Table 1. DMBA metabolism and cytochrome P-450 content in microsomes from immature and mature female rat adrenals*

Treatment	Cytochrome P-450 content (nmoles/mg protein)†	DMBA metabolism (pmoles formed/ min/mg protein)‡	P
Immature rats			
Control	0.18	48.3 ± 1.1	—
+ ACTH	0.29	91.8 ± 1.2	< 0.001
+ ACTH/DMBA	0.28	84.9 ± 1.7	< 0.01
+ DMBA	0.19	37.0 ± 0.1	< 0.01
Mature rats			
Control	0.49	86.3 ± 5.4	—
+ ACTH	0.43	97.2 ± 10.6	NS§
+ ACTH/DMBA	0.39	83.4 ± 1.1	NS
+ DMBA	0.45	85.8 ± 5.8	NS

* Rats were treated with ACTH and/or DMBA as indicated. The concentration of DMBA was 50 μM.

† Typical results from single experiments with microsomes from pooled adrenals from five rats as indicated in the text.

‡ Mean ± S.D.; duplicate experiments with microsomes from pooled adrenals from five rats as indicated in the text.

§ No significant difference.

cytochrome P-450 and AHH in these adrenals provide an explanation for the diminished sensitivity to DMBA. The fact that adult rats are unaffected by ACTH in these respects indicates that maturation is associated with a marked enhancement of the capacity of the adrenal to metabolize polycyclic aromatic hydrocarbons and drugs.

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Recovery of prostacyclin production by cultured bovine smooth muscle cells after aspirin inhibition: effect of serum replacement and concentration in culture medium

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Arachidonic acid (AA) is converted to PGI_2 in the cells of the vessel wall. This conversion requires a cyclooxygenase-catalyzed step. Aspirin irreversibly inactivates cyclooxygenase, thus inhibiting PGI_2 generation [1]. After aspirin treatment vascular cells recover their ability to produce PGI_2 by synthesizing new enzyme [2]. There are still wide discrepancies however as regards the time for regeneration of PGI_2 synthesis when cultured vascular cells are used. In cultured endothelial cells from the human umbilical vein it has been reported to take 36 hr by one group [2] and 2 hr to recover 50% by another [3]. Cultured porcine aortic endothelial cells seem to require 24 hr [4] and smooth muscle cells from the rat aorta 1 hr [5]. The serum concn in culture media greatly influences cellular events such as protein synthesis by different cells [6, 7]. The aim of the present study was to assess whether supplementation of fresh serum at different concns influenced the recovery of PGI_2 synthesis in aspirin-treated vascular

cells. The effect of serum on the basal production of PGI_2 was evaluated.

Materials and methods

Primary cultures. Bovine smooth muscle cells were derived from aortas pretreated with collagenase to remove endothelial cells. The cells were isolated by enzymatic treatment of the outer part of the media as described previously [8].

Culture media. The cells were grown in 55-mm^2 Leighton tubes [Nunc tissue culture tubes (Libco Europe)] in Eagle's minimum essential medium, with Hank's balanced salt solution supplemented with HEPES (20 mM), penicillin (100 U./ml), streptomycin (100 U./ml) and 5% fetal calf serum and 5% new-born calf serum. These cells were fed twice a week. Fresh culture medium was given to the cells 12–16 hr before use.

Subcultures. The cells were subcultured in a ratio of 1:2 by brief exposure to a 0.05% trypsin–0.02% EDTA solution. The experiments reported here were carried out with cells in the sixth and seventh passages at confluence.

PGI_2 assay conditions. At the beginning of the experiment the cells were washed twice with phosphate buffer saline (Grand Island Biological Co., Grand Island, NY), Ca^{2+}·Mg^{2+}-free, and incubated with 6.6 μM final concentration of AA (Nu Check Prep., Elysian, MN) in 1 ml of buffer (with added 1 mM $CaCl_2$ and $MgCl_2$) for 20 min at 37°. After that period the buffer was removed and stored at −20° until assayed for 6-keto-$PGF_{1\alpha}$ by a radioimmunoassay according to Czervionke *et al.* [9]. Fresh culture medium was added to the cells, and aspirin (200 μM) [in the form of the water-soluble lysine salt (Flectadol, Maggioni, Italy)] or an equal amount of buffer was added. After 30 min the culture medium was removed, 6.6 μM AA was added for 20 min and the cells were tested for 6-keto-$PGF_{1\alpha}$ production as described earlier. Thereafter fresh culture medium, either serum-free or containing 2 or 10% new-born calf serum, was added to the cells. After 1 hr the medium was removed and replaced with fresh material after testing the cells for 6-keto-$PGF_{1\alpha}$ production.

The procedure was repeated after 3 and 6 hr. In some experiments cycloheximide [3 μg/ml (Sigma Chemical Co., St. Louis, MO)] was added to the medium of control and aspirin-treated cells and 6-keto-$PGF_{1\alpha}$ was tested 30 min later and after 3 and 6 hr as described.

At the end of all the experiments the cells were detached by 0.05% trypsin and 0.02% EDTA and counted in a hemocytometer.

The tubes contained approximately 5×10^5 cells. In all instances the cell number did not differ significantly at the beginning and end of the experiment. The data presented in the figures are means of three to five replicate experiments for the same cell preparation. S.D.s, omitted in the graphs, never exceeded 20% of the means. Each figure reports the data from one cell culture. Similar results were obtained from at least three different cell cultures. Statistical analysis was by SPLIT-PLOT design analysis of variance.

Results

The first series of experiments was made to investigate whether the serum concn in culture media influenced the recovery of 6-keto-$PGF_{1\alpha}$ production after aspirin. Fig. 1 shows that after aspirin treatment 6-keto-$PGF_{1\alpha}$ production from exogenous AA was almost completely suppressed (more than 90% of control values). Synthesis of 6-keto-$PGF_{1\alpha}$ was recovered faster when the cells were cultured with 10% of serum than with 2 or 0% ($P < 0.01$). With 10% serum recovery was complete within 3 hr of incubation while at that time with 2 or 0% it reached about 70 and 20% of control. By 6 hr the cells, incubated with 10% serum, produced 1.7 times the concn of 6-keto-$PGF_{1\alpha}$ produced before aspirin, while the cells incubated with 2 and 0% serum remained at 85 and 25% respectively. Fig. 1 also shows the results when the incubation media were replaced each time not with fresh material but with the medium

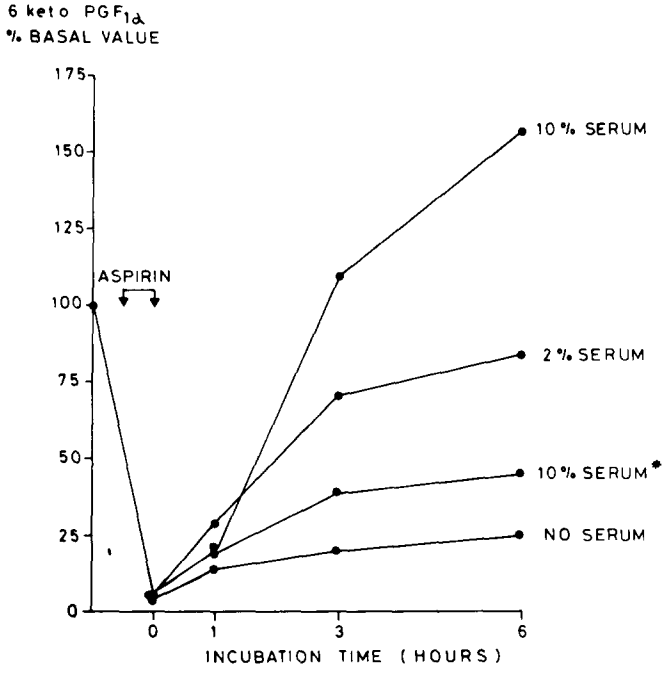

Fig. 1. Recovery of 6-keto-$PGF_{1\alpha}$ production by bovine aorta smooth muscle cells after aspirin treatment as a function of serum replacement and concn. Ability to synthetize 6-keto-$PGF_{1\alpha}$ from exogenous AA was measured at intervals after removal of aspirin. Fresh culture medium with different percentages of serum was used each time except in the curve marked with an asterisk where, instead of fresh material, the medium exhausted by 18 hr contact with untreated cells was used.

exhausted by 18 hr contact with untreated cells. In this condition the effect of 10% serum was markedly lower than when the medium containing either 10 or 2% serum was replaced at each interval ($P < 0.01$ in each condition).

Fig. 2 shows that different percentages of serum in the culture media markedly influenced the production of 6-keto-$PGF_{1\alpha}$ in cells not exposed to aspirin too. When the cells were incubated with 10% serum after 6 hr 6-keto-$PGF_{1\alpha}$ production was 1.8 times more than at the beginning of the experiment. In contrast, the absence of serum resulted in rapid and almost complete loss of 6-keto-$PGF_{1\alpha}$-synthesizing capacity; 2% serum did not significantly modify the production of 6-keto-$PGF_{1\alpha}$ throughout the experiment but the production was lower ($P < 0.01$ at 3 and 6 hr) in comparison to 10% serum. When exhausted medium was used a significant reduction in the cells' ability to produce 6-keto-$PGF_{1\alpha}$ was observed at 3 and 6 hr ($P < 0.01$ vs 2 and 10% serum).

When the cells were not stimulated by AA the production of 6-keto-$PGF_{1\alpha}$ was undetectable at any time and at any serum concn in the media (data not shown).

As shown in Fig. 3 cell treatment with cycloheximide resulted in complete prevention of the recovery of 6-keto-$PGF_{1\alpha}$ production after aspirin (left panel). Untreated cells exposed to cycloheximide lost the 6-keto-$PGF_{1\alpha}$-synthesizing capacity almost completely within 3 hr (right panel). In both experiments media containing 10% serum were used.

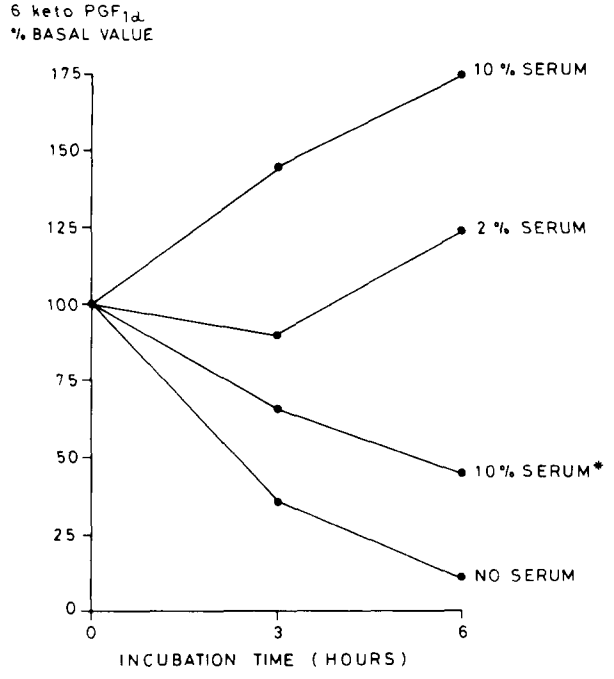

Fig. 2. Time course of 6-keto-$PGF_{1\alpha}$ production by untreated cells as function of serum replacement and concn. The cells were stimulated at intervals with exogenous AA. Each time fresh medium was used, with different percentages of serum except in the curve marked with an asterisk, where medium exhausted by 18 hr contact with untreated cells was used.

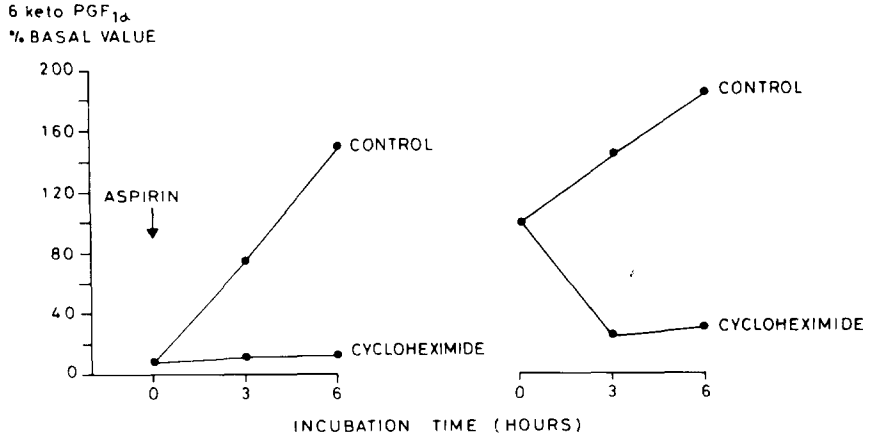

Fig. 3. Effect of cycloheximide on the production of 6-keto-$PGF_{1\alpha}$ by untreated (left panel) or aspirin-treated (right panel) cells. Cells were cultured with 10% serum. The medium was replaced at each interval after stimulation with AA.

Discussion

The data reported here indicate that the capacity of smooth muscle cells in culture to recover their ability to produce 6-keto-$PGF_{1\alpha}$ after aspirin inhibition is related to the serum concn in culture media. Recovery was much faster when medium contained 10% serum than 2 or 0%. This stimulating capacity of serum was much more apparent when the cells were exposed several times to fresh serum as compared to cells for which the serum, even at high concn, was not renewed during the experiment. Whether this is due to replacement of rapidly exhausted stimulating substances or to removal of accumulating inhibitors remains to be determined.

The capacity of 10% serum to stimulate 6-keto-$PGF_{1\alpha}$ synthesis was also apparent in cells not exposed to aspirin. This concentration of fresh serum almost doubled 6-keto-$PGF_{1\alpha}$ production by the cells within 6 hr. The rebound of 6-keto-$PGF_{1\alpha}$ synthesis observed in Fig. 1, and reported by other authors [2, 5] after aspirin, may therefore not be due to accelerated synthesis of the enzyme after inhibition by this drug but rather to experimental conditions favouring enzyme synthesis or activity. A minimum amount of serum is required not only for aspirinated cells to recover their 6-keto-$PGF_{1\alpha}$-synthesizing capacity but also for untreated cells to maintain their basal enzymatic activity.

Treatment of quiescent cells in culture with fresh serum in the presence of adequate nutrients stimulates a series of cellular events like protein phosphorylation, transport and stimulation of RNA and protein synthesis [6, 7]. It is conceivable that fresh serum similarly stimulates cyclooxygenase activity both in untreated and aspirin-treated cells, because it stimulates the synthesis of new enzyme.

This is supported by the observation that cycloheximide, an inhibitor of protein synthesis, prevented the recovery of enzymatic activity after aspirin, at the same time inducing the loss of 6-keto-$PGF_{1\alpha}$ production in untreated cells. The data shown in Fig. 3 suggest that cyclooxygenase is continuously inactivated and resynthesized by the cells. In our experimental conditions a complete turnover of the enzyme seems to be achieved within 3 hr. Since no direct measurement of protein synthesis could be made in the present study the possibility of some additional side effects of cycloheximide (though unlikely at the concns used) cannot be excluded. The stimulating effect of serum on prostaglandin synthesis in different experimental systems has already been reported. Baenziger *et al.* [10] demonstrated that fetal calf serum is able to stimulate the recovery of histamine-mediated PGI_2 synthesis in human endothelial cells in culture, a phenomenon not inhibited by cycloheximide. Hong and Levine [11] and Coughlin *et al.* [12] showed that human serum stimulates prostaglandin production in MC5-5 fibroblasts or bovine vascular cells. They attributed this activity to the platelet-derived growth factor contained in serum which has been shown to stimulate cellular phospholipase A_2 and release of endogenous AA. In our experimental conditions, however, serum did not significantly stimulate 6-keto-$PGF_{1\alpha}$ production when exogenous AA was not added to the cells. This suggests that the increased 6-keto-$PGF_{1\alpha}$ production we observed is due more to a better capacity of the cells to convert exogenous AA to this prostaglandin than to a phospholipase A_2 stimulating activity of serum.

In summary we have shown that fresh bovine serum is able to stimulate AA conversion to 6-keto-$PGF_{1\alpha}$ and to accelerate recovery of this activity after aspirin treatment in cultured smooth muscle cells. The nature of this serum-stimulating property is still unknown. However, the ability of vascular cells to convert AA to 6-keto-$PGF_{1\alpha}$ is regulated in several steps at extracellular level too. The time of recovery after aspirin inhibition could differ dramatically depending not only on intracellular activity but also on the extracellular environment.

Therefore any estimation of the time required for vascular cells to recover their PGI_2-synthetizing capacity *in vivo* after aspirin treatment, based on data obtained from cultured cells, must be made with caution.

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