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## MECHANISM OF ACROLEIN-INDUCED VASCULAR TOXICITY

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Acrolein, an environmental pollutant and a lipid peroxidation product, is implicated in vascular pathogenesis. Although evidence indicates a link between vascular pathogenesis and acrolein, no direct studies relating to effects of acrolein on vascular function and responses are known. This study investigated the effects of acrolein on vascular function to understand the underlying mechanism of acrolein-induced vascular responses. Male Sprague-Dawley rats were treated with acrolein (2 or 4 mg/kg; i.p.) for 3 or 7 days. Urine and blood samples were collected. Changes in systolic blood pressure (SBP) and responses to acetylcholine and phenylephrine were determined. Acrolein (4 mg/kg, 7 days) significantly increased SBP by 25%, phenylephrine vasoconstriction by 2-fold, but decreased urinary excretion of nitrite by 25%. Acrolein inhibited generation of cyclic guanosine 3'5'-monophosphate (cGMP) by 98%, and did not alter expression of nitric oxide synthase (eNOS). Acrolein increased the generation of lipid hydroperoxide in plasma and aortic tissue by 21% and 124% respectively, increased glutathione-S-transferase (GST) and glutathione peroxidase (GSH-Px) activities. Acrolein up-regulated the expression of GST by 2 fold. These data suggest that induced SBP and altered vasoconstriction/vasodilatation in acrolein treated rats may be due to reduced availability of NO via increased free radical generation and reduced antioxidant defense.

*Key words: acrolein, reactive oxygen species, NO and NOS system, glutathione, lipid peroxide*

### INTRODUCTION

Acrolein, an  $\alpha,\beta$ -unsaturated aldehyde is produced by a variety of synthetic and natural processes including; incomplete combustion of fuels and other organic compounds, petrol, coal, and wood; engine exhausts; production and manufacturing processes such as the plastic industry; photochemical oxidation of airborne hydrocarbons; and cigarette smoke (1-4). In the biological system,

acrolein is produced as a metabolic product of allyl alcohol, allylamine, spermine, spermidine, and the widely used anticancer drug cyclophosphamide (5-7). Additionally, acrolein is produced as a result of lipid peroxidation of membrane lipids, LDL, and cyclopentenone prostaglandins (8-11).

Toxicity of acrolein on respiratory system, kidney and in cell cultures has been widely reported where most studies indicate that acrolein effects are due to GST catalyzed GSH depletion (12,13). Once target cells are depleted of a threshold cytosolic GSH level, acrolein can directly react with other nucleophilic groups including proteins and alter their function.

Although acrolein has been linked to altered vascular reactivity and atherosclerosis (14,15), the precise cellular and molecular mechanism of acrolein-induced vascular toxicity is not known. Increased free radical generation and an impairment of glutathione-dependent anti-oxidant system by acrolein have been reported (15,13). Increased free radicals have been linked to deterioration of the nitric oxide/nitric oxide synthase (NO/NOS) system in vasculature (16-18). Since humans are exposed to acrolein through a variety of environmental situations, especially as a component of smoke and automobile exhaust, it is important to understand the cellular and molecular effects of this reactive aldehyde in details. In this study we investigated the effect of acrolein on vascular reactivity and examined the possible involvement of ROS in the alteration of cardiovascular effects via the ROS-sensitive NO/NOS system.

## MATERIALS AND METHODS

Male Sprague-Dawley rats, (300-350 grams) (Harlan Sprague-Dawley, Houston, TX) were used in this study. Animals were randomly divided into groups; control and treatment. The treatment group received acrolein dose of 2 mg/kg/day or 4 mg/kg/day i.p for 3 or 7 days ( $n \geq 4$  for each treatment). The control group ( $n \geq 4$ ) received an equal volume of vehicle (0.9% saline solution) for the same duration.

This study was approved by the Texas Southern University Animal Care and Usage Committee and was performed according to NIH guidelines for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985) and Animal Welfare Act. At the end of treatment period, animals were anesthetized with pentobarbital sodium (50 mg/kg, ip). After a midventral laparotomy, the aorta was removed, gently cleaned from surrounding fat and connective tissues. Cleaned aorta was either stored at  $-80^{\circ}\text{C}$  for biochemical analysis or in ice-cold Krebs's buffer for aortic ring study.

24 hour urine was collected before and after treatment and stored at  $-80^{\circ}\text{C}$  until analysis. Blood samples were collected via cardiac puncture on the day of experiment and plasma was separated and stored at  $-80^{\circ}\text{C}$  until analysis.

*Measurement of Systolic Blood Pressure (SBP).* Blood pressure was measured before and after treatment periods by tail-cuff plethysmography using an electrophysiograph (Apollo 170, IITC, USA).

*Aortic Ring Study.* The thoracic aorta was removed, cleaned of adhering fat and connective tissue, and cut into 3-mm-wide transverse rings. Aortic rings were hung from a stainless steel hook in an organ bath filled with 10 ml of Krebs's buffer [118; KCl, 4.75;  $\text{CaCl}_2$ , 1.9;  $\text{KH}_2\text{PO}_4$ , 1.19;  $\text{MgSO}_4$ , 1.19; glucose, 11.1;  $\text{H}_2\text{CO}_3$ , pH. 7.2 ( $37^{\circ}\text{C}$ )]. The buffer was continuously bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Aortic ring was equilibrated to 2 g of tension for 90 minutes, while charged

with  $10^{-7}$  M phenylephrine (PE) every 30 minutes. Baseline was established and vasoconstrictor response to PE ( $10^{-9}$  to  $10^{-5}$  M) was determined. Vasorelaxation response to acetylcholine ( $10^{-8}$  to  $10^{-5}$  M) was also examined in PE ( $10^{-7}$  M) constricted ring. Tension was measured isometrically by using a force transducer connected to signal manifold (Transbridge, Model TBM 4M, World Precision Instrument, Sarasota, FL, USA) and displayed on the computer by data acquisition system (model DI 720 and DI-205; DataQ Instruments, Akron, OH, USA). All responses were measured as differences between basal and peak responses.

### *Biochemical Analysis*

Urinary excretion of nitrite was determined by Greiss Assay (19). Plasma cGMP level was determined using an enzyme immunoassay kit (Cayman chemical Comp. Ann Arbor, MI, USA) following manufacturer protocol.

*Measurement of Lipid Peroxidation Products.* Content of lipid peroxidation products was measured in plasma and tissue homogenates by using a Lipid Peroxide (LPO) kit (Cayman Chemicals, Ann Arbor, MI, USA). Plasma level of 8-isoprostane, an indicator of free radical activity was also measured by an EIA kit (Cayman Chemicals, Ann Arbor, MI). Samples were used either immediately after collection or lipid peroxides were extracted and extracts were stored at  $-80^{\circ}\text{C}$  until used for analysis. The final volume of the assay (1 ml) containing 500  $\mu\text{l}$  sample extract, 450  $\mu\text{l}$  of chloroform-methanol mixture, and 50  $\mu\text{l}$  of freshly prepared chromogen was used to measure absorbance at 500 nm in quartz cuvettes (ThermoSpectronic, Genesys 5, Rochester, NY, USA). Standards of 0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 nmole/ml were used to determine lipid peroxide concentration of samples. Standards of 0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 pg/ml were used to determine 8-isoprostane concentration of plasma samples.

*Preparation of Aortic Tissue Extracts.* Aortic tissue extracts were prepared based on the procedures described by Simons and Vander Jagt (20) with minor modifications. Briefly, after a midventral laparotomy, the aorta was removed, gently cleaned from surrounding fat and tissues and immediately rinsed with ice-cold Krebs's buffer. All subsequent procedures were performed at  $4^{\circ}\text{C}$ . The aorta was cut in small pieces, placed in homogenizing buffer (10 mM potassium phosphate, pH 7.0) and homogenized using a Polytron homogenizer. The homogenate was centrifuged at 10000x g,  $4^{\circ}\text{C}$  for 15 min. The supernatant was used either immediately or stored at  $-80^{\circ}\text{C}$  to be analyzed later for different assays. Protein concentration was determined in supernatant from aortic homogenates using a Micro BCA kit (Pierce, Rockford, IL, USA) and following manufacturer procedures.

*Glutathione Peroxidase (GSH-Px) Assay.* GSH-Px activity in tissue homogenates were measured by using a kit from Sigma Chemical Company. The kit measures enzyme containing a selenium moiety by the use of tert-butyl hydroperoxide (t-BuOOH) as the substrate. The final assay mixture (0.2 ml) was composed of 10  $\mu\text{l}$  of the tissue supernatant, 180  $\mu\text{l}$  of assay buffer, 10  $\mu\text{l}$  of NADPH and 10  $\mu\text{l}$  of t-Bu-OOH. The decrease in absorbance at 340 nm was measured using plate reader (EL808, Ultra microplate-reader) with a kinetic program over 5 minutes period. The activity is expressed as  $\mu\text{mol}/\text{min}/\text{mg}$  protein.

*Glutathione S-transferase (GST) Assay.* GST activity was measured in aortic tissue homogenates using a GST Assay Kit from Cayman Chemical which measures the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione.

*Purification and Immunoblotting of GSTs.* Supernatant from centrifuged tissue homogenate was used for affinity purification of GSTs using a MicroSpin GST Purification Module from Amersham Biosciences (Piscataway, NJ, USA). Equal volumes of purified proteins were loaded onto 12% SDS-polyacrylamide gel and subjected to electrophoresis. Separated proteins were blotted onto PVDF membranes (Amersham Biosciences). Membranes were then blocked with blocking buffer [5%

milk in tris-buffered saline containing 0.1% tween (TBST)]. After blocking, the membranes were probed with polyclonal anti-GST P1-1 antibody (1:1000, dilution Calbiochem-Novabiochem Corp. San Diego, CA. USA) in blocking buffer overnight at 4°C. The membranes were washed extensively with TBST and then incubated with anti-rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution, Amersham Biosciences) for one hour. After extensive washing with TBST, membranes were subjected to enhanced chemiluminescence detection using ECL-Plus from Amersham Biosciences. Signals were captured on X-ray films and quantitation measurements were performed by using a Personal Densitometer SI scanner (PDSI) and Image Quant V 5.0 software from Molecular Dynamics.

*Western Blotting of eNOS.* For the detection of eNOS, western blot analysis was performed as described above for GSTs, except that blocking was for 2 hours at room temperature. Following blocking, the membrane was probed with polyclonal anti-eNOS antibody (1:1000 dilution, Sigma, USA) for 2 hours. After washing, membranes were incubated with an anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution) (Amersham Biosciences) for one hour. The membrane was washed extensively with TBST before subjecting to chemiluminescence as described for GSTs. Quantitative measurements were performed using a PDSI scanner and Image Quant V 5.0 software.

*Statistics Analysis.* Data are presented as Mean  $\pm$  SEM. Comparisons were made within each group and between groups using ANOVA and student t-test for significant differences. P value  $<0.05$  was considered as significant.

## RESULTS

In this study, 30 male Sprague-Dawley rats were divided in 6 groups. For the three-day treatment, the mean initial weights of rats treated with vehicle, 2 or 4 mg/kg of acrolein were  $317\pm 0.89$ ,  $318\pm 3.5$ , and  $314\pm 1.2$  respectively. After three days, weights increased by 6%, 5%, and 4%. For seven-day study, baseline weights were  $315\pm 1.8$ ,  $320\pm 1.5$ , and  $322\pm 1.1$  for vehicle, 2 or 4 mg/kg of acrolein respectively. After seven days of treatment the weights increased by 10%, 6%, and 8% (Table 1).

*Changes in Systolic Blood Pressure (SBP).* Baseline blood pressure in vehicle, 2 and 4 mg/kg of acrolein were  $125\pm 4.0$ ,  $116\pm 5.2$ , and  $117\pm 4.3$  mmHg respectively. After 3 days of treatment acrolein increased systolic blood pressure by 12% and 22% respectively. For the seven-day treatment, the basal blood pressures were  $122\pm 5.8$ ,  $121\pm 4.5$ , and  $120\pm 1.2$  mmHg for vehicle, 2 or 4 mg/kg

Table 1. Distribution of animals, changes in body weight, and systolic blood pressure during the study

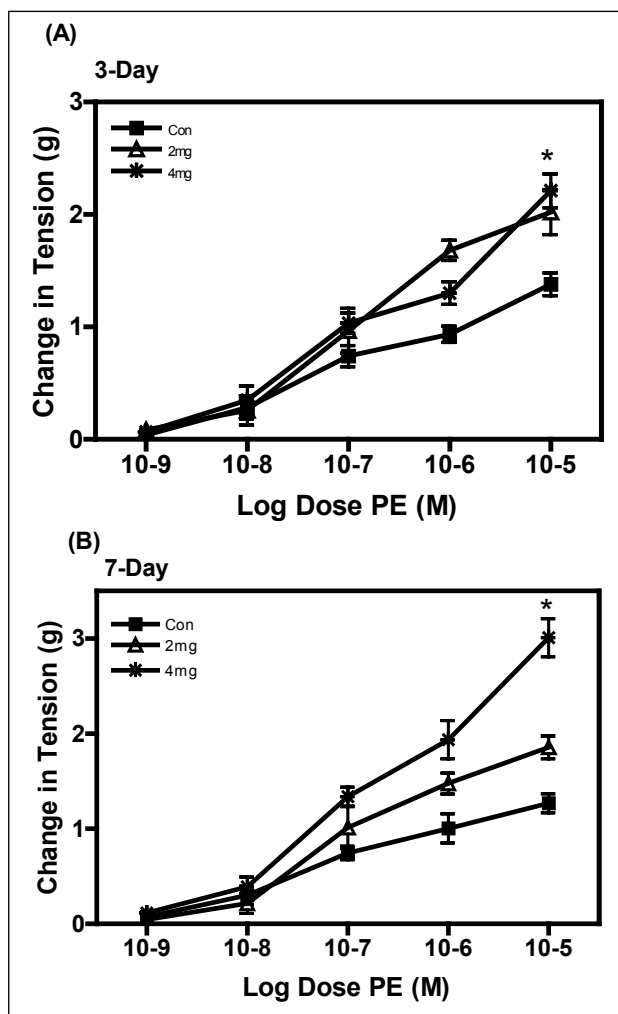
Treatment	Body Weight (g)			Systolic Blood Pressure (mmHg)		
	0 Day (8)	3 Day (4)	7 Day (4)	0 Day (8)	3 Day (4)	7 Day (4)
Control	$316\pm 0.89$	$331\pm 2.3$	$345\pm 2.1$	$123\pm 4.0$	$123\pm 1.8$	$125\pm 4.4$
Acrolein 2 mg/kg	$319\pm 3.5$	$335\pm 1.5$	$340\pm 1.4$	$118\pm 5.2$	$130\pm 2.2$	$133\pm 2.2$
Acrolein 4 mg/kg	$318\pm 1.2$	$327\pm 2.1$	$349\pm 1.7$	$118\pm 4.3$	$143\pm 2.6^*$	$148\pm 3.1^*$

N.B: Values in parentheses are the number of animals in each experimental group. \* $p<0.05$  versus vehicle treatment for the same duration. Values are Mean  $\pm$  SEM.

of acrolein. This was increased by 10%, 25% in 2 and 4 mg/kg acrolein treated groups, respectively (Table 1).

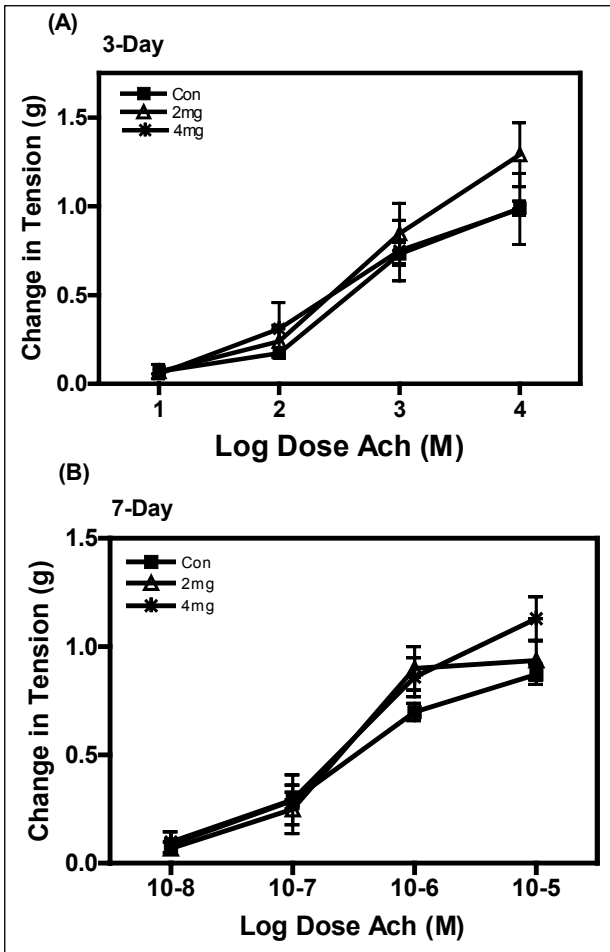
*Effect of Acrolein Treatment on PE Vasoconstriction.* After 3 days of treatment, mean increased in PE vasoconstriction was 1.2 and 0.6 fold respectively in 2 and 4 mg/kg acrolein groups. While in 7 days treatment it was 0.6 and 2 fold increase respectively (Fig. 1).

*Effect of Acrolein on Ach Vasodilation.* The effect of acrolein treatment on the vasodilatory response was investigated by measuring the reactivity of aortic rings to acetylcholine (Ach) (Fig. 2). Treatment with 2 or 4 mg/kg acrolein for 3 and 7 days, exhibited no significant changes in vasodilatory response to Ach compared to vehicle-treated animals.



*Figure 1.* Vasoconstriction Response of Isolated Aortic Rings to Phenylephrine (PE).

Sprague-Dawley rats were treated with acrolein in an aqueous solution at 0, 2, and 4 mg/kg concentration daily (i.p) for 3 (1.A) and 7 (1.B) days. Thoracic aorta was removed and cut into 3-mm-wide transverse rings. Aortic rings were hung in an organ bath filled with 20 ml of Kreb's buffer and equilibrated to 2 g of tension then stimulated with 10<sup>-7</sup> M PE. After washing out PE and adjustment of the baseline, vasoconstrictor response to PE (10<sup>-9</sup> to 10<sup>-5</sup> M) was studied. Tension was measured isometrically. Values are expressed as the mean  $\pm$  s.e.m. of n=4 with duplicates for each sample.



*Figure 2.* Vasodilation Response of Isolated Aortic Rings to Acetylcholine (Ach). Sprague-Dawley rats were treated with acrolein in an aqueous solution at 0, 2, and 4 mg/kg concentration daily (i.p) for 3 (2.A) and 7 (2.B) days. Thoracic aorta was removed and cut into 3-mm-wide transverse rings. Aortic rings were hung in an organ bath filled with 20 ml of Krebs's buffer and equilibrated to 2 g of tension then stimulated with  $10^{-7}$  M PE. After washing out PE and adjustment of the baseline, vasorelaxation response to acetylcholine (Ach,  $10^{-8}$  to  $10^{-5}$  M) was examined after pre-constriction of rings with PE ( $10^{-7}$  M) and adjustment of baseline to 0 (1.B). Tension was measured isometrically. Values are expressed as the mean  $\pm$  s.e.m of  $n=4$  with duplicates for each sample.

*Effect of Acrolein on Nitric Oxide and cGMP Reduction.* To determine whether the acrolein-related increase in vasoconstriction response is associated with alterations in NO, NO synthesis was determined in urine samples and the levels of cGMP, a second messenger molecule in NO signaling, was determined in plasma. Animals treated with 2 mg/kg acrolein for 3 and 7 days did not show any significant change in urinary excretion of nitrite. Although treatment with 4 mg/kg of acrolein for 3 days did not exhibit significant effect on urinary nitrite content, it significantly decreased urinary nitrite excretion in animals treated for 7 days (*Fig. 3*).

*Figure 4* shows that acrolein treatment resulted in different effects on the levels of plasma cGMP in response to different acrolein concentrations. Compared to vehicle-treated controls, animals treated daily with 2 mg/kg acrolein

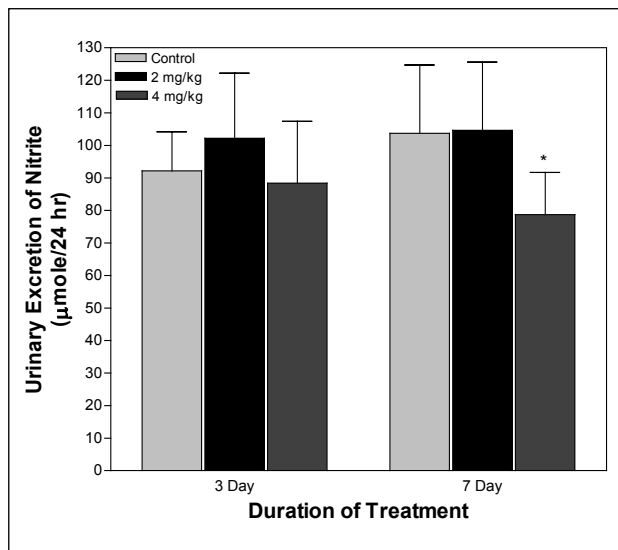


Figure 3. Effects of Acrolein on Urine Nitrite Concentration.

Sprague-Dawley rats were treated with acrolein in an aqueous solution at 0, 2, and 4 mg/kg concentration daily (i.p) for 3 and 7 days and nitric oxide content of urine was measured using Greiss reagent. A 250 µl aliquot of urine sample was mixed with equal amount of Greiss reagent and was used to quantify nitrite concentration at wavelength 540 nm using a plate reader. Values are expressed as the mean  $\pm$  s.e.m of n=4 with duplicates for each sample. \*P<0.05 compared to control.

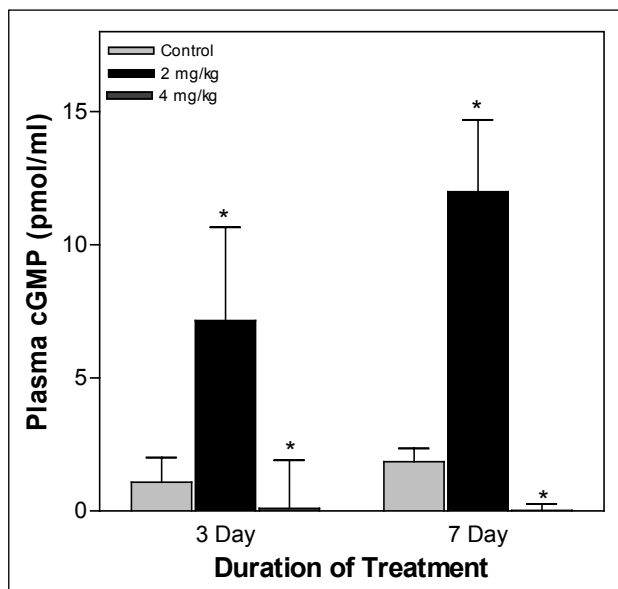
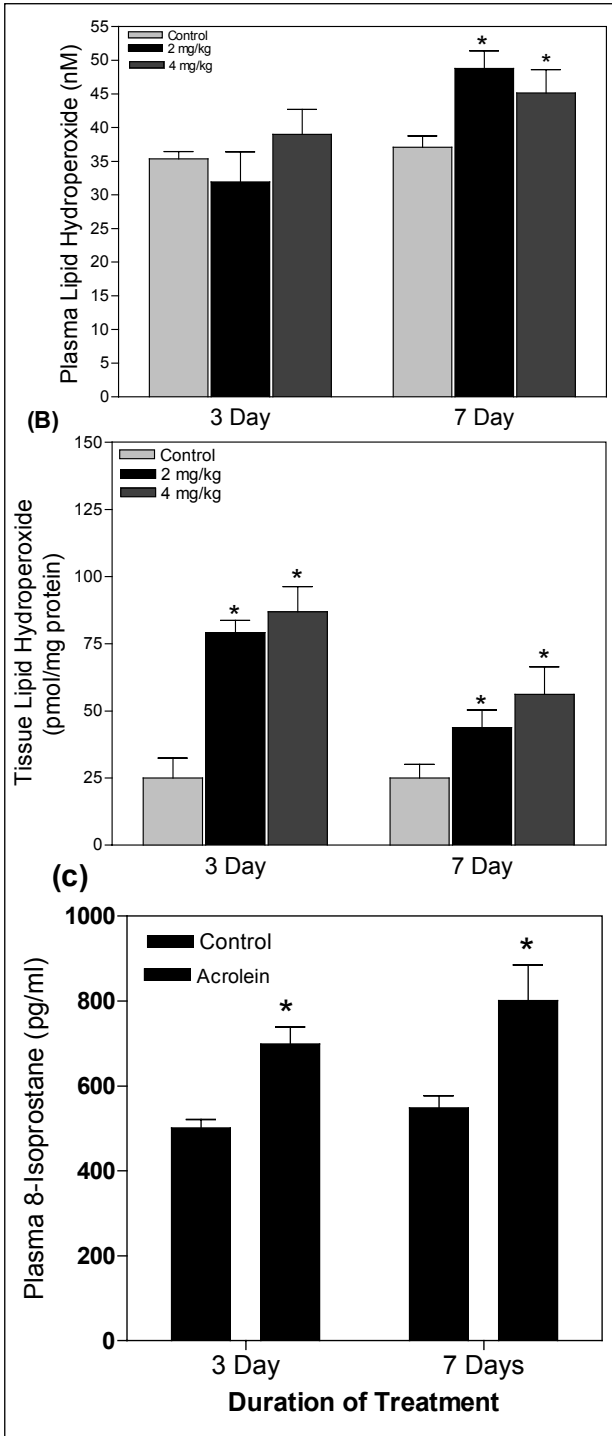


Figure 4. Effects of Acrolein on cGMP Concentration.

Sprague-Dawley rats were treated daily with 0, 2, and 4 mg/kg acrolein for 3 and 7 days. At the end of treatment, blood was collected and plasma was separated. cGMP content in plasma was determined using an enzyme immunoassay kit. Values are expressed as the mean  $\pm$  s.e.m of n=4 with duplicates for each sample. \*P<0.05 compared to control.

for 3 and 7 days exhibited about 7 and 11 fold increases in plasma cGMP content, respectively. In contrast, the cGMP concentration was significantly reduced in animals treated with 4 mg/kg acrolein by 91% and 98% respectively after 3 and 7 days treatment.

*Effects of Acrolein on Plasma and Tissue Lipid Hydroperoxide Generation*  
Treatment with acrolein 2 or 4 mg/kg for 3 days did not alter plasma lipid



*Figure 5.* Measurement of Lipid Peroxidation Products in Plasma and Tissue.

Sprague-Dawley rats were treated with acrolein in an aqueous solution at 0, 2, and 4 mg/kg concentration daily (i.p) for 3 and 7 days and lipid peroxide was measured by a Lipid Peroxide (LPO) Assay Kit in plasma sample (4.A) or tissue homogenates (4.B). Figure 4.C is the measurement of 8-Isoprostane in plasma using a kit from Cayman. The final volume of assay (1 ml) containing 500 µl plasma, 450 µl chloroform-methanol mixture, and 50 µl of freshly prepared Chromogen was mixed and absorbance was measured at 500 nm. Values are expressed as the mean ± s.e.m of n=4 with duplicates for each sample. \*P<0.05.



hydroperoxide (Fig. 5.A). On the other hand, after 7 days treatment, both 2 and 4 mg/kg acrolein increased plasma lipid hydroperoxide generation by 31% and 21% respectively. For tissue lipid hydroperoxide generation both 2 and 4 mg/kg acrolein treatment for 3 and 7 days significantly increased it by 215% and 247% (3 days) and 75% and 124% (7 days) respectively (Fig. 5.B).

Figure 5C reveals that exposure of animals to 4 mg/kg acrolein for 3 and 7 days causes 39% and 46% increase in 8-Isoprostane level in plasma respectively.

**Effect of Acrolein on Glutathione Peroxidase (GSH-Px) Activity.** Treatment with 2 mg/kg of acrolein for 3 days increased GSH-Px activity by 190% but 7 days treatment showed no significant change in activity (Fig. 6). Animals treated with 4 mg/kg of acrolein exhibited a significant change in GSH-Px activity only in group treated for 7 days (212%).

**Effect of Acrolein on Glutathione S-transferase (GST) Activity.** Figure 7 shows that 4 mg/kg of acrolein increased GST activity in rats treated for 3 and 7 days by 70% and 150% respectively. On the other hand 2 mg/kg had no significant effect.

**Effect of Acrolein on GST and eNOS expression.** Figure 8 reveals that both 2 and 4 mg/kg of acrolein increased GST expression by 42% and 100%

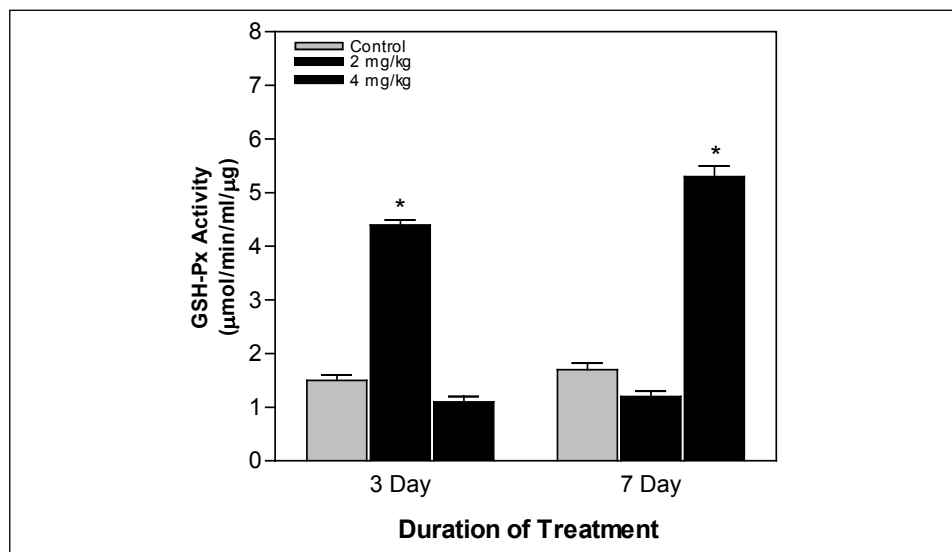


Figure 6. Effect of Acrolein on GSH-Px Activity.

Sprague-Dawley rats were treated with acrolein in an aqueous solution at 0, 2, and 4 mg/kg concentration daily (i.p) for 3 and 7 days. Tissue homogenate and a kit from Sigma Chemical Company were used to measure GSH-Px activity. The final assay mixture (0.2 ml) was composed of 10 µl of the tissue supernatant, 180 µl of assay buffer, 10 µl of NADPH, and 10 µl of t-Bu-OOH. Oxidation of NADPH to NADP was measured by following the decrease in absorbance at 340 nm wavelength as the determination of GSH-Px activity. GSH-Px activity is expressed as µmol NADPH min/µg. \*P<0.005 compared to control.

respectively. On the contrary, acrolein was without any effect on eNOS expression (result not shown).

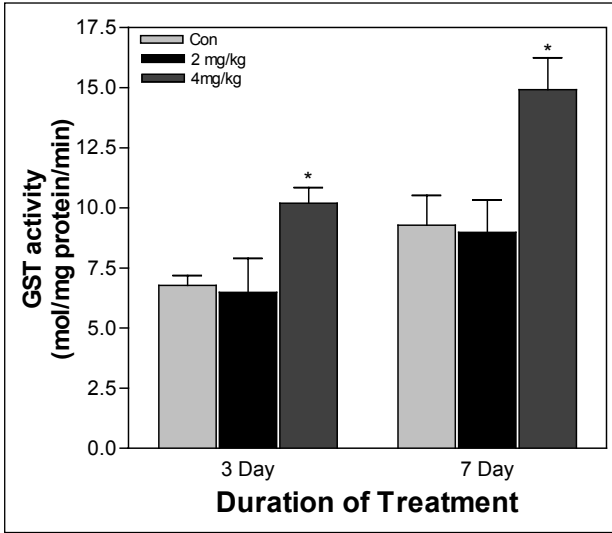


Figure 7. Induction of Total GST Activity after Treatment with Acrolein. Sprague-Dawley rats were treated with 0, 2, and 4 mg/kg acrolein for 3 and 7 days and aortic tissue homogenates were assayed for GST activity toward 1-chloro-2,4-dinitrobenzene. Each experiment was performed in triplicate. Values are expressed as the mean  $\pm$  s.e.m of n=4 with duplicates for each sample. \*P<0.05 compared to control.

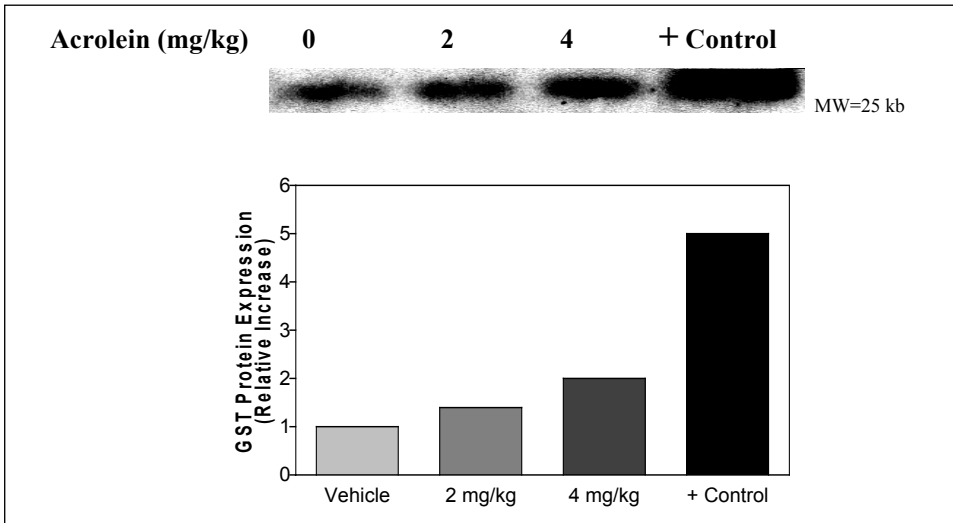


Figure 8. Effect of Acrolein Treatment on GST Expression. Western blot analysis was performed by using purified total GST from vehicle and acrolein treated tissue extracts, equivalent to 0.5 mg of protein. The extracts were prepared and subjected to immunoblotting using anti-GST P<sub>1</sub> as described in materials and methods. Commercial preparation of purified GST was used as a positive control. A representative immunoblot is displayed. Intensity of signals were measured and quantified using PDSI scanner and Image Quant V 5.0 software from molecular Dynamics.

## DISCUSSION

Humans are constantly exposed to a variety of environmental situations, particularly the components of smoke, which are chief sources of acrolein. Although the acute toxicity of acrolein has been extensively investigated, the effect at subtoxic levels is limited, particularly with regard to vascular function and response. Therefore, the present study determined the profile of acrolein concentration-dependent toxicity, to identify the subtoxic concentration of acrolein in Sprague-Dawley rats, which was identified to be between 2 to 5 mg/kg body weight. Based on this information, dose-dependent studies were performed using two concentrations, 2 and 4 mg acrolein/kg body weights.

In this study, acrolein produced an increased in blood pressure that was associated with a decrease in NO availability, an increase in lipid peroxidation, and a decrease in cGMP. Furthermore, an increased in detoxifying enzymes, GST and GST-Px, was observed. As the result of these changes, an increased in vascular response to PE and ultimately an increased in blood pressure was noticed.

One of the consequences of acrolein effects on the cardiovascular system is the alteration of systolic blood pressure (SBP). There are contradicting studies reporting either an increase (21,22) or decrease in SBP (23-25). Our findings are in agreement with Kutzman and colleagues (21,22) who observed a dose dependent increase in blood pressure in Dahl selected rat lines. In the present study, a significant increase in SBP was observed in group treated with 4 mg/kg of acrolein after 7 days of treatment. Consistent with the blood pressure changes, this study demonstrates an increase in PE vasoconstriction after treatment with different doses of acrolein. The most unexpected finding of the aortic ring study was the absence of alteration in Ach-induced vasodilatation. Although we were expecting a reduced vasodilatory response to Ach in acrolein-treated animals, our observation of unchanged Ach action indicated a different mechanism of acrolein action. It seems that administration of Ach was not sufficient enough to evoke a difference over increased vasoconstriction tone in response to PE in acrolein treated rats. The present study has demonstrated reduction in NO production/availability in response to acrolein treatment. This may explain the absence of vasodilatory response to Ach in acrolein treated animals since Ach evoked vasodilatory responses are NO-dependent. Additionally, other studies have reported that it is the increase in vasoconstriction response and not the failure to elicit vasodilatation that regulates SBP effectively (26).

The importance of nitric oxide in the biological system is well documented. NO, produced by endothelial cells, plays an important role in the regulation of cardiovascular functions such as vasorelaxation, platelet aggregation, VSMC proliferation and cardiovascular homeostasis. Depending on the concentration and its interaction with other ROS, NO exhibits a dual redox function, antioxidant and pro-oxidant. At low doses, it functions as an antioxidant and protects cells

against insults of peroxides whereas at higher doses, it causes deleterious effects via nitrosative stress. In vascular diseases, bioavailability of NO depends on its rate of production along with its rate of removal which can result in loss of NO signaling and increased production of new radicals. NO can react with molecular oxygen, superoxide anion, other ROS, transition metals and thiols leading to formation of reactive nitrogen species (RNS) such as  $N_2O_3$ , peroxynitrite, nitrosyl-metal complexes, and S-nitrosothiols (27,28). Since one of the proposed mechanisms of acrolein toxicity is through production of free radicals by modifying thiol groups of important proteins such as NAD(P)H oxidase and xanthine oxidase (29,30), production of peroxynitrite as the result of interaction between NO and  $O_2^{\cdot-}$  will not be surprising. In a recent study by Jaimes et al (31) exposure of cultured endothelial cells to acrolein caused an increase in production of superoxide through activation of NAD(P)H oxidase and authors concluded that reduced bioavailability of NO in their model was due to interaction between NO and  $O_2^{\cdot-}$ . Based on these studies, a free radical scavenger or a compound that increases endogenous antioxidant enzyme activity that can restore NO bioavailability would be beneficial. There are studies that suggest the benefits of compounds such as pentoxifylin (PTX) for their capability to enhance NOS activity, attenuate oxidative metabolism, generate proinflammatory cytokines and preserve antioxidant enzyme activity such as SOD and GSH (32).

In the present study, acrolein treated rats exhibit concentration-dependent endothelial dysfunction, as demonstrated by the increased vasoconstriction response to PE (2 mg/kg for 3 days,  $p < 0.05$ ; 4 mg/kg for 7 days,  $p < 0.05$ ). On the other hand, no significant change in vasodilatory response to Ach was observed. Although the molecular basis of endothelial dysfunction is not completely understood, numerous studies suggest the loss of NO biological activity and/or biosynthesis as a central mechanism. It appears that NO-related mechanisms are involved in endothelial dysfunction caused by acrolein treatment. It is interesting that measurement of NO based on urine nitrite content and plasma cGMP levels, indicate a differential effect depending on acrolein concentration and exposure time. While there was no change in urine nitrite content, there was significant increase in cGMP level in plasma irrespective of treatment time, in response to an acrolein concentration of 2 mg/kg body weight. This observation suggests that at this concentration, acrolein causes activation of NO signaling but does not significantly alter nitrite content. Although the reason for this difference is not clear, it is possible that these responses may be associated with low-dose related protective effects of NO. In contrast to the effect of 2 mg/kg body weight, animals treated with an acrolein dose of 4 mg/kg body weight exhibit significant inhibition of NO-mediated signaling by reducing cGMP level irrespective of exposure time. On the other hand, significant reduction in nitrite content was observed only in animals treated for 7 days. The reason for the difference in nitrite and cGMP content in response to acrolein is not clear. A decrease in NO production/availability can be due to destruction of NO by ROS or its conversion

to RNS, inhibition of NOS via protein modification by ROS and RNS, reduction of NO mediator, cGMP by direct modification of guanyl cyclase through nitrosylation, or inhibition of NOS activity by acrolein. In our study, increased LPOs were observed in aortic tissue which supports the first two possibilities. In contrast to alterations in nitrite and cGMP content in response to acrolein treatment, no significant change in eNOS expression was detected by western analysis, suggesting that change in NO may not be related to eNOS expression.

Like NO, NOS enzymes are sulfhydryl-containing enzymes that are susceptible to altered redox states. Reduced cGMP, as well as reduced NO availability due to acrolein treatment suggest a reduced NOS activity or protein expression. Our western analysis data indicates no significant alterations in eNOS expression in response to acrolein treatment. Inactivation of eNOS or reduction in availability of NO can lead to an impaired vascular reactivity and an increase in blood pressure. Although expression of eNOS is not changed in our model, its function may have been modified by free radicals or acrolein itself, resulting in eNOS-acrolein adduct formation. Although we have no evidence, these modifications are possible, which may cause inactivation of eNOS and then alter NO production. Alternatively, it is possible that NO is produced in normal levels but decreased in its availability to the tissues because of its reaction with reactive oxygen species generated by acrolein. Irreversible loss of NOS function is possible, as a consequence of oxidative and nitrosative stress and oxidation of critical amino acids and polypeptide backbone, which are common effects of interaction with ROS and RNS. Although endothelium expresses mainly eNOS, under certain conditions such as inflammation, it can express iNOS, the inducible form of NOS. Recent studies are indicating that role of NO depends on its source of production also and can be either beneficial or detrimental. These studies are suggesting that iNOS production of NO which is in large amount and is sustained for a long period of time, is likely to be detrimental, while NO from eNOS which is in lesser amount and is short living, is considered to be beneficial (33,34,28). Although iNOS expression was not checked in this study, but the level of NO in this study suggests that iNOS was unchanged.

Increased lipid peroxidation as a result of oxidative stress is known to be involved in the pathogenesis of cardiovascular diseases, including atherosclerosis. To overcome the insults of oxidative stress caused by ROS, cells have developed a number of defense strategies including various protein disulfide reductase enzymes as well as multifunctional DNA repair and thiol-reducing proteins such as the thioredoxin reductase/thioredoxin synthase system. There are also antioxidant mechanisms such as GSH, radical-scavenging vitamins E and C and ROS-metabolizing enzymatic systems such as superoxide dismutase, catalase and GSH-Px.

The results of the present study indicate that acrolein-induced vascular impairment and increase in SBP is related to increase in oxidative stress. This observation is in agreement with the findings of Adams and Klaidman (35) who

have reported an increase in the level of oxygen free radicals upon treatment with acrolein as the result of formation of glutathionyl propionaldehyde. Furthermore, they have reported that acrolein is oxidized by xanthine oxidase and aldehyde dehydrogenase to produce acrolein radicals and  $O_2^{\cdot-}$ . Additionally, Awasthi and Boor (36) have proposed that toxicity due to AAM, whose major metabolite is acrolein, is due to acrolein-induced lipid peroxidation and oxidative stress in rats treated with AAM. GS-propionaldehyde, produced from reaction of acrolein and GSH, produces oxygen free radicals and hydroxyl radicals in the presence of xanthine oxidase and aldehyde dehydrogenase. Formation of these radicals causes lipid peroxidation and further production of reactive oxygen species. The results of the present study provide evidence for acrolein-related oxidative stress in the form of generation of LPOs, and activation of detoxification enzymes. However, the extent of expression of these responses in relation to acrolein dose and exposure time is complicated. Acrolein's influence on multiple cellular targets can cause: (1) depletion of cellular GSH by upregulating GST, causing ROS generation, (2) lipid peroxidation reaction of the ROS with membrane lipids, and (3) generation of acrolein along with other reactive aldehydes from the lipid peroxidation, which further enhances oxidative stress by attacking proteins and DNA. In this study animals exposed to low dose of acrolein (2 mg/kg) for 3 days responded by generating LPOs, but in 7-day treated animals, there was a reduction in LPOs but no change in GST activity. Although the reason for these differences is not clear, changes related to 2 mg/kg acrolein treatment appear to be low-dose-associated protection response. In agreement with this possibility, no significant change in SBP and vascular response was observed at this dose of acrolein. In contrast, at higher concentrations of acrolein (4 mg/kg) in 3 and 7 day treated animals, both generation of LPOs and activation of the detoxification enzyme, GST, were increased. These observations clearly indicate acrolein, by generating additional ROS, impairs the redox state of the vasculature, alters the NO / NOS system, and ultimately translate into impaired vascular reactivity.

The investigation of one of the important components of the antioxidant defense system, glutathione S-transferase (GST), indicates a significant increase in its activity. An increase in GST activity might be a physiological response to increased free radical level. Since glutathione is utilized by the body to detoxify xenobiotics and remove free radicals through its direct antioxidant effect, the toxic levels of acrolein stimulate induction of GST activity, thereby depleting GSH and causing direct damage to the vasculature due to free radicals accumulation. To further investigate activation of GST, expression of GST protein was measured by western blot and an increase in the expression of this protein was found. In support of our findings, other investigators (37,38,39,13) have also shown induced expression of GST protein in VSMC cultures and hepatoma cells treated with acrolein and other  $\alpha,\beta$ -unsaturated aldehydes.

Based on the results obtained in this study, it is concluded that acrolein-induced increased systolic blood pressure and altered vascular reactivity may be

due to an impaired NO and NOS system. This alteration of NO production / availability is probably via increased generation of ROS as well as disturbed antioxidant defense.

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