HYPOXIA ENHANCES PROSTAGLANDIN SYNTHESIS IN RENAL MESANGIAL CELL CULTURES

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ABSTRACT

In view of recent findings which suggest that renal prostaglandins mediate the effect of hypoxia on erythropoietin production, we have studied whether hypoxia is a stimulus for in vitro prostaglandin synthesis. Studies were carried out in rat renal mesangial cell cultures which produce erythropoietin in an oxygen-dependent manner. Production rates of PGE_2 and in specified samples also of 6-keto-$PGF_{1\alpha}$, as a measure of PGI_2, and $PGF_{2\alpha}$ were determined by radioimmunoassay after incubation at either 20% O_2 (normoxic) or 2% O_2 (hypoxic) in gas permeable dishes for 24 hrs. Considerable variation in PGE_2 production was noted among independent cell lines. PGE_2 production appeared to be inversely correlated to the cellular density of the cultures. In addition, PGE_2 production was enhanced in hypoxic cell cultures. The mean increase was 50 to 60%. $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ increased by about the same rate. These results indicate that hypoxia is a stimulus for in vitro prostaglandin production.

INTRODUCTION

Renal prostaglandins are thought to be involved in the mechanisms by which hypoxia induces the elaboration of erythropoietin. Prostaglandins of the E-type as well as prostacyclin (PGI_2) and its metabolite, 6-keto-PGE_1, enhance the production of erythropoietin in experimental animals (1) and in glomerular mesangial cell cultures (2). On the other hand, inhibitors of prostaglandin synthesis like indomethacin attenuate the hypoxia-induced production of erythropoietin in vivo (1, 3) and in vitro (2, 3). The constriction of the renal artery in dogs stimulates both erythropoietin and PGE_2 release (4). We have recently shown that the elaboration of erythro-

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poietin increases in mesangial cell cultures grown from rat glomeruli, when the oxygen concentration is lowered in the incubator (5, 6). It was the objective of the present study to investigate whether the synthesis of prostaglandins is also stimulated under these experimental conditions.

METHODS

Mesangial cell cultures

Glomeruli were isolated from male Sprague-Dawley rats (70 - 100 g) by the sieving technique described in (5). About 60 glomeruli/cm^2 were plated in 75 cm^2 tissue flasks (Greiner, Nürtingen) with 15 ml of medium. The medium consisted of RPMI 1640 with 10% fetal bovine serum (Boehringer, Mannheim), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.7 IU/ml insulin. Medium was changed every 2 - 3 days. Glomerular outgrowths were subcultured after 21 days in order to obtain homogeneous mesangial cell cultures (7). Usually 0.5 - 1.0 x 10^6 cells in 5 ml medium were seeded per 25 cm^2 culture dish. Dishes with a gas permeable bottom part were used (Petriperm®, Heraeus, Hanau). Incidentally, cells were also used for study after further passages. On day 7 of subculture and 24 hrs after the last medium change, the medium was carefully removed and substituted by fresh medium that had been pre-equilibrated in the incubator for 3 days. Cells were then incubated at 37° C in a humidified atmosphere containing 5% CO_2 and either 20% or 2% O_2 (O_2 and CO_2 controlled incubators, Heraeus). 24 hrs later, samples of culture medium were taken and frozen in liquid nitrogen. They were then kept at -60° C until assayed for prostaglandins and lactate.
In an additional experiment, the effect of cobalt chloride (10 µmol/l) on PGE_2 production was also assessed in culture. This experiment was carried out because cobalt is a potent stimulus for erythropoietin production *in vivo* (8) and in mesangial cell cultures (6).

Assay of prostaglandins

Prostaglandins in culture media were quantified by direct radioimmunoassays without prior extraction. Fresh incubation medium was used for blanks and binding controls.
PGE_2 was determined by a commercially available (^{125}I) radioimmunoassay kit (New England Nuclear, Dreieich, FRG).
Specific antibodies against 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ were generated in rabbits as previously described (9, 10). The radioimmunoassays were performed as published (9, 10) using 5, 8, 9, 11, 12, 14, 15-^3H-6-keto-$PGE_{1\alpha}$ (specific activity 120 Ci/mmole) and 5, 6, 8, 9, 11, 12, 14, 15-^3H-$PGF_{2\alpha}$ (specific activity 150 Ci/mmole, both New England Nuclear, Dreieich, FRG) as the respective tracers. Free and

antibody-bound fractions were separated using a charcoal suspension. The sensitivities of the assays (detection limit defined as 10% displacement of tracer) were 3 pg/ml for PGE_2, 16 pg/ml for 6-keto-$PGF_{1\alpha}$ and 10 pg/ml for $PGF_{2\alpha}$.

Prostaglandin concentrations were related to the number of cells per dish and are expressed in ng/10^6 cells. In order to disaggregate the cells, culture media were removed and 1 ml of trypsin solution was added (2.5 mg/ml trypsin - 0.18 mg/ml EDTA in Hank's balanced salt solution). Microscopic evaluation showed that the cells were completely disaggregated after 10 min of incubation. Aliquots were then taken from the cell suspensions and cell numbers determined in a Coulter Counter (Coulter Electronics, Krefeld).

Assay of lactate

Lactate production was determined as an index of cellular hypoxia. A commercial L-lactate assay kit was used (Boehringer, Mannheim). The values were corrected for lactate added with the culture media (2 µmol/ml).

Statistical evaluation

Data are expressed as the mean ± standard error. Student's t-statistic was used to compare two group means for significance of difference. The Wilcoxon signed-ranks test was used to compare related groups. P was considered significant at the < 0.05 level.

RESULTS

Fig. 1 depicts PGE_2 production rates in eight independent mesangial cell lines. PGE_2 production was significantly greater in cultures maintained at 2% O_2 than at 20% O_2 (Wilcoxon test applied to compare cell line means). There was considerable variation in PGE_2 production among the different cell lines. As reported below, prostaglandin synthesis depended on cell density. Therefore, it is noteworthy here that the numbers of cells per dish were not significantly different after incubation at 2% O_2 when compared to those of cultures from the same cell line after incubation at 20% O_2. The production of lactate was significantly greater in cultures maintained at 2% O_2 (10.2 ± 0.9 µmol/10^6 cells and 24 hrs, n = 25) than at 20% O_2 (6.5 ± 0.3 µmol/10^6 cells and 24 hrs, n = 27).
Fig. 2 shows production rates of PGE_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ which were determined in three serial subcultures of the line, D. At 20% O_2, 3.6 ng PGE_2, 2.5 ng $PGF_{2\alpha}$ and 0.9 ng 6-keto-$PGF_{1\alpha}$ were produced (mean values per 10^6 cells and 24 hrs). At 2% O_2, prostaglandins formation increased significantly. Their percent

increase was very similar: PGE_2 : + 56%, $PGF_{2\alpha}$: + 53% and 6-keto-$PGF_{1\alpha}$: + 41%. Furthermore, prostaglandin formation was enhanced when arachidonic acid (10 µmol/l) was added to cells of the same line maintained at 20% O_2. Here, 8.3 ng PGE_2, 5.1 ng $PGF_{2\alpha}$ and 1.9 ng 6-keto-$PGF_{1\alpha}$ were produced per 10^6 cells and 24 hrs (mean of 2 determinations with closely similar results).
Cobalt chloride (10 µmol/l) did not significantly stimulate the formation of PGE_2 in this cell line (3.8 ± 0.8 ng/10^6 cells and 24 hrs; n = 7).

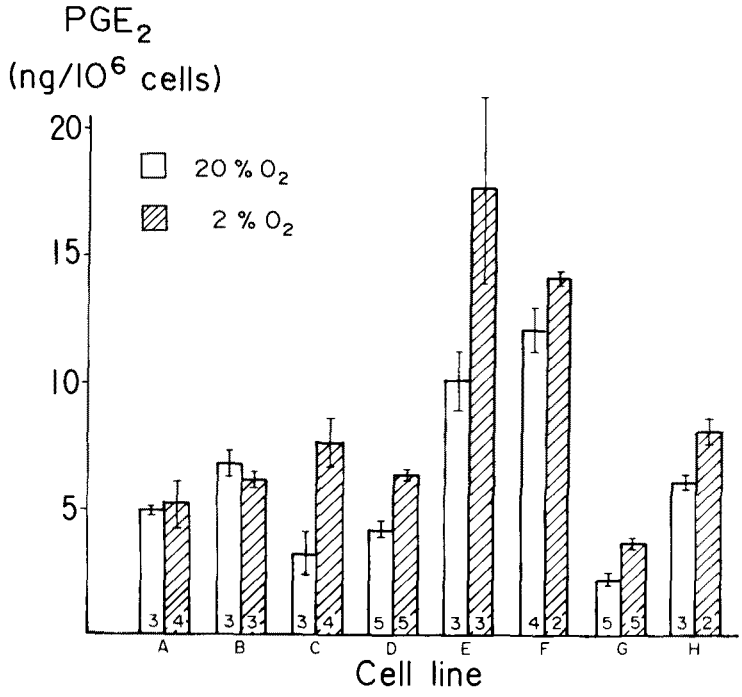

Fig. 1 PGE_2 production in 8 independent mesangial cell lines during 24 hrs of incubation at 20% or 2% O_2. No. of measurements are given at the bottom of each bar. Hypoxic incubation caused a significant increase in the mean PGE_2 production rate (Wilcoxon signed-ranks test).

Possibly, the relatively large scatter of PGE_2 formation among similarly treated cell lines (Fig. 1) resulted from differences in

the cell density. Fig. 3 shows that the formation of PGE_2 tended to be lower in cultures with high cell density. The relationship between cell concentration and PGE_2 formation per number of cells could have resulted from feedback inhibition of PGE_2 synthesis. This possibility was further tested in experiments in which mesangial cells were incubated with 1 ml instead of with 5 ml medium. The results shown in Table 1 indicate that the formation of PGE_2 per number of cells was indeed inhibited when the cell number to medium volume ratio was increased.

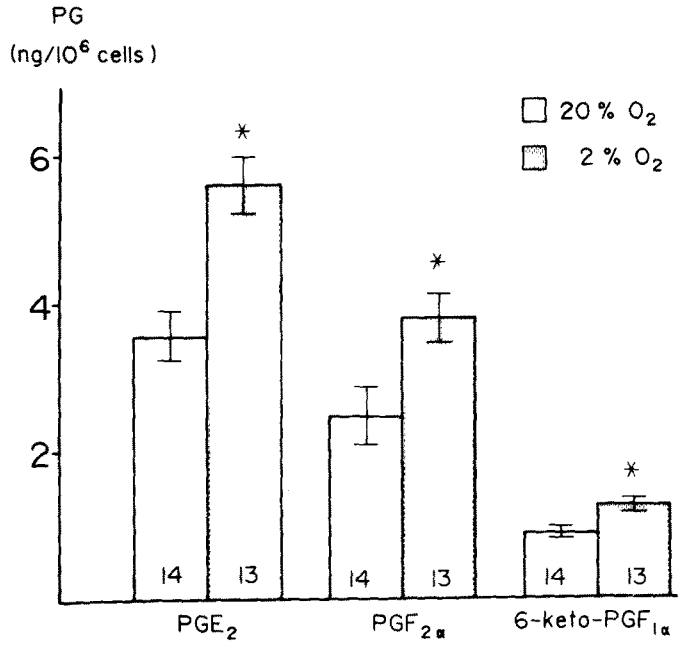

Fig. 2 Prostaglandin production in a mesangial cell culture (line D) during 24 hrs of incubation at 20% or 2% O_2. Asterisks indicate a significant increase at $P < 0.05$ (Student's t-test, No. of measurements are given at the bottom of each bar).

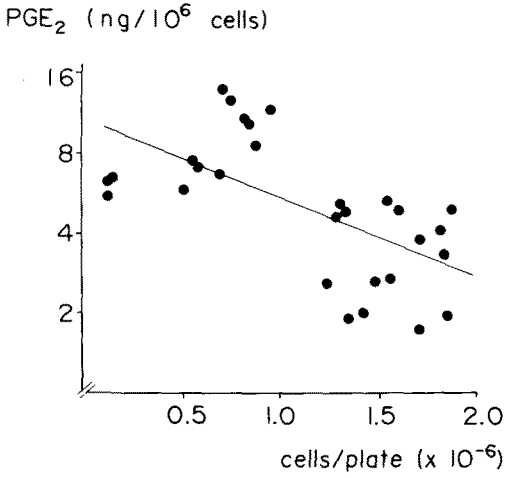

Fig. 3 PGE_2 production related to the cellular density in the culture dish (24 hrs of incubation; 20% O_2). The correlation coefficient, r, is -0.63 in this semilogarithmic production.

Table 1. Influence of cell number to medium volume ratio on PGE_2 formation (20% O_2).

	Medium volume ml	Cells/dish (x 10^{-6})	PGE_2 ng/ml	PGE_2 ng/10^6 cells and 24 hrs	Expt.No.
Expt. 1	5	1.44 ± 0.08	0.63 ± 0.05	2.21 ± 0.20	5
	1	1.31 ± 0.11	1.52 ± 0.12*	1.18 ± 0.08*	4
Expt. 2	5	0.14 ± 0.01	0.17 ± 0.01	6.15 ± 0.27	3
	1	0.14 ± 0.00	0.29 ± 0.03*	2.15 ± 0.26*	3

* significantly different from the respective 5 ml value
Mean ± SEM

DISCUSSION

Earlier studies have shown that isolated renal glomeruli (11 - 13) and cellular outgrowths from glomeruli produce PGE_2, $PGF_{2\alpha}$ and PGI_2 (13, 14). A new finding in the present study was the increase in prostaglandin synthesis by glomerular mesangial cell cultures when the O_2 concentration in the incubator was lowered from 20% to 2%. Lactate production, as an index of cellular hypoxia, also increased during incubation at 2% O_2. Previous investigations have demonstrated enhanced PGE_2 formation by the intact kidney in dogs following renal artery constriction (4) or following the induction of hypoxemia (15). Isolated canine kidneys respond to hypoxemic perfusion with an increase in 6-keto-$PGF_{1\alpha}$ and thus probably PGI_2 production (16). Because PGI_2 titers are also elevated in the perfusate of hypoxic hearts (17, 18), O_2 deficiency appears to be a common stimulus for prostaglandin production. Vasodilatatory prostaglandins produced in response to hypoxia may in turn improve the cellular O_2 supply (19).

Several hypotheses have been proposed to explain the mechanism by which hypoxia could affect prostaglandin synthesis (19). Our results show that PGE_2, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ increased by about the same rate during hypoxic incubation of mesangial cells. Thus hypoxia would not seem to interfere beyond the endoperoxide, PGG_2, level in the synthesis pathway of prostaglandins. It is more likely that the availability of free arachidonic acid increases under hypoxic conditions. Obviously the availability of arachidonic acid was limiting prostaglandin synthesis in our cultures, because elevated prostaglandin titers were found when exogenous arachidonic acid was added to the cells. There are several possibilities by which hypoxia could affect the pool of free arachidonic acid, including an activation of phospholipases because of an increase in the cytosolic Ca^{2+} concentration (19) or a decrease in the rate of fatty acid incorporation into glycerolipids because of a lowered ATP availability (20).

Both PGE_2 and PGI_2 stimulate the production of erythropoietin in mesangial cell cultures (2, 3). It remains to be proven whether the moderate increase of prostanoids production in hypoxic mesangial cell cultures is sufficient to stimulate the elaboration of erythropoietin. However, taken together with our previous observation that indomethacin inhibits the hypoxia-induced production of erythropoietin in mesangial cell cultures (2, 3), a link between the prostaglandin system and erythropoietin production appears likely. Hagiwara et al. (21) have recently demonstrated an important role of endogenous PGE_2 in the mechanism of erythropoietin production by cultured renal carcinoma cells. As pointed out by these authors (21), the possibility still exists that the effect of prostanoids is unspecific, in that prostanoids are essential for renal cells to maintain their functions, including the production of erythropoietin.

The effect of cobalt on erythropoietin production is apparently not mediated by prostaglandins. Cobalt did not stimulate the production of PGE_2 by mesangial cells. It has already been shown that cobalt does not affect PGE_2 synthesis in rabbit kidney medulla slices (22). In addition, the effect of cobalt on erythropoietin production in experimental animals is not attenuated by indomethacin (3).
The amount of PGE_2 produced per culture dish increased with increasing mesangial cells density (Table 1). Hagiwara et al. (23) have already shown that erythropoietin production in human renal carcinoma cells in culture increases in parallel with increasing cell density. However, it was also observed in the present study that the rate of PGE_2 production per number of cells was lowered when the culture medium to cell number ratio was increased. This result indicates a feedback inhibition of PGE_2 synthesis. Declining PGE production at increasing cell density has already been demonstrated in porcine aortic smooth muscle and endothelial cell cultures (24). It is also reported in that study that the addition of fresh culture medium stimulated prostaglandin production, which leveled down, at the latest, after 24 hrs of culture (24). Thus in order to demonstrate more pronounced effects of stimuli of prostaglandin synthesis, experimental devices may be more suitable in which cultured cells are continuously perfused with fresh medium instead of being maintained in a small volume of resting medium.

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