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KINETIC ANALYSES OF THE EFFECTS OF HYPEROXIA AND HYPOXIA ON VASCULAR CYCLOOXYGENASE ACTIVITY IN VITRO

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SUMMARY Kinetic analyses were performed to understand the mechanism of hyperoxic induced inhibition of prostacyclin synthesis by human umbilical arteries. Brief exposure of arterial segments to oxygen resulted in over 30% decrease in Vmax of cyclooxygenase in treated vessels. In contrast, cyclooxygenase from hypoxic arterial segments showed approximately a 49% increase in Vmax. There were no significant differences in apparent Km values. These studies suggest that the decreased production of prostacyclin by hyperoxic tissue is due to cyclooxygenase inactivation.

Exposure to high oxygen concentrations is used as a therapeutic modality both in the treatment of respiratory distress in the neonate and adult. This exposure induces morphological as well as biochemical alterations both in pulmonary endothelium in vivo and in cultured vascular endothelium in vitro (1-3). The clinical manifestations of the acute phase of oxygen toxicity include vasoconstriction, thrombus formation and occlusion of the vascular lumen (4). Since prostacyclin produced by normal vasculature is a potent vasodilator and inhibitor of vascular thrombus formation (5), we reasoned that the pathological changes seen during the acute phase of oxygen toxicity might result in part from inhibition of prostacyclin synthesis. We have recently reported that exposure of human umbilical arterial segments to hyperoxia caused inhibition in the release of immunoreactive prostacyclin, and that the decrease in the production of prostacyclin appeared to be due to the inhibition of vascular cyclooxygenase (6). In the present study we have investigated the mechanisms by which changes in oxygen tension affect cyclooxygenase.

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MATERIALS AND METHODS Umbilical cords were obtained from normal full-term vaginal deliveries where there was no evidence of maternal ingestion of aspirin or other drugs within two weeks of delivery. The cords were dissected and the umbilical arteries isolated and cleaned immediately in cold Hank's buffered salt solution (HBSS) without Ca^{++} and Mg^{++} pH 7.4. Segments of approximately 25-40 mg each were prepared and were opened longitudinally prior to use. Arterial segments were placed in 16 x 100 mm glass tubes containing 4.0 ml of 25 mM tris buffered HBSS, pH 7.4. In 4 paired experiments the tissue was subjected for 20 min exposure either to an air stream (control) or to a stream of 95% oxygen-5% carbon dioxide gas mixture (hyperoxia). The gas streams were regulated to maintain agitation at the surface of the buffer without disturbing the tissue at the bottom of the tube. Mean pO_2 values from buffers subjected to such treatment were 160 $\pm$ 2 (1SE) mm Hg (control) and 663 $\pm$ 10 (hyperoxia). pH values were 7.42 for controls and 7.41 for hyperoxic samples. In four further experiments, paired arterial segments were subjected for 20 min to either aeration, or to a 95% nitrogen - 5% carbon dioxide gas mixture (hypoxia). Mean pO_2 values from buffers bathing vascular segments subjected to hypoxic conditions was 56 $\pm$ 10 mm Hg versus 172 $\pm$ 8 in control aerated segments. pH of the buffer differed by less than 0.1 after these treatments. Following exposure of the paired umbilical arterial segments for 20 min to the respective gasses described above, vascular segments were rinsed once with 4 ml isotonic saline and were then homogenized in 50 mM tris buffer, pH 8.0, containing 2 mM reduced glutathione, and 250 µM hydroquinone, and microsomal fractions prepared (7). Microsomal pellets were suspended in 50 mM tris buffer, pH 8.0 containing 2mM reduced glutathione, 250 µM hydroquinone and 1 µM hemin (complete buffer) equilibrated with air. Protein content was determined by a dye binding procedure (8). Microsomes were incubated with varying concentrations of [^{14}C]arachidonic acid (AA) (50-60 mCi/mmol, Amersham) in complete buffer at 37°C. The reaction products were extracted after acidification twice with 2.5 vol of ethylacetate. The combined ethylacetate fractions were dried over anhydrous magnesium sulfate and evaporated under nitrogen. The residue was dissolved in 50 µl of chloroform and analysed by thin layer chromatography on silica gel G plates in a solvent system of ethylacetate-acetic acid (99:1, v/v) (9) with appropriate standards including AA and the prostaglandin metabolites $6KPGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, PGD_2 and TXB_2 (obtained from Upjohn Co.). Following radioautography, the bands containing radioactive metabolites were assayed by liquid scintillation counting. Overall recovery was 92 $\pm$ 8% of radiolabeled AA added, and degree of metabolism was not affected by any of the treatments. Prostaglandin production was calculated from the specific radioactivity of the added substrate and assumes no dilution from endogenous AA. The total cyclooxygenase activity was expressed as the sum of $6KPGF_{1\alpha}$, the stable, nonenzymatic hydrolysis product of prostacyclin, PGE_2 and $PGF_{2\alpha}$ production. No PGD_2 or TXB_2 was detected in these experiments.

Statistical evaluation was performed by the paired t test in all sets of experiments.

RESULTS Conditions for the kinetic analyses of cyclooxygenase from human umbilical arteries were established from the experiments described in Figs. 1 and 2. Fig. 1 demonstrates that cyclooxygenase product formation increased linearly for the first five

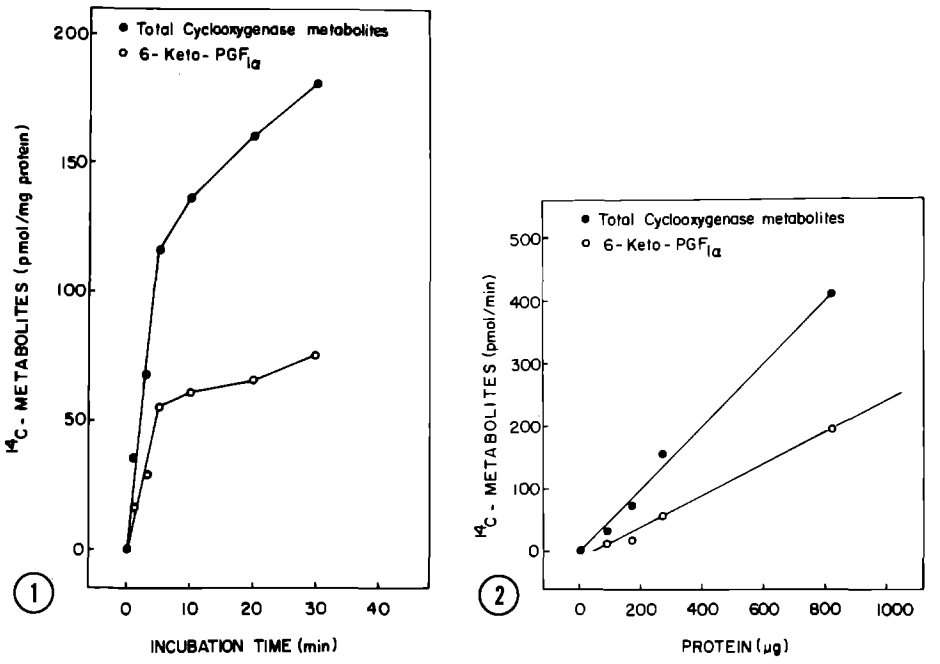

Fig. 1: Time course of the conversion of arachidonic acid to prostaglandins by umbilical arterial microsomes: microsomal protein (500 μg) was incubated with 5 μm ^{14}C-arachidonic acid in 1 ml of complete buffer at 37°C for indicated times. The products were analysed by TLC-radioautography liquid scintillation. Cyclooxygenase products equal the sum of $6KPGF_{1\alpha}$, PGE_2 and $PGF_{2\alpha}$. Values are the means of duplicate determinations. The deviations from the means were always less than 10%.

Fig. 2: Conversion of arachidonic acid to prostaglandins by aortic microsomes: Different concentrations of aortic microsomes were incubated with 5 μm ^{14}C-arachidonic acid in 1 ml of complete buffer for 10 min at 37°C. The products were analysed by TLC-radioautography-liquid scintillation.

min. Thus subsequent initial rate values were determined at 5 min. As depicted in Fig. 2, prostaglandin production increased linearly with added microsomal protein up to 850 μg per assay.

When human umbilical arterial segments were exposed to elevated oxygen tension, the effects persisted in microsomes isolated from those arterial segments. The maximum rate (Vmax) of conversion of arachidonic acid to prostaglandins was decreased by approximately 30% (Fig. 3A). The affinity for arachidonic acid (Km) of the enzyme however, did not change significantly. The results of four such experiments are seen in Table 1. The mean Vmax decreased by nearly 33% ($p < 0.02$) while the Km was not signi-

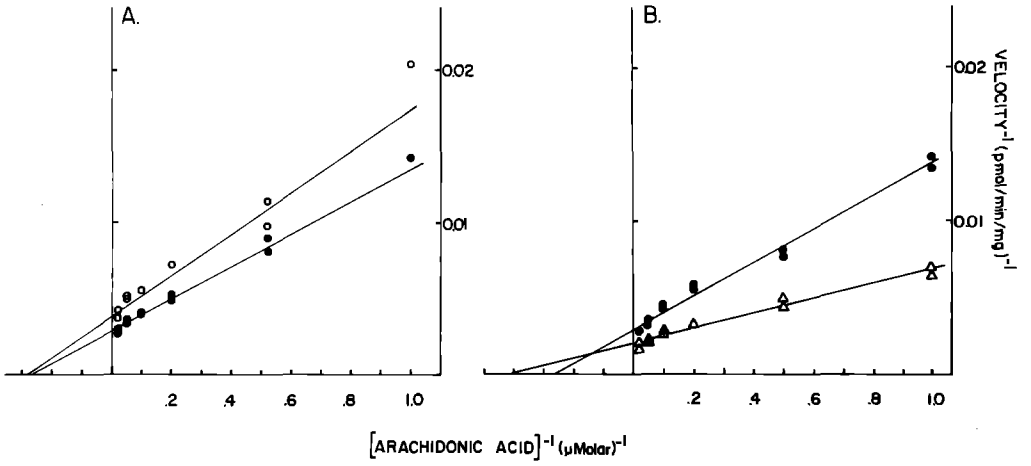

Fig. 3: Effects of exposure to hyperoxia and hypoxia on kinetics of microsomal cyclooxygenase: Microsomes (250 μg) from aerated arterial segments (●) or oxygenated segments (○) (panel A); aerated segments (●) or nitrogenated segments (△) (panel B) were incubated with varying concentration of ^{14}C-arachidonic acid in 500 μl complete buffer (equilibrated with normal room air) at 37°C for 5 min. The products were analysed by TLC-radioautography-liquid scintillation.

ficantly changed. Such changes were seen in each of the four cord arteries analyzed. The relative production of 6KPGF$_{1\alpha}$, PGE$_2$ and PGF$_{2\alpha}$ was not significantly altered by exposure to elevated oxygen tension (Table 2).

Hypoxia produced the opposite effects. As depicted in Fig. 3B, in a typical experiment, exposure of arterial segments to

Table 1. Effects of hyperoxia and hypoxia on kinetic parameters of microsomal cyclooxygenase

Treatment of Arterial Segment	Vmax (pmol/min/mg)	Km app (μM)
Air	260 ± 54	5.9 ± 1.5
Oxygen (n=4)	173 ± 37 ($p < .02$)	3.7 ± 0.5 (NS; $p > .1$)
Air	271 ± 35	5.1 ± 0.7
Nitrogen (n=4)	404 ± 56 ($p < .01$)	3.3 ± 0.3 (NS; $p > .1$)

Table 2. Effects of hyperoxia and hypoxia on products of microsomal cyclooxygenase

Treatment of Arterial Segment	$6KPGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	Total
Air	123 ± 14 (62±5)	38 ± 7 (19±2)	39 ± 10 (19±3)	200 ± 28 (100)
Oxygen	79 ± 22 (56±8)	34 ± 14(24±5)	28 ± 8 (20±5)	142 ± 39 (100)
Air	129 ± 12 (60±3)	42 ± 14(19±5)	46 ± 3 (21±3)	218 ± 24 (100)
Nitrogen	197 ± 36 (59±7)	59 ± 14(18±5)	74 ± 11 (22±4)	331 + 30 (100)

Paired arterial segments were exposed to the indicated gas stream for 20 min at room temperature. Following treatment, microsomes were prepared and incubated with 10 μm [^{14}C]arachidonic acid for 5 min at 37°C in 500 μl complete buffer equilibrated with room air. The products were analyzed by TLC-radioautography-liquid scintillation. Data represent pmol/mg/min (% total products) of cyclooxygenase metabolites. All values are ± 1SD of four experiments.

hypoxia led to an enhancement of cyclooxygenase activity in isolated microsomes. This enhancement resulted primarily from an increase in the Vmax (although some increase in apparent affinity for arachidonic acid was also observed). The mean values for 4 such experiments (Table 1) showed a 49% increase ($p<0.01$) in maximum rate of cyclooxygenase activity in microsomes from arteries subjected to hypoxic conditions. A shift in the apparent Km to lower concentrations was also observed but this was not found to be statistically significant. Again no shift in the distribution of cyclooxygenase products was observed (Table 2).

These results suggest that exposure of umbilical cord arterial segments for short term to differing oxygen tensions can result in altered levels of active microsomal cyclooxygenase.

DISCUSSION We have previously demonstrated that when human umbilical arterial segments were exposed to altered oxygen tensions, significant alteration was seen in their ability to release immunoreactive prostacyclin. Elevated oxygen tension led to

decreased production of cyclooxygenase products while exposure to reduced oxygen tension increased the subsequent formation of these metabolites. A similar stimulation of prostacyclin production after hypoxic treatment has also been reported in dog lung (10). We found that these effects persisted when the metabolism of exogenous [^{14}C]arachidonic acid by microsomes prepared from treated arterial segments was studied, suggesting that the effect of alteration in oxygen tension was operating at the level of cyclooxygenase, rather than fatty acid availability from membrane phospholipids (6).

In this study we have performed kinetic analyses of cyclooxygenase activity in microsomes prepared from vascular segments exposed to altered oxygen tensions, in order to elucidate the mechanism of the observed changes in activity. The conversion of arachidonic acid to the endoperoxides PGG_2 and PGH_2 by cyclooxygenase cannot be directly assayed since those products are rapidly converted to the prostaglandins E_2, $F_{2\alpha}$ and prostacyclin by enzymes in the microsomes, and to prostaglandins D_2 and E_2 by nonenzymatic breakdown (11). However, cyclooxygenase does appear to be the rate-limiting step in the various conversions. Thus, the use of the sum of cyclooxygenase products should be a useful approximation of total cyclooxygenase activity. Our results suggest that the inhibition of cyclooxygenase by oxygen resulted from a decrease in the amount of active enzyme (a 30% decrease in Vmax) rather than the accumulation of a competetive inhibitor which persisted in isolated microsomes (apparent Km was not increased). Conversely, an increased Vmax for cyclooxygenase was seen in microsomes prepared from segments exposed to hypoxic conditions.

Alteration of the rate of autocatalyzed destruction of cyclooxygenase, might well account for our observed changes in the

maximal level of enzyme activity. In vesicular gland microsomes, Smith and Lands (12) demonstrated that active cyclooxygenase leads to its own inactivation by a turnover-dependent mechanism distinct from end product inhibition. At oxygen concentrations below 30 μM (pO_2 approximately 20 mm/Hg) cyclooxygenase is less active (13), thus its autocatalyzed inactivation rate would be expected to be reduced. As a result, subsequent assay would reveal greater remaining activity. At normal oxygen tension the enzyme would be more active, and its destruction more rapid. While the partially purified enzyme does not appear to be stimulated by oxygen concentrations above 30 μM (13), it is possible that in arterial segments, where oxygen gradients exist the intracellular oxygen concentration does not reach the concentration of the bathing buffer. Exposure to oxygen might thus stimulate both cyclooxygenase activity and its destruction.

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