The Effects of Nitrogen Dioxide and Nitrite on Lung and Plasma Enzymes of the Rabbit

by

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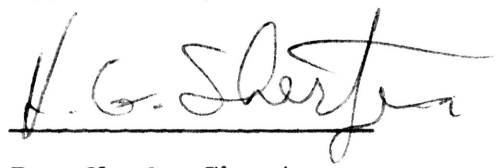

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ABSTRACT

Nitrogen dioxide and nitrite toxicity were investigated using lactate dehydrogenase and pyruvate kinase as indicators of cell damage. Lung tissue enzyme levels were concurrently studied to assess enzyme release. No change occurred in pyruvate kinase or lactate dehydrogenase activities during exposure to 2 ppm nitrogen dioxide or 2% nitrite. Pyruvate kinase and lactate dehydrogenase activities varied greatly in control animals and, therefore, were poor indicators of cell damage in rabbits.

INTRODUCTION

When a tissue is damaged through injury or disease, cellular constituents may be released. The release of specific enzymes from damaged tissues is a valuable diagnostic tool in the detection of certain clinical disorders. For example, the appearance of high levels of serum glutamic oxaloacetic transaminase, lactate dehydrogenase, and creatine phosphokinase indicates myocardial infarction (1, 2, 3). Serum enzyme changes have been used in the diagnosis of hepatitis (4), liver metastasis (5), pancreatitis (6, 7), ovarian neoplasia (6, 7), and other disorders.

Certain disorders produce characteristic alterations in the total activity or isozyme patterns of various enzymes.

* The format of this thesis is basically that of The Journal of Biological Chemistry

(Isozymes are the different forms of enzymes.) A regression to fetal isozymes (dedifferentiation) seems characteristic of neoplastic tissues (8). While such alterations may be random, they may be associated with enhanced survival of tumor cells. Thus, the observed increase of total lactate dehydrogenase activity and differential increase of the M_4 isozyme of lactate dehydrogenase in tumor cells (9) has selective advantage for the developing tumor. The M_4 form of lactate dehydrogenase supports the anaerobic degradation of glucose through glycolysis (10), is characteristic of tissues dependent upon glycolysis (10), and is readily inducible by exposure to hypoxic conditions (11, 12, 13). Since some tumor cells are frequently exposed to hypoxic conditions and rely primarily upon glycolysis to meet energy demands (8), such alterations may assist in tumor cell proliferation. Similarly, the K-isozyme of pyruvate kinase is associated with tissues exposed to hypoxic conditions (8) and is associated with tumor development (8, 14, 15). The shift to the K-isozyme occurs *in vivo* after four days of injecting extracts of tumor cells into mice (14) and *in vitro* following a two hour perfusion of rat liver with blood from rats with tumors (15). Since such changes precede other indicators of tumor development (8), enzymatic alterations appear to be intimately associated with carcinogenesis.

Tissue enzyme changes and serum enzyme alterations are important disease associated events which could have

additional applications. Since diseases of environmental origin are increasingly important in metropolitan areas (16), enzyme changes following pollutant exposure could be clinically and diagnostically significant. Previous investigations suggest that oxidant gases produce such changes. Exposure of guinea pigs to high levels of nitrogen dioxide increased serum lactate dehydrogenase and aldolase activity (17). Similarly, hyperbaric oxygen caused lactate dehydrogenase release from the isolated perfused rat lung (18). The appearance of damage at low levels of oxidant exposure is indicated by: the loss of cilia and hypertrophy of the bronchiole epithelium in rats exposed to 2 ppm nitrogen dioxide (19), hyperplasia of alveolar type 2 cells in rats and guinea pigs exposed to 2 ppm nitrogen dioxide (20, 21), the increased appearance of injected tritiated serum proteins in the lung lavage fluid of mice exposed to 5 ppm nitrogen dioxide (22), qualitative alterations in serum proteins of guinea pigs exposed to 1 ppm nitrogen dioxide (23), and the formation of lipid peroxides in rats exposed to 1 ppm nitrogen dioxide (24). The decreased activity of lung tissue enzymes following oxidant exposure (25, 26) may be interpreted as either a chemical inactivation or a release of enzymes from the lung. However, there is no significant change in the lactate dehydrogenase activity of brain homogenates directly exposed to 7.6 atmospheres of 100% oxygen for three hours *in vitro* (27). Conversely, rats exposed to .75 ppm (26) or 2.5 ppm (25) ozone *in vivo*

show a greater than 10% loss of lactate dehydrogenase activity in lung tissue homogenates. An *in vivo* release of lactate dehydrogenase would explain the discrepancey between *in vivo* and *in vitro* results. Thus, existing studies indicate the release of enzymes from the lung at all exposure levels studied. These studies suggest the feasibility of serum enzyme techniques in the diagnosis of oxidant damage.

As one of the more common pollutants of industrial areas, nitrogen dioxide (NO_2) is an oxidant gas suitable for use in developing such techniques. In aqueous solution NO_2 is converted to nitrite (NO_2^-). Nitrite is also used as a preservative in cured meats (28, 29) and is a normal salivary constituent. Atmospheric nitrogen dioxide and dietary nitrite are common pollutants to which humans are exposed, and, therefore, a comparison of the effects of these environmental contaminants would be relevant. Elevated serum levels of enzymes following nitrogen dioxide or nitrite exposure would be of diagnostic value in detecting tissue damage in laboratory animals during toxicological studies. This study represents a preliminary investigation of pyruvate kinase and lactate dehydrogense activities in the plasma and lung tissue of rabbits exposed to 2 ppm atmospheric nitrogen dioxide or 2% dietary nitrite.

MATERIALS AND METHODS

Materials

Reduced nicotinamide adenine dinucleotide (NADH), pyruvic acid, phosphoenol pyruvate, lactate dehydrogenase, and adenosine diphosphate (ADP) were purchased from Sigma Chemical Co. (St. Louis, MO). Nitrogen dioxide was obtained from Air Products Inc. (Tamque, PA). Sodium nitrite was from J. T. Baker Chemical Co. (Phillipsburg, NJ). All NADH came from the same bottle since a study of NADH from four commercial sources revealed the presence of varying amounts of a lactate dehydrogenase inhibitor in all preparations (30). The use of NADH from a single bottle is the recommended method for reducing resultant variability (30, 31, 32).

Laboratory rabbits

Male New Zealand White rabbits weighing about 2 kg were used in all experiments. All animals were given food and water ad libitum. Nitrite-fed rabbits had approximately 2% nitrite in their food. Control and nitrogen dioxide exposed rabbits were given food which was essentially nitrite-free.

Food preparation

Food was prepared by grinding nitrite-free rabbit chow in a Waring blender. The food was moistened with water and nitrite was added to the food of the nitrite-

fed animals. Control and experimental rabbit chow were heated at 80°C until dry.

Exposure of rabbits

Nitrite-fed rabbits were given food containing nitrite for ten days prior to sacrifice. Nitrogen dioxide-exposed rabbits were placed in an exposure chamber for sixteen hours per day for ten days prior to sacrifice. The nitrogen dioxide was mixed with filtered and humidified air at the chamber entry port and a vacuum pump at the exit port pulled the air through the chamber. Flow meters monitored the flow of air and nitrogen dioxide to insure proper humidity and a 2 ppm nitrogen dioxide concentration.

Collection of plasma

Plasma was collected using either EDTA or heparin as the anticoagulant. Red cells were immediately removed by centrifugation. Plasma was not frozen due to the freeze-lability of some of the lactate dehydrogenase isozymes (33, 34). The enzyme activity of plasma was assayed as soon as possible due to the instability of unpurified pyruvate kinase (35).

Preparation of lung cytosol

Rabbits were sacrificed by removal of blood via heart puncture. Lungs were immediately removed, perfused with Hank's Balanced Salt Solution for two hours to remove red blood cells, and homogenized in a Waring blender for 20 sec. This sample was homogenized in a Potter-Elvejhem

glass homogenizer with a motor driven Teflon pestle. The homogenate was spun for 10 min at 27,000 g in a Sorvall RC2B refrigerated centrifuge at 4°C, using a SS34 rotor, and the supernatant was then spun at 105,000g for 45 min in a Beckman L5-50 centrifuge at 4°C using a 60 Ti rotor. The resultant supernatant comprised the lung cytosol fraction. The cytosol was not frozen and was assayed as soon as possible on the day of collection.

Biochemical analyses

The activities of pyruvate kinase and lactate dehydrogenase were determined by established methods (36) except that pyruvate kinase was assayed at pH 7.0 instead of pH 7.6. Protein was determined by the method of Lowry, et al (37). Other information appears in the legend with each table.

RESULTS

Enzyme activity in heparinized plasma

In clinical enzymology, serum is normally collected by allowing blood to clot in a test tube, rimming the test tube with a glass rod, removing the supernatant, and centrifuging the supernatant to remove whole cells (32, 38). However, ozone and nitrogen dioxide exposure increases the osmotic fragility of erythrocytes exposed in vitro (39). This may be due to lipid peroxides that appear responsible for the lysis of erythrocytes (40) or erythrocyte ghost

vesicles (40, 41) following *in vitro* superoxide (40, 41) or hydrogen peroxide (40) exposure. Since lipid peroxides are significantly increased in rats exposed to 1 ppm nitrogen dioxide *in vivo* (24), nitrogen dioxide could increase the fragility of erythrocytes. Since blood cells contain about one hundred times the lactate dehydrogenase activity of normal serum (32), and since erythrocytes from oxidant-exposed rabbits could be susceptible to hemolysis, traditional clinical techniques used for collection of blood could lead to apparent high enzyme activities in serum from oxidant-exposed andimals. Further, the lability of pyruvate kinase (35) suggests that a rapid technique is necessary for collecting blood.

Heparin initially appeared to be an ideal anticoagulant for this study. Heparinized plasma may be collected by immediate centrifugation of blood from heparin injected animals. Furthermore, heparinized plasma differs from serum only by the presence of heparin and coagulation factors normally precipitated in the clotting process. Most importantly, the lactate dehydrogenase activity in heparinized plasma does not significantly differ from the activity in serum (38).

However, the pyruvate kinase and lactate dehydrogenase activities of heparinized plasma (Table 1) were characteristically quite variable. No significant differences were observed in the activity of either enzyme following nitrogen dioxide or nitrite exposure. A very large blank

activity was noted during some of the lactate dehydrogenase assays.

Enzyme activity in plasma collected with EDTA

Due to the large variability in enzyme activity in heparinized plasma, other anticoagulants were investigated. EDTA was selected as an anticoagulant because it is not an inhibitor of lactate dehydrogenase (32) and because of the absence of the large and variable blank activity present in the lactate dehydrogenase assays of heparinized plasma.

Krebs and Eggleston investigated the effect of diet on rat liver pyruvate kinase activity (42). A low carbohydrate diet or starvation decreased the pyruvate kinase activity in liver. A high carbohydrate diet increased pyruvate kinase activity. Animals on a high carbohydrate diet had about ten times the pyruvate kinase activity of animals on a low carbohydrate diet. Pyruvate kinase

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Table 1. Enzyme activity in heparinized plasma

The enzyme activities are expressed in International Units (μ moles of NADH converted to NAD per minute at 25°C) per ml heparinized plasma. The number of animals in each exposure group is in parentheses. Values are expressed as the mean ± S.E.M.

Exposure	LDH	PK
Control (4)	.090 ± .029	.067 ± .01
$NaNO_2$ in food (2)	.066 ± .004	.50 ± .40
NO_2 gas (2)	.082 ± .039	.11 ± .05

is inhibited by ATP and Ca^{++} (42) and activated by K^{+} and fructose-1,6-diphosphate (FDP) (8, 42, 43, 44). Krebs and Eggleston suggested that both changes in the rate of pyruvate kinase synthesis and in the concentration of effectors caused the differences in pyruvate kinase activity in rats on different diets.

Variations in the activity of the liver form of pyruvate kinase (L-isozyme) could result from altered eating habits following nitrogen dioxide or nitrite exposure. The K-isozyme is the predominate isozyme in rat and human lung tissue (45, 46), rat intestine (45), and rat stomach (45), the organs directly exposed to nitrogen dioxide and nitrite. EDTA is a negative effector of both the K-isozyme and the L-isozyme (35, 47). The FDP-activated L-isozyme purified from human erythrocytes looses 60% of its activity in the presence of EDTA. The FDP-activated form of the K-isozyme purified from pig platelets looses less than 30% of its activity in the presence of EDTA (47). Since EDTA prevents conversion to different allosteric forms, some K-isozyme activity is lost in the presence of EDTA but EDTA eliminates variability caused by conversion to other allosteric forms. Furthermore, EDTA is a chelator of calcium which strongly inhibits pyruvate kinase (42). Therefore, EDTA might inhibit purified pyruvate kinase but not reduce the activity of the unpurified enzyme exposed to Ca^{++}.

In this study, EDTA did not inhibit lactate dehydrogenase or pyruvate kinase (Table 2). Since EDTA could reduce the

Table 2. Effects of EDTA on serum enzymes

Serum was collected by allowing blood to clot in a test tube, rimming the test tube, and centrifuging to remove remaining cells. The controls were diluted with equal volumes of Hank's Balanced Salt Solution prepared without any divalent cations. A 5 mM EDTA solution was prepared in the same Hank's Balanced Salt Solution and serum was diluted with equal volumes of this EDTA solution. Activity is expressed in International Units per ml of diluted serum.

	LDH	PK
Control	0.142	0.147
EDTA solution	0.152	0.160

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variability of pyruvate kinase activity, it was used as anticoagulant in all remaining pyruvate kinase assays.

Neither nitrogen dioxide nor nitrite caused any significant change in lactate dehydrogenase or pyruvate kinase activity (Table 3). The lactate dehydrogenase and pyruvate kinase activity of plasma collected with EDTA varied greatly.

If a single cell type with a characteristic lactate dehydrogenase-pyruvate kinase ratio released different amounts of enzymes in different rabbits, this ratio would remain stable although the total activities of enzymes might vary greatly between rabbits. If a different cell type with a different ratio released large amounts of enzymes into the blood, the lactate dehydrogenase-pyruvate kinase ratio would change. This ratio was calculated for each animal to determine if it was affected by nitrite or nitrogen dioxide (Table 3). The ratio was not significantly affected by either oxidant.

Table 3. Plasma enzyme activity

Syringes were filled to half capacity with Hank's Balanced Salt Solution prepared without any divalent cations and containing 5 mM EDTA. Blood was drawn into the syringe from the median ear artery. Plasma was immediately separated from whole cells by centrifugation. Lactate dehydrogenase and pyruvate kinase activities are expressed in milli International Units per mg protein. All values are listed as the mean ± S.E.M. The number of animals in each exposure group is given in parentheses.

	LDH	PK	PK/LDH
Control (10)	1.39 ± .24	1.44 ± .24	1.22 ± .23
$NaNO_2$ in food (3)	1.62 ± .50	1.70 ± .54	1.39 ± .44
NO_2 gas (3)	1.25 ± .48	0.84 ± .24	1.07 ± .02

Enzyme activity in lung tissue

Like the enzyme activities in plasma, pyruvate kinase and lactate dehydrogenase activities in lung tissue varied greatly. There were no significant changes in the activity of either enzyme following nitrogen dioxide or nitrite exposure (Table 4).

Table 4. Lung cytosol enzyme activity

Activity is expressed in International Units per mg protein. Each value represents the mean ± S.E.M. The number of animals in each exposure group is given in parentheses.

	LDH	PK	PK/LDH
Control (3)	1.39 ± .24	1.44 ± .24	1.22 ± .23
$NaNO_2$ in food (4)	1.62 ± .50	1.70 ± .54	1.39 ± .44
NO_2 gas (2)	1.25 ± .48	0.84 ± .24	1.07 ± .02

DISCUSSION

In contrast to previous reports, plasma lactate dehydrogenase and pyruvate kinase activities failed to increase following nitrogen dioxide exposure. However, the large variance in enzyme activities may have obscured actual changes. For instance, plasma lactate dehydrogenase and pyruvate kinase activities of nitrite-fed rabbits were more than 15% higher than the activities of control rabbits (Table 3). Due to the large variance, these changes were not significant.

Variability in rabbit plasma lactate dehydrogenase has been previously reported. Wróblewski and Gregory (48) noted extreme variation in rabbit plasma lactate dehydrogenase activity. They observed tremendous fluctuations in total activity and lactate dehydrogenase isozyme patterns of the same control rabbit. These fluctuations were consistently observed in control rabbits assayed at weekly intervals for lactate dehydrogenase activity and isozyme distribution. Interestingly, these authors also investigated plasma lactate dehydrogenase after experimentally inducing lung tumors in two rabbits. There was no consistent change in the lactate dehydrogenase activty or isozyme pattern after 6½ weeks, at which time one of the rabbits died. The authors did note an increased variability in the isozyme patterns. Significantly, the authors achieved consistent results with lactate dehydrogenase from human

plasma.

Additionally, lactate dehydrogenase release following oxidant exposure may be a variable event. Nishiki, et al (18) noted a release of lactate dehydrogenase from the isolated perfused rat lung during hyperbaric oxygen exposure. The standard error of the mean at different time points ranged from 20 to 33% of the mean. However, the lactate dehydrogenase activity steadily increased in the perfusate with increasing exposure time. Nevertheless, lactate dehydrogenase release in lungs from normal rats was not significantly different from lactate dehydrogenase release in lungs from rats deficient in tocopherol, a known antioxidant. Further, no data was reported for lactate dehydrogenase activity at various times during rat lung perfusions in the absence of oxidant exposure. Conversely, lactate dehydrogenase and total protein levels increase in control rabbit lung perfusates as perfusion continues (49). Therefore, lactate dehydrogenase release from the isolated perfused lung during oxidant stress is variable and may be a perfusion artifact. Variability in lactate dehydrogenase release following in vivo oxidant exposure is not at all surprising.

Drastic conditions appear necessary to induce changes in lactate dehydrogenase of rabbit serum. Thorling and Jensen (50) noted that the M subunit contributed from 21 to 45 percent of the serum lactate dehydrogenase activity of ten control rabbits. Three of six rabbits made anemic by removing 30 to 40 ml of blood a day for five days had a

higher M subunit activity. Similarly, one of two rabbits injected with 20 mg of cobalt had a higher M subunit activity than any control. However, the authors performed no statistical analysis of this data and did not entirely present the raw data. Therefore, even these conditions may have been statistically nonsignificant. However, the authors noted that all of the nine rabbits exposed to hypoxic conditions (10% O_2 and 90% N_2) for 24 hours remained within the control range.

Conditions employed in this experiment were comparatively mild. While the limit for nitrogen dioxide exposure in industrial workers is 5 ppm nitrogen dioxide (51), rabbits were exposed to 2 ppm nitrogen dioxide in this study. Nitrite concentrations used in this study were high relative to concentrations in human foods. While 150 to 200 ppm nitrite is added to human foods (28), rabbits consumed 2% (20,000 ppm) nitrite in this study. However, even higher concentrations may be found in some livestock forage (52). Nevertheless, any tissue damage produced by 2% nitrite failed to significantly raise plasma lactate dehydrogenase and pyruvate kinase activity in rabbits.

The small number of animals examined for tissue enzyme changes limits the conclusions which can be drawn from this data. Due to the lower enzyme activity in plasma, a release of enzymes from the lung would become significant in plasma before loss was detected in lung tissue. Since enzyme activity did not significantly change in plasma, alterations

in enzyme activity in lung tissue would have to be caused by chemical inactivation or a biochemical adaptation. Such changes are not apparent at these exposure doses in the rabbit.

However, one consistent pattern was observed during this experiment. The levels of both lactate dehydrogenase and pyruvate kinase in EDTA collected plasma increased following consumption of nitrite (17% and 18% respectively). The lung cytosol levels of lactate dehydrogenase and pyruvate kinase simultaneously dropped (35% and 56% respectively). The conclusion that consumption of nitrite produces lung damage initially appears unlikely. However, nitrosamines may be formed from nitrite in vivo in the stomach (29) and in vitro during cooking of food (28). At high levels, nitrosamines are known hepatotoxins (28). Their metabolism depends upon a microsomal enzyme system (53, 54, 55, 56) found in the lung as well as the liver (57, 58). Since there are many forms of this microsomal enzyme system (59), metabolism of a given nitrosamine could preferentially occur in the lung. Metabolites could cause tissue destruction at the metabolism site just as dimethylnitrosamine produces hepatotoxicity (28). While highly tentative, this data may suggest the in vivo formation of nitrosamines from nitrite, nitrosamine transport to the lung, and lung tissue damage by the nitrosamine metabolites. Certainly, this indicates a need for further research.

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