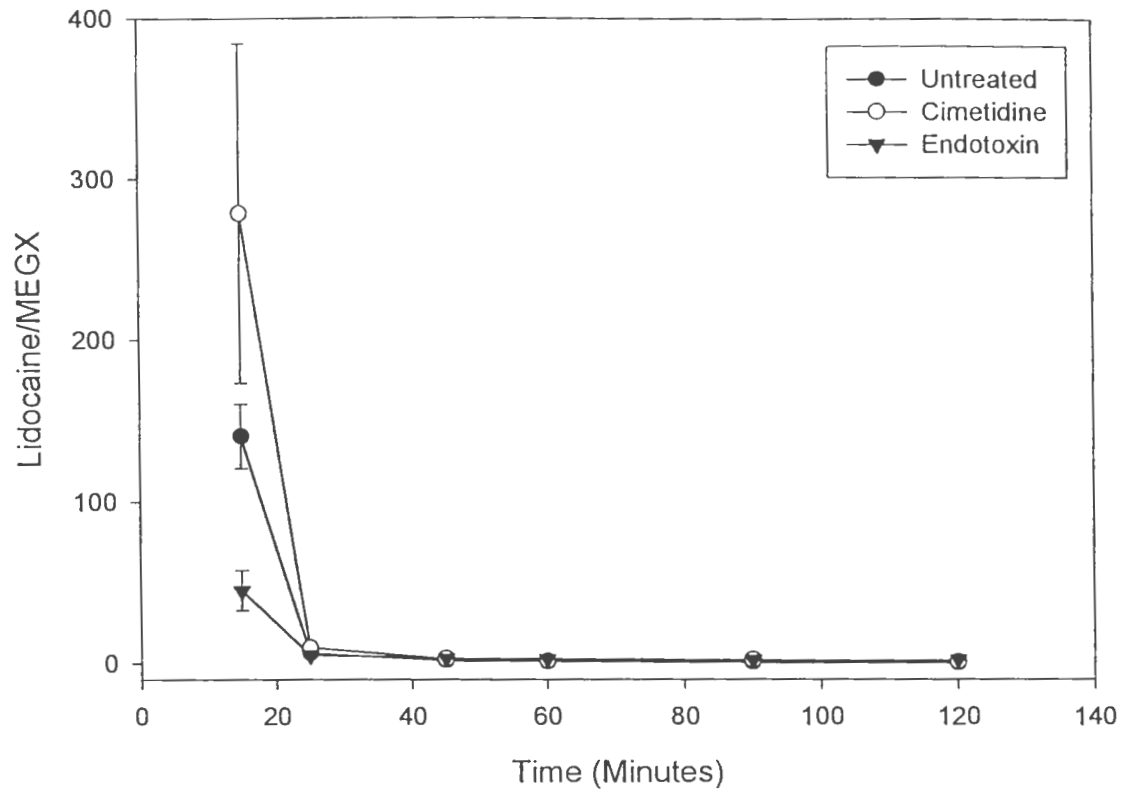
Figure 6. Lidocaine concentrations of the two sub-populations of endotoxin treated rats (six hour pre-treatment, LD_{100}), shock (n=4), and non-shock (n=3), over the course of two hours. Lidocaine (2mg/kg) was administered and serum samples were taken at 15, 25, 45, 60, 90, and 120 minutes after dosage (* $p<0.05$ Shock vs. Control at 45, 60, 90, and 120 minutes) (+ $p<0.05$ Non-Shock vs. Control at 45, 60, 90, and 120 minutes). Means $\pm$ SEM are reported.
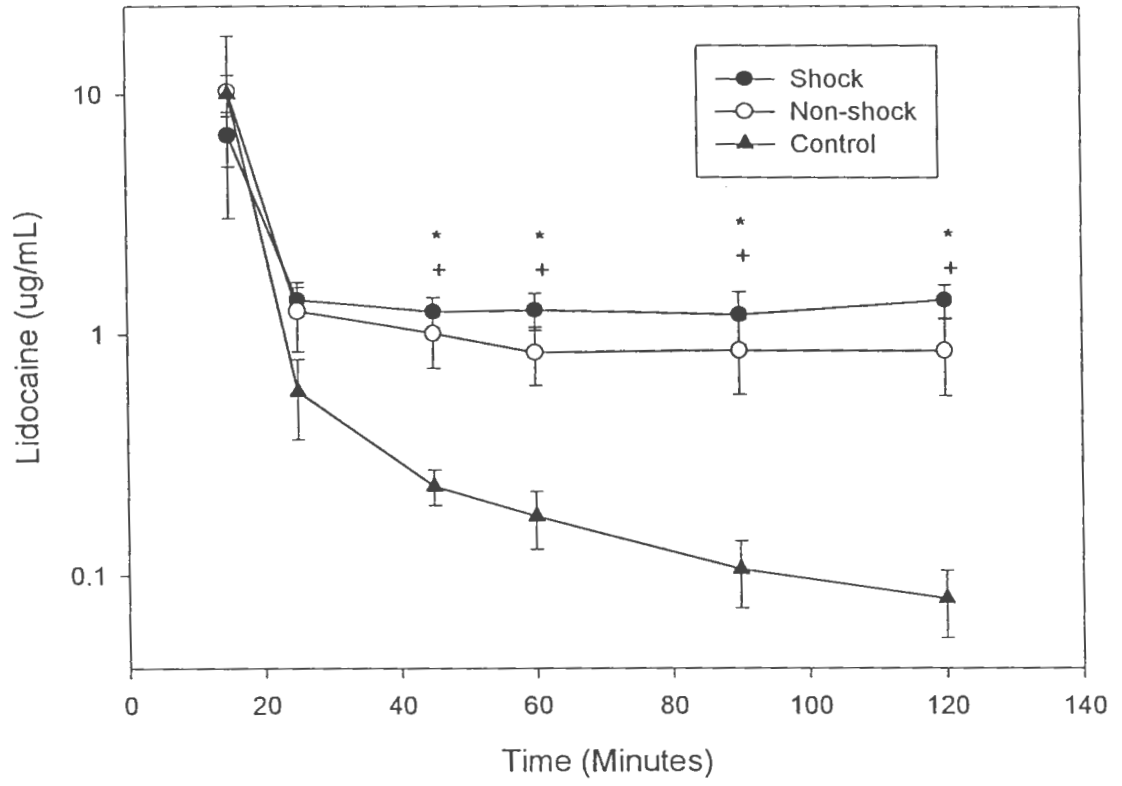
Figure 7. MEGX concentrations of the two sub-populations of endotoxin treated rats (six hour pre-treatment, LD_{100}), shock (n=4), and non-shock (n=3), over the course of two hours. Lidocaine (2mg/kg) was administered and serum samples were taken at 15, 25, 45, 60, 90, and 120 minutes after dosage (* $p<0.05$ Shock vs. Control at 15, 25, 45, 60, 90, and 120 minutes) (+ $p<0.05$ Non-shock vs. Control at 15, 25, 45, 60, 90, and 120minutes) (-$p<0.05$ Non-shock vs. Shock at 25, 45, 60, and 90 minutes). Means $\pm$ SEM are reported.
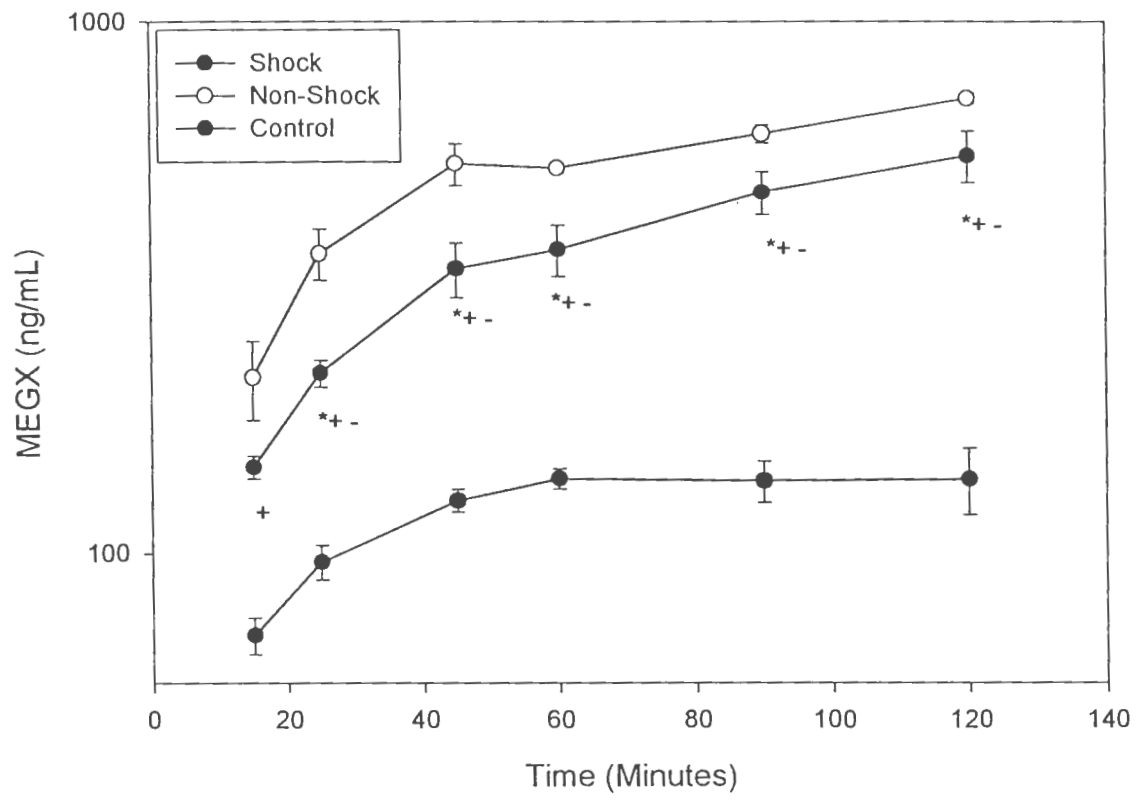
Figure 8. Lidocaine/MEGX ratios of the two sub-populations of endotoxin treated rats (six hour pre-treatment, LD_{100}), shock (n=4), and non-shock (n=3), over the course of two hours. Lidocaine (2mg/kg) was administered and serum samples were taken at 15, 25, 45, 60, 90, and 120 minutes after dosage (* $p<0.05$ Shock vs. Control at 15, 60, and 120 minutes) (+ $p<0.05$ Non-Shock vs. Control at 15 min). Means ± SEM are reported.
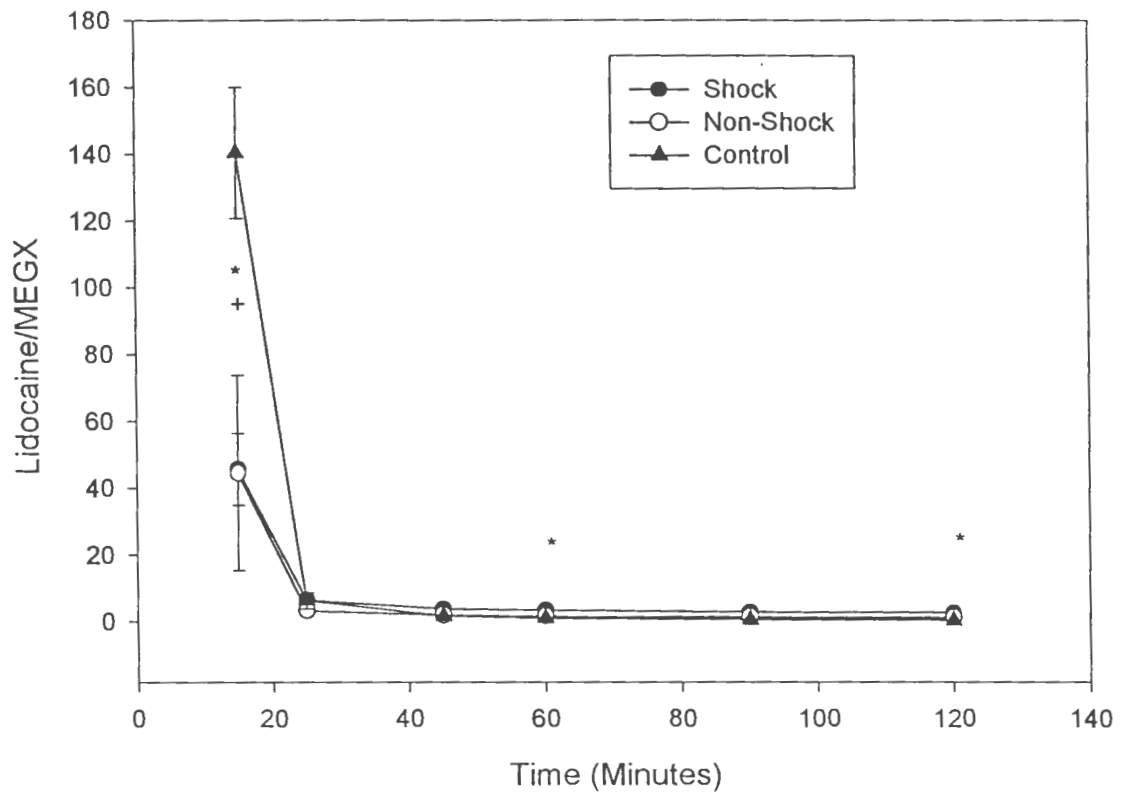
Discussion

There was a significant decrease in the blood pressure between the untreated and endotoxin groups. Many previous models have examined the effects of low dose endotoxin administration chronically and acutely. (Fish, et. al., 1986; Keeler, et. al., 1981; Dedichen, 1972; Suffredini, et. al., 1989) Although these models showed some effect of low dose endotoxin on hemodynamics, we were interested in the effects of a 100% lethal dose, and the immediate effects on blood pressure. Previous studies in rats using a low dose of endotoxin, 1 mg/kg/day, elicited insignificant drops in mean arterial pressures after 6 hours, but after 30 hours of chronic administration there was a significant change in pressure. (Fish, et. al., 1986) Keeler, et. al. found no significant change in mean arterial pressure after an acute 4 hour infusion of 2.5 mg/kg/hr endotoxin, but saw significant changes after a chronic infusion of 2.4-2.9 mg/kg over 4 days. (Keeler, et. al., 1981) In a canine model, aortic blood pressures dropped between control and endotoxin animals one hour after endotoxin administration, and continued to fall over the course of the next six hours. (Dedichen, 1972) Endotoxin response on blood pressure has been shown to significantly decrease mean arterial pressure after five hours in humans. (Suffredini, et. al., 1989) Although blood pressure changes occurred in these previous experiments, the degree of magnitude was less than that seen in this experiment. This can be attributed to the differences in dosages between experiments, and leads us to believe that an extremely large dose of endotoxin can cause severe and lethal hypotension within eight hours after administration.

Two distinguishable sub-populations of endotoxin rats were shown, shocked and non-shocked. The non-shocked animals had very small decreases in pressure, less than

20 mmHg, which were comparable to the insignificant acute responses of blood pressure to endotoxin previously seen. (Fish, et. al., 1986; Keeler, et. al., 1981) The shocked animals had blood pressures so low that life was unsustainable after the eight hours.

The clearance of lidocaine from the serum was reduced in the endotoxic model. After 20 minutes, the endotoxin group showed very little change in lidocaine concentrations, compared to the control groups. This reduction in clearance could possibly be due to alterations in the liver enzymes reducing the rate of lidocaine metabolism. Lidocaine is metabolized via P450 2C11, 2B1, and 3A2 to MEGX in the rat. (Nakamoto, et. al., 1997; Oda, et. al., 1989; Imaoka, et. al., 1990) In normal rats the levels of 2C11 and 3A2 are 139 and 84 pmol/mg protein, compared to 6.9 pmol/mg protein for 2B1. (Nakamoto et. al., 1997) Nakamoto, et. al., showed that upon induction of liver enzymes with phenobarbital, the rate at which MEGX is formed is increased. 2B1 levels were increased to 360 pmol/mg protein, and 3A2 levels rose to 390 pmol/mg protein, while 2C11 levels stay the same. This suggests that the more 2B1 and 3A2 there is in the rat liver, the greater the formation of MEGX *in vivo* will be. (Nakamoto, et. al., 1997) It has been shown in rats that after six and twelve hours, P4502C11 (Morgan, 1989) and P4503A2 (Sewer, et. al., 1996) respectively are down regulated after a single 1mg/kg dose of endotoxin. P450 2B1 was also inhibited in mice by low dose endotoxin as well. (Stanley, et. al., 1988) It is known that the systemic response to endotoxin is expressed by a variety of cytokine mediators. Using a higher dose of endotoxin than used in previous experiments, it could be assumed that there would be a much larger systemic response. Upon this assumption one might assume a greater degree of down-regulation of P450 due to the increase of cytokine mediators, leading to greater inhibition.

Cimetidine was used as a positive control of P450 inhibition because it has been shown in rat to inhibit lidocaine clearance in vivo (Fruncillo, et. al., 1983), and in the perfused liver. (Bruck, et. al., 1990) This inhibition in lidocaine clearance was seen, however, the resultant decrease in lidocaine clearance was only intermediate to that shown by endotoxin. The specificity of metabolism of the P450 isoenzymes may explain the lower lidocaine clearance in the endotoxin animals. Cimetidine has been shown to inhibit 2C11, but not members of the 2B or 3A family in rats. (Levine, et. al., 1998) In untreated animals, lidocaine metabolism to its major metabolite, MEGX, is dependent on both 2C11 and 3A2. (Nakamoto, et. al., 1997) Therefore, only one of the two main isoforms of P450 responsible for lidocaine metabolism is inhibited. Thus, cimetidine would have less of an effect on lidocaine clearance than during endotoxic shock where both forms are inhibited. Endotoxin inhibits P450 isoforms responsible for lidocaine metabolism, but physiologic changes in the hemodynamics also might intensify the alterations seen.

Hepatic blood flow is an important aspect of lidocaine elimination, and clinical manifestations that reduce hepatic blood flow would also be expected to rate lidocaine would be eliminated. (Zito, et. al., 1978) Indocyanine green (ICG) elimination is dependent exclusively on hepatic blood flow. (Zito, et. al., 1978) It has been shown using a different model of sepsis, cecal ligation and puncture (CLP), that hepatic blood flow increases at early time points (2-10 hours), but is markedly decreased after 20 hours. (Wang, et. al., 1991) The later time points, those after 16 hours, of the CLP model are similar to the high dose endotoxin model in this study in that they both represent a hypodynamic model of sepsis. Hepatocellular dysfunction, tested by ICG clearance, was

seen at both the early and later time points with a higher degree of dysfunction at 20 hours. (Wang, et. al., 1991) Since lidocaine in this study was distributed via the hepatic artery, a decrease in blood flow could significantly alter the clearance of the drug from the blood.

The concentration of MEGX was significantly higher in septic animals compared to control and cimetidine, and cimetidine animals compared to controls. During the early time points all three groups have an increasing slope, but while the slopes of the cimetidine and control groups level off at later time points, the MEGX levels of the endotoxin group still increase at these later time points. It has been shown in the acute and chronic endotoxic rat there is a decrease in renal blood flow. (Fish, et. al., 1986) Endotoxin also results in impaired renal oxygenation and perfusion in the dog model, which is due to central hypotension and the local effects of endotoxin on the kidney. (Gullichsen, et. al., 1989) The decrease of renal blood flow, and the resultant renal failure, may be a cause of the accumulation of MEGX in the serum at the later time points. These data seem rather anomalous when looked at in conjunction with the lidocaine concentrations. A possible reason for the increased concentration of MEGX could be due to liver cell dysfunction and the poor elimination of drug metabolites from the blood.

During sepsis, hepatocellular dysfunction occurs within 2 hours (Wang, et. al., 1995), as well as a reduction in bile flow. (Bolder, et. al., 1997) This loss of function decreases the amount lidocaine metabolites being eliminated by the bile duct, and in conjunction with poor renal perfusion, there could be an increase of lidocaine metabolites in the serum in the endotoxic model. It has been recently shown in a severe ischemia-

reperfusion injury rat model that the fluorescence immunoassay used in this experiment, is not selective in detecting only MEGX, but also detects other lidocaine metabolites, particularly the free, and conjugated forms of hydroxy-MEGX, which accumulated in the plasma. (LeClercq, et. al., 1997) If renal perfusion and bile flow, the two main routes of metabolite excretion, are lower during sepsis, there will be an accumulation of metabolites in the rat. If septic rats have higher levels of lidocaine metabolites in their serum due to accumulation the amounts will be higher than what is being generated at that time. When analyzed, the immunoassay will detect all the MEGX and hydroxy-MEGX generated and not excreted, resulting in higher values than control and cimetidine.

Since levels of MEGX were shown to be elevated during sepsis in our rat model, we wanted to see if we could use a lidocaine:MEGX ratio as a marker for drug metabolizing capabilities instead of just looking at the metabolite. As was stated earlier, lidocaine clearance was significantly lower in the endotoxin group, whereas MEGX formation was higher. Ratios of serum levels of lidocaine:MEGX were significantly different for all three groups at 15 minutes. Measurements at this point are more variable than measurements of serum ratios taken at a time interval when the clearance and metabolite might be reaching a steady state. In this experiment a steady state started to occur between 45 and 60 minutes, and a ratio of parent to metabolite might make it possible to assess the livers drug metabolizing capabilities, rather than at an earlier time point. Elin, et. al. looked to see if there was a difference in parent:metabolite ratios using lidocaine, as an assessment tool to determine liver function in patients with chronic viral hepatitis but found no significant difference, which was not surprising since the

differences in lidocaine and MEGX concentrations were not significantly different either. (Elin, et. al., 1997) Therefore this is a novel approach that needs further examination in various other types of disease states, as well as in hypodynamic sepsis, to validate its usefulness.

The two sub-populations of the endotoxin group gained our interest. We examined these two groups separately to see if there was any difference between those rats that had a drop in blood pressure, compared to those that did not. Both groups had a significantly lower clearance of lidocaine, compared to the control group, but were not significant compared to each other. Although we did not see a significant effect, there is the possibility that there is a significant difference between the shock and non-shock animals, and a type II statistical error might have been committed with our small sample size. Further experimentation would support the role that altered hemodynamics might play in the elimination of lidocaine during septic shock.

MEGX concentrations between the two sub-populations were significantly higher for the non-shock than shock group. This supports the idea previously mentioned in regards to the hepatocellular dysfunction inhibiting bilary excretion of the metabolite compounds. The renal blood flow in the non-shocked animals most likely is not changed, so the accumulation of metabolite could possibly be due to a reduction in bile flow. This would lead to an accumulation of MEGX and the free and conjugated forms of hydroxy-MEGX, which could not be distinguished with the TDx immunoassay.

In conclusion, endotoxin produces many negative physiologic changes that are seen systemically, in specific organs, and cellularly. Endotoxin can cause severe hypotension. Endotoxin inhibits the P450 drug-metabolizing enzyme system, which is

responsible for removing xenobiotics from the body non-toxically. The specific isoforms which, are capable of metabolizing lidocaine, are inhibited by endotoxin. This inhibition reduces the clearance of lidocaine from the serum in septic rats, and the systemic effects led to an accumulation of lidocaine metabolites in the serum, which can possibly be indistinguishable using the TDx analyzer. Due to these conflicting results, the ratio of lidocaine:MEGX is being postulated as a possible tool determining drug metabolizing capabilities during sepsis, but further validation is needed in this model of hypodynamic sepsis.

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Appendix I

The metabolism of lidocaine by rat liver microsomes has been studied extensively. (Nakamoto, et. al., 1997; Oda, et. al., 1989; Imaoka, et. al., 1990) Lidocaine is N-deethylated to give monoethylglycinexylidide (MEGX), its major metabolite. (Figure 9) This reaction is catalyzed by various P450 enzymes in the rat liver, but the isoforms which have shown the highest turnover for MEGX formation are largely P450 2B1, 2C11, and 3A2. (Nakamoto, et. al., 1997; Oda, et. al., 1989; Imaoka, et. al., 1990) Endotoxin administration has been shown to inhibit many P450 isoforms, specifically P450 3A2, and 2C11, (Sewer, et. al., 1996; Morgan, et. al., 1993) in rats, and 2B1 in mice (Stanley, et. al., 1991), which are the same isoforms that metabolize lidocaine. In this study we examined the effects of an acute, lethal dose of endotoxin on the metabolism of lidocaine in isolated rat liver microsomes.

Male Sprague Dawley rats, ranging from 250-550g, were administered endotoxin, cimetidine, or no treatment. Cimetidine, 80mg/kg, was injected IP, daily for three days prior to surgery, was used as a positive control for inhibition of cytochrome P450. (Levine, et. al., 1998) Endotoxin (E. Coli 026:B6 LPS, lot #91608), 45.6 mg/kg, was injected IP six hours before the liver was removed and homogenized.

Rats were sacrificed, and the front two lobes of the liver were excised. Livers were weighed and homogenized 1:3 in phosphate buffered solution (PBS) containing NaCl 136.9mM, KCl 2.2 mM, NaH_2PO_4 8.1 mM, KH_2PO_4 1.5 mM, pH 7.4. The homogenates were centrifuged at $4^{\circ}C$ for 20 minutes at 10,000 x g. Supernatant was decanted off, and 5 mL of ice cold PBS was added. The supernatant was then centrifuged in a Beckman Ultracentrifuge, using a 42.1 rotor, at $4^{\circ}C$ for one hour at 35,000 x g. The

supernatant was poured off and discarded. Fifteen mL of ice cold PBS was added, and the microsomal pellet was vortexed away from the glycogen, and resuspended using a glass homogenizer. The homogenate was centrifuged again, to reduce the amount of hemoglobin, at 4°C for one hour at 35,000 x g. Supernatant was discarded, and the pellet was resuspended in 7.0 mL of PBS. The microsomal solution was pipetted out into 1.0 mL aliquiots and frozen at –80°C. Total microsomal protein was determined by bicinchoninic acid (BCA) assay. (Smith, et. al., 1985)

Microsomal solution, 50 μL, was incubated in 0.1 mol/L sodium phosphate (NaPi) buffer, pH 7.4, in a final volume of 400 μL, in the presence of 4 mM reduced nicotinamide adenine dicucleotide. Lidocaine was then added in a final concentration of 50 μM. The reaction was incubated at 37°C for 20 minutes and then stopped by the addition of 10 μL of 70% perchloric acid. Denatured protein was removed by centrifugation, and the supernatant was analyzed for lidocaine and MEGX by the TDx analyzer. (Abbott laboratories)

There was no significant difference between the different treatment groups in the amount of lidocaine consumed. The control group consumed 2.7 nmol of lidocaine, while the endotoxin and cimetidine group consumed 3.3, and 2.9 nmol of lidocaine respectively. There was no significant difference in MEGX formation between any of the treatment groups. (Figure 10)

Lidocaine is metabolized by many P450 isoforms, but those that possess the highest activity for the catalyzation of lidocaine to MEGX are 2C11, and 3A2, in rat liver microsomes. (Nakamoto, et. al., 1997) In our experiment we found there was no diffference between groups. We expected there to be a significant difference between the

endotoxin and untreated groups, as well as between the cimetidine group and the untreated group, in the amounts of lidocaine metabolized, and the amount of MEGX formed. We had expected to see more lidocaine and less MEGX in the endotoxin groups because all three of the isoenzymes responsible for lidocaine metabolism are inhibited by endotoxin. (Morgan, 1990; Sewer, et. al., 1996) It has been shown *in vivo* and in the perfused rat liver that lidocaine clearance is inhibited by cimetidine. (Bruck, et. al., 1990; Fruncillo, et. al., 1983) This decrease in lidocaine clearance can be due to cimetidine inhibition of P4502C11, but not 3A2. (Levine, et. al., 1998) In this experiment, we expected to see some difference between the control and the cimetidine groups in regards to MEGX formation, and lidocaine elimination. The inhibition was expected to be less than that seen with endotoxin because of cimetidines incomplete inhibition of all lidocaine metabolizing P450 isoforms.

Previous studies have shown that MEGX formation was much greater than that shown in this experiment. (Nakamoto, et. al. 1997; Oda, et. al., 1989) Our values were very minute, between $1\text{-}8 \times 10^{-5}$, while in other studies there was a 100 times greater amount of MEGX formed. This comparison with other researchers results leans us to believing the microsomes used in the experiment were not as active. The methodologies used by these groups worked with microsomes stored in 250 mM sucrose. We stored our isolated microsomoes in PBS, as described by Muller, et. al. All other procedures were similar, leading us to believe it is this difference in the storage which led to the faulty results. (Muller, et. al., 1996)

Figure 9. Metabolic pathways of lidocaine. (Drawn from published data. Oda, et. al, 1989; Chandel, et. al, 1995)

Lidocaine

3-OH Lidocaine   Me-OH Lidocaine   MEGX

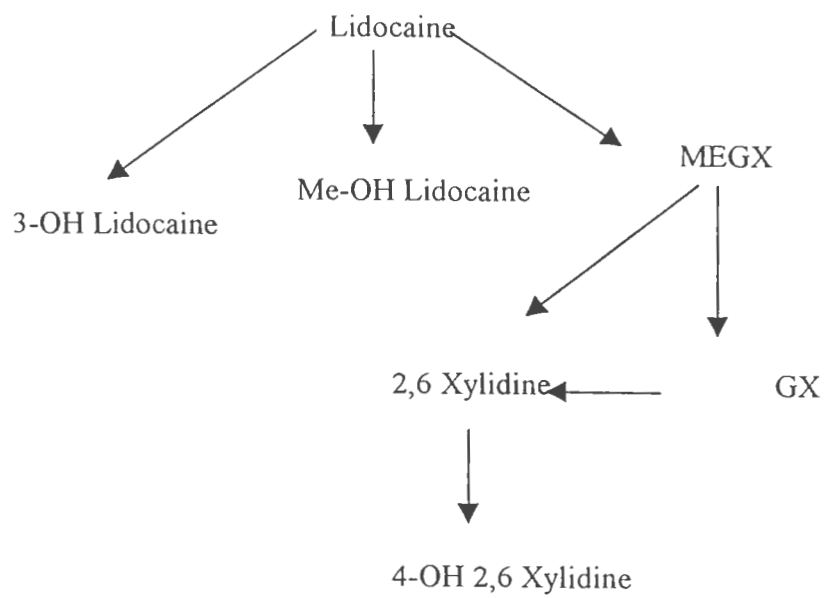2,6 Xylidine   GX

4-OH 2,6 Xylidine

Figure 10. In vitro MEGX concentrations (ng/ug protein). 50 µM Lidocaine was added to 50 µL of microsomes containing 50 µL PBS, 0.1M NaPi, and 2mM NADH, and incubated at 37°C for 20 minutes. Assay was centrifuged and supernatant was analyzed for MEGX concentration by flourescence immunoassay. Values represent pmoles of MEGX formed per minute per µg protein.
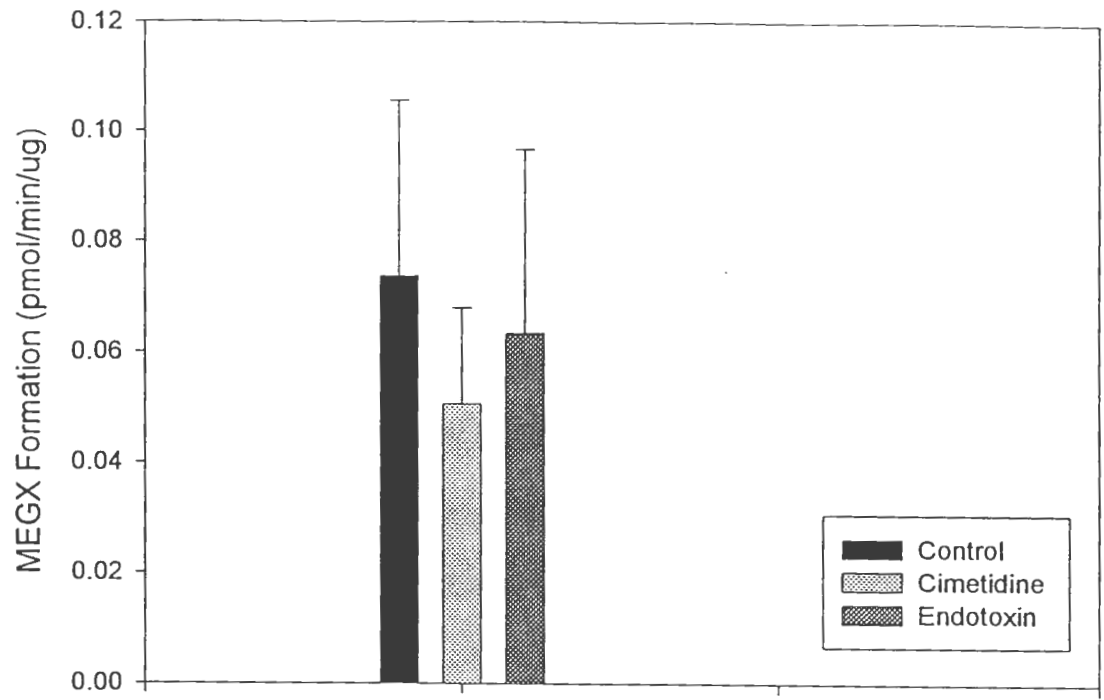
Appendix II

Infections in humans and animals are capable of altering the expression and activity of the enzyme isoforms that make up the cytochrome P450 drug metabolizing enzyme gene superfamily. There are at least 14 families and 26 sub-families which belong to the P450 enzyme system in mammals. (Nelson, et. al., 1996) Many xenobiotics as well as physiologically produced substrates are bioactivated or biotransformed, or detoxified by this enzyme system, so the regulation of this system is very important. Although there are many different genes, only a select few are responsible for the metabolism of most drugs. In humans these isoforms are P450's 1A2, 2A6, 2C8, 2C9, 2C11, and 3A4. (Spatzenegger, et. al., 1995) In rodents and other mammals, there are homologous counterparts to these isoenzymes. (Imaoka, et. al, 1990) Potential alterations in these enzymes as a result of an infection have the potential to change serum levels of therapeutic compounds.

In a serious infection, like sepsis, there is an overwhelming inflammatory response that occurs on the systemic level. This systemic inflammatory response is correlated with the generation and release of various cytokines. (Jacobs, et. al., 1989) These cytokines are capable of altering the hepatic metabolism of drugs through suppression of the aforementioned P450 isoforms, making the understanding how these cytokines alter P450 activity clinically relevant.

Endotoxin, or lipopolysaccharide (LPS), found on the cell wall of the gram-negative bacteria, is used as a model for bacterial sepsis. Endotoxin administration causes activation of the inflammatory response system, which results in high concentrations of cytokines released into the bloodstream. (Cannon, et. al, 1990; Hack, et.

al, 1989) In the endotoxin model, these high concentrations of cytokines, as well as the LPS itself, centralize near the hepatocyte. (Billiar, et. al, 1992)

The release of tumor necrosis factor α (TNF-α) from macrophages is the primary step in response to LPS administration in animals. (Lowry, 1993) TNF-α then up-regulates the release of itself and interleukin-1 (IL-1), which in conjunction with TNF-α, stimulates the release of interleukin-6 (IL-6), as well as many other mediators of the inflammatory system. Since the liver has its own macrophages, the Kupffer cells, endotoxin can act directly in the liver to generate the immune response, releasing cytokines directly at the liver. Results of this are the high levels of liver localized cytokines. (Billiar, et. al. 1992)

Overall administration of LPS has effects on the activity and regulation of the P450 system. LPS causes decreases in P450 protein and mRNA expression in rat hepatocytes. (Carlson, et. al, 1996; Sewer, et. al., 1997) Although the effect can be through the endotoxin acting directly on these hepatocytes to cause these changes, the activation of the Kupffer cells, and subsequent release of cytokines, may also mediate these changes. Since LPS causes a rise in cytokine levels, and these cytokines play a role in the inflammatory response, the effects of certain cytokines on P450 regulation have been described.

Several studies have described the effects of independent administration of TNF-α, IL-1, and IL-6 on drug metabolism and P450 levels have been looked at. In the rat model, total levels of P450 are suppressed by TNF-α, and IL-1. (Pous, et. al., 1990; Wright, et. al., 1991; Nadin, et. al., 1995) Rats treated with TNF-α had decreased levels of activity, mRNA expression, and protein content of P450's 2C11 and 3A2 after one and

three days of treatments. (Nadin, et. al., 1995) Further evidence supporting the involvement of TNF-α in the suppression of some P450 isoforms came from the administration of pentoxifylline to rats. Pentoxifylline blocks TNF-α formation in response to LPS. When administered to rats given LPS, the LPS induced suppression of 2E1 and 3A2 in rat liver was blocked. (Monshouwer, et. al., 1996)

Administration of IL-1 is also selective for inhibition of various isoforms of P450. A single dose of IL-1 to rats suppressed the total hepatic P450 content as well as the metabolizing activities of many xenobiotics, 24 hours after administration. (Kurokohchi, et. al., 1992) Daily doses of IL-1α elicited a reduction of activity, but not expression of P450 3A2, 2C11, and 1A1 in male rats. (Ferrari, et. al., 1993) Decreases in the expression of 2C11 and 3A2 after two IL-1 injections were also seen in the male rat liver after 24 hours. (Morgan, et. al., 1994)

IL-6 also can alter the activity and expression of P450. (Wright, et. al, 1991; Morgan, et. al. 1994; Chen et. al., 1992) Suppression of the protein and mRNA of P450 2C11, and the mRNA of 2E1, was seen after three injections of IL-6. (Morgan, et. al. 1994) There were no alterations in the levels of P450 3A2 or 2E1 mRNA or protein after 24 hours. (Morgan, et. al. 1994)

The effect cytokines have on cells, and the regulation and activity of intracellular processes, are through receptor activated second messengers. The response each of these cytokines previously mentioned has on hepatocytes is played out through these second messengers. One type of second messenger in liver cells are sphingolipids. Ceramide, a product of the hydrolysis of sphingomyelin, by sphingomyelinase, is thought to act as a second messenger in many cell types. It has been shown that IL-1 and TNF-α activate

sphingomyelinase to produce ceramide. (Hannun, et. al., 1994) Incubation of hepatocytes

with either sphingomyelinase or a short chain ceramide, (C_2-ceramide) has shown

suppression of 2C11, mimicing the effects of IL-1. (Chen, et. al., 1995) This suggests

that sphingolipids can act in determining the expression of hepatic P450 genes. The

question still remains as to whether it is ceramide carrying out the effects in the cell, or if

it is the products, sphingosine and sphingosine-1-phosphate, of ceramide metabolism.

The metabolites of ceramide have been researched, and it has been shown that at doses of

IL-1, capable of suppressing 2C11, and maximize the activity of sphingomyelinase,

ceramide is barely detectable. (Chen, et. al., 1995)

Another second messenger in cells is nitric oxide (NO). NO is a product of nitric

oxide synthase, which forms NO from arginine. There are two forms of nitric oxide

synthase. The one relevant to LPS, is the inducible form that is found in macrophages

and hepatocytes upon an inflammatory stimulation. (Bredt, et. al., 1994; Morris, et. al.,

1994) NO is capable of binding to any heme containing protein, and cytochrome P450 is

no exception. Upon binding to P450, the catalytic activity is altered, and the inhibition is

only partially reversible. (Wink, et. al., 1993) Several studies have investigated the role

of P450 inhibition by nitric oxide after LPS administration. The addition of a nitric oxide

synthase inhibitor to the drinking water of rats four days before LPS administration

partially prevented a decline of 2B1 and 2B2 activity, and mRNA, seen in endotoxemic

animals pretreated with phenobarbitol to induce the concentrations of 2B1 and 2B2.

(Khatsenko, et. al., 1993; Khatsenko, et. al., 1994)) It was postulated from these

experiments that the decrease in P450 catalytic activity is due to the increase of NO in the

cell, and this increase of NO also might be a contributing factor to the degradation of the

P450 enzymes. (Khatsenko, et. al., 1993) Muller, et. al. also showed that the the administration of either two different nitric oxide synthase inhibitors in normal or phenobarbital treated rats inhibited the decreases seen in the LPS generated decline of P450 content, as well as ethylmorphine N-demethylase, and the metabolism of midazolam. (Muller, et. al, 1996) In rats, the enzymes involved in the metabolism of ethylmorphine are P450 2B1, 2C6, 2C11, and 3A2, so many of the isoforms responsible for drug metabolism are inhibited by NO.

In summary, LPS causes a widespread systemic inflammatory response. This response is mediated by various cytokines, and the effects of these cytokines on different cell types is degradative. It has been shown in hepatocytes, endotoxin, can interact and alter the expression and activity of the P450 system. Other studies have shown corraborative evidence that the mediators IL-1, IL-6, and TNF-α, all have the ability to suppress different P450 isoforms important to the metabolism of drugs. The formation of second messengers from receptor mediated binding of these cytokines has also been investigated. Increased levels of ceramides have been shown to inhibit P450 expression. Nitric oxide has also been suggested as a culprit in the inhibtion of catalytic activity of the P450 enzymes. In closing, although much work has been done to examine the mechanisms of regulation of the P450 system during endotoxemia, there is much more research of unanswered questions to be done.

Appendix III

Conclusions and Future Investigations

Conclusions:

1.) An LD_{100} dose of endotoxin causes severe hypotension in rats after eight hours.

2.) Lidocaine clearance is decreased in this rat model of endotoxin induced septic shock, compared to controls, after 25 minutes of lidocaine administration.

3.) Increased concentrations of the metabolite MEGX was seen in septic rats, compared to controls, 120 minutes after administration of lidocaine.

4.) Levels of MEGX increased during septic shock, and a definitive reason for this increase is still unknown, since the TDx assay for MEGX also detects hydroxy-MEGX (LeClercq, et. al., 1997). The lidocaine:MEGX ratio could be a useful indicator of drug metabolism inhibition, but further validation is needed in a model where accurate concentrations of MEGX have been determined.

Future Investigations:

1.) It has been shown that the TDx assay for MEGX is also sensitive to hydroxy-MEGX. (LeClercq, et. al., 1997) Examination of the serum concentrations of MEGX and hydroxy-MEGX using high performance liquid chromatography to examine the concentrations of each of these two metabolites is needed to determine the actual concentrations of metabolites.

2.) Microsomal amidases are capable of rapidly hydrolyzing MEGX. (Hollunger, et. al., 1960) Investigation to see whether these amidases are inhibited by endotoxin, would

add further explanation to the increase of serum MEGX levels seen in our septic model.

3.) Reduction in bile flow has been shown in endotoxic rats. (Bolder, et. al., 1997) Since bile flow is one possible route for lidocaine metabolite excretion, investigation of the role this reduction in bile flow has on the increased levels of MEGX in the serum seen in the endotoxic model is needed.

4.) Since lidocaine is a high extraction compound, blood pressure plays an important role on its clearance. Examination of the extent to which severe hypotension alters the clearance of lidocaine, and the excretion of MEGX, is necessary.

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