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Effect of Methyl Oleate Ozonide, a Possible Ozone Intermediate, on Normal and G-6-PD Deficient Erythrocytes

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While ozone (O_3) has long been recognized as a respiratory tract irritant (STOKINGER 1957, 1965; YOUNG & SHAW 1964; MENZEL 1976), it has also been unequivocally established that it causes a wide variety of systemic effects including chromosomal aberrations in circulating lymphocytes (ZELAC et al. 1971, 1971a; MERZ et al. 1975), alters liver metabolism (GARDNER 1979; GRAHAM et al. 1982), and changes in erythrocyte metabolism indicative of oxidant stress (LARKIN et al. 1978; BUCKLEY et al. 1975). The mechanism by which O_3 affects such systemic changes is unknown. However, it has been hypothesized that O_3 exerts its toxicity via intermediates (MENZEL et al. 1975). While the nature of such intermediate(s) is not known, theoretical support exists that toxicity may be mediated by O_3 induced formation of systemic ozonides and/or hydroperoxides (MENZEL 1979). The present study was designed to evaluate the effects of a possible ozone intermediate [i.e. methyl oleate ozonide (MOO)] on the red blood cells of normal and glucose-6-phosphate dehydrogenase (G-6-PD) deficient humans. G-6-PD deficient human erythrocytes have been previously hypothesized as being at greater risk to O_3 (CALABRESE et al. 1977) but as yet no direct testing of that hypothesis has been published.

METHODS AND MATERIALS

Using a sample size of five per group, each subject group was compiled as follows:

1. Normal Humans: Volunteers without blood dyscrasias from the campus community at the University of Massachusetts, Amherst.

2. G-6-PD Deficient Humans: Out-patient volunteers clinically diagnosed as A-variant, Mediterranean or Worcester variant G-6-PD deficients from St. Vincent's Hospital, Worcester, Massachusetts.

Blood was obtained by venipuncture, collected in heparinized vacutainers and chilled on ice. The samples were prepared after the fashion of MENZEL et al. (1975). After centrifuging at 900G for 10 minutes, the buffy coat was removed. The cells were then resuspended in a solution of 0.154 M NaCl, 0.02 M KH_2PO_2, pH 7.0, and 0.05 M glucose (Menzel Cell Buffer, MCB). The cells were then centrifuged and resuspended twice more, dis-

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carding the supernatant each time and resuspending to a hematocrit of 5% after the final centrifugation. The cell suspension was aliquoted into culture tubes and placed on ice. One tube provided the pre-exposure parameter values while the other tubes were innoculated with one dose of either 1.6×10^{-3}M, 8.0×10^{-4}M or 4.0×10^{-4}M of MOO and incubated on a rotary shaker at 37°C. After incubation for 40 minutes the post-exposure parameter values were determined.

The dosage levels used were patterned after the concentrations used by MENZEL et al. (1975) to elicit dose-response Heinz body formation in normal human blood and were bracketed about a concentration (8.0×10^{-4}M) that produced a 50% reduction in G-6-PD level in normal human blood in pretesting.

Production of methyl oleate ozonide. Production of the methyl oleate ozonide (MOO) follows the procedure of D.B. MENZEL (personal communication).

1.0 gm methyl oleate was dissolved in 25.0 ml dry n-pentane in a 50 ml Ehrlenmeyer flask. The reaction solution was out-gassed with dry, oxygen-free nitrogen, stoppered and placed in an ice bath to temperature equilibrate at 0°C while preparing the liquid ozone.

Liquid ozone was prepared for reaction by freezing the compound out on dustless Silica Gel (mesh 18-28) in the U-tube apparatus suspended in a dry ice/isopropanol bath (-80°C). The ozone was generated by a Welsbach Laboratory Ozonator, Model T-408 (Welsbach Ozone Systems Corp., Philadelphia) coupled to a Zero-Air Generator. The liquid ozone was blue.

After approximately 2.0 gm of ozone was collected, the gas flow was carefully switched to dry, pure nitrogen to gently sweep out the residual oxygen. The drawn tip of the U-tube was introduced into the ester solution. The reaction was initiated by slowly warming the ozone to room temperature. The MOO formed was a white precipitate in a 1:1 molar ratio to the ozone. The excess ozone and solvent were removed under vacuum at 0°C .

The MOO was purified by quantitative thin layer chromatography using Silica Gel G plates run at 0°C in 10% diethyl ether in n-pentane. Replating of product ozonide, developed under the same conditions, showed a virtually pure product with traces of compounds consistent with peroxidic breakdown products.

Production of the ozonide required a U-tube apparatus formed from 10 mm combustion tubing of the following dimensions: the "U" was 4 inches deep and 3 inches wide with a 2 inch extension set at 90° from one side and a 4 inch extension with drawn tip angled at 60° from the opposite side. The curvature of the base was filled with dustless Silica Gel (mesh 18-28) between glass-wool plugs.

Of paramount importance during ozonide production was temperature control of the liquid ozone. If warmed too rapidly, liquid ozone is violently explosive. N_2 flow should be adjusted to a rate of 1-2 bubbles per second. Any hissing or popping is a danger signal and the tube must be chilled immediately. To facilitate production trials, the liquid ozone may be collected in several U-tubes and stored at -80°C (approximate loss of 5% per day).

Evaluation of Samples. The hematological parameters measured were selected primarily on the basis of their being widely accepted indicators of oxidative stress. The parameters included: methemoglobin (MetHb), levels of reduced glutathione (GSH). MetHb was measured according to the method by BROWN (1973) using potassium ferricyanide and potassium cyanide as reagents and measuring changes in optical density at 630 nm. A colorimetric reaction employing 5,5'-dithiobis nitrobenzoic acid (DTNB) was used to measure the amount of GSH in blood according to PRINS & LOOS (1969) at 421 nm. The measurement of G-6-PD activity was based on an ultraviolet kinetic enzyme assay kit packaged by Princeton Biomedix, Inc., Princeton, NJ 08540. Measurements were made with a spectrophotometer with a temperature controlled flow cell and automatic printer; calculator.

STATISTICAL ANALYSIS

The data collected during experimental trials were reduced to report the mean effect of a reactant on a subject group for a prescribed parameter. The descriptive statistics were generated using the BMDP1S-Simple Data Description program of the BMDP statistical package (University Computing Facility, University of California, Los Angeles. Copyrighted; The Regents of the University of California).

The BMDP1S program generated a Z-score for each individual response which was used to test the data as belonging to a normally distributed population. All responses were within a two-tailed 95% confidence interval ($\alpha = 0.05$) as described by: $-1.96 \leq Z \leq 1.96$. Judging from these results there were no spurious individual responses to treatment (DIXON & MASSEY 1969) and the data were not transformed. The reported values for relative variation from the untreated baseline were calculated as the percent decrease for the G-6-PD and GSH parameters, or as the factor increase for percent methemoglobin formation.

A non-parametric statistical method was used to evaluate the population behavior of the subject groups against the treatments, a two-way classification problem (HOLLANDER & WOLFE 1973). A Wilcoxon Rank Sums Test was used to evaluate the differential responsiveness of the subject groups and to test the dose-response assumption for each reactant. The former was a test concerning all possible pairs and the latter a test of treatment versus control, e.g. baseline values (WILCOXON & WILCOX 1973). The test hypothesis was that the populations were equal.

RESULTS

Response of the G-6-PD Deficient Human Erythrocyte. The erythrocytes of G-6-PD deficient humans displayed evidence of being overwhelmed by the MOO. This was best exemplified by a total elimination of measurable G-6-PD activity in the three MOO incubation concentrations down from a baseline G-6-PD activity of 0.48 ± 0.23 IU/2mHb. These differences were all significantly different from the baseline level at $p < 0.01$. GSH levels were decreased significantly from baseline ($p < 0.01$) level for each MOO concentration. The response also illustrated a dose-related manner ($p < 0.01$). MOO incubation resulted in highly statistically significant ($p < 0.05$) increases in metHb (%) above baseline. As in the case with GSH responses, there was a definite dose-response relationship ($p < 0.05$). (Tables 1 and 2).

Response of the Normal Human Erythrocyte. Erythrocytes from normal humans were shown to be sensitive to MOO in a dose-dependent manner that paralleled the responses of the G-6-PD deficient erythrocytes. In contrast to the total elimination of G-6-PD activity caused by MOO in the G-6-PD deficient erythrocytes, the normal human red cells displayed a dose-related ($p < 0.01$) percent decrease of 38%, 46%, and 58% from a baseline value of 7.36 ± 2.69 IU/gmHb. The normal human erythrocyte response, as measured by decreases in GSH levels and increases in % metHb, were significantly ($p < 0.01$) different from baseline values and followed a dose-dependent response ($p < 0.01$). (Tables 1 and 2).

Relative Erythrocyte Reactivity of G-6-PD Deficient and Normal Humans. The G-6-PD deficient human erythrocytes was significantly more sensitive to oxidant stressing by MOO than was the normal human erythrocyte, as measured by G-6-PD activity and percent increase of metHb. This behavior was consistent whether the data were tested empirically or as the percent decrease in enzyme activity or the percent increase in metHb formation.

Response of the two subject groups to GSH level produced an anomaly. Regarded empirically, there was no difference between the subject groups ($p > 0.10$). However, the baseline GSH level for the enzyme deficient humans was approximately one-half that of the normal humans (66.68 mg/100 ml RBC and 110.10 mg/100 ml RBC, respectively). In light of this fact, percent decrease in GSH level was a more realistic indicator of sensitivity. Evaluation in this context revealed that the G-6-PD deficient human erythrocytes were significantly more resistant to GSH depletion than the normal human ($p < 0.05$).

DISCUSSION

These findings represent an initial attempt to evaluate the hypothesis (CALABRESE et al. 1977) that G-6-PD deficient individuals may be at enhanced hemolytic risk to ambient ozone exposure using human cells. It appears quite clear that normal and G-6-PD deficient erythrocytes respond quite differently as measured

TABLE 1

The Effect of MOO on MetHb Formation (%)[1,2]

Exposure Group	Level 1 4.0×10^{-4}M	Level 2 8.0×10^{-4}M	Level 3 1.6×10^{-3}M
Normal Human	28.5	49.3	72.0
G-6-PD Deficient Human	91.0*	94.0*	95.0*

[1]adjusted for control

[2]Table values are critical difference values for the Wilcoxon Rank Sum Test.

*significantly different from normal human values at $p < 0.01$

TABLE 2

The Effect of MOO on GSH Levels as Measured by % Decrease From Control Values

Exposure Group	Level 1 4.0×10^{-4}M	Level 2 8.0×10^{-4}M	Level 3 1.6×10^{-3}M
Normal Human	57	76	81
G-6-PD Deficient Human	42*	58*	81

*significantly different from normal human values at $p < 0.05$

[1]Table values are critical difference values for the Wilcoxon Rank Sum Test.

by changes in metHb and GSH to the levels of stress caused by MOO at the given concentrations. Of considerable relevance for the further evaluation of the above mentioned hypothesis would be the additional testing of the responses of these different cell types to incubation of MOO at much lower and presumably more realistic concentrations.

It must be emphasized that it isn't definitely known whether MOO is a toxic intermediate of ozone and, if so, how much is formed following ambient O_3 exposures, and what its biological half-life is. Nevertheless, an excellent theoretical foundation supports the premise that O_3 acts via one or more toxic intermediates (MENZEL et al. 1975) and that a fatty acid ozonide such as MOO is a likely candidate. Finally, the use of possible ozone intermediates such as MOO for in vitro studies offers a potentially valuable tool for the elucidation of ozone toxicity mechanisms.

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