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Studies on the interaction of minoxidil with prostacyclin synthase *in vitro*

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Minoxidil (6-(1-piperidinyl)-2,4-pyrimidinediamine-3-oxide, Lonolox®) is a potent antihypertensive agent in man [1] and has been implicated to act as a direct smooth muscle vasodilator by a yet undefined biochemical mechanism [2]. Interestingly, as a side effect the drug promotes hair growth in man for which the underlying mechanism is also obscure (cf. [3] and literature cited herein). Recently, Kvedar *et al.* reported that minoxidil selectively inhibits prostacyclin (PGI_2)* synthesis of cultured endothelial and smooth muscle cells from bovine aorta [3]. Prostacyclin synthase had been previously characterized as a cytochrome P-450 protein [4] and therefore spectral changes could be expected with minoxidil. In the present study we have investigated the interaction of minoxidil with solubilized prostacyclin synthase in order to demonstrate that the target for the inhibitory action of minoxidil is the heme active site of prostacyclin synthase.

Materials and methods

Minoxidil was a gift from the Upjohn Co. (Kalamazoo, MI). Piperidine and pyrimidine were obtained from Aldrich-Chemie (Steinheim, F.R.G.) and imidazole, 6-keto-$PGF_{1\alpha}$ and tranylcypromine from Sigma-Chemie (Deisenhofen, F.R.G.). [1-^{14}C]PGH_2 was prepared as described [5]. Bovine aortic microsomes were isolated according to published procedures, resuspended in 10 mM KP_i buffer (0.1 mM BHT, 0.1 mM DTT, 0.1 mM EDTA, 20% glycerol, pH 7.5) and solubilized with 0.5% cholate/0.2% lubrol PX (v/v) for 30 min at 0–4° followed by centrifugation for 60 min at 100,000 *g*. The supernatant, referred to as solubilized prostacyclin synthase, contained 1.61 nmol/ml cytochrome P450 (cf. [6]). Optical difference spectra for determining the binding affinity of minoxidil were recorded with a Cary 118 spectrophotometer (Varian, Darmstadt, F.R.G.) between 350 and 500 nm [7]. The inhibitory effect of minoxidil on prostacyclin synthase activity was assayed upon incubation of [1-^{14}C]PGH_2 with the enzyme followed by HPLC radiochromatographic analysis of [1-^{14}C]-labelled 6-keto-$PFG_{1\alpha}$, the stable hydrolysis product of PGI_2.

Results and discussion

The addition of minoxidil to solubilized prostacyclin synthase (Fig. 1) caused a concentration-dependent increasing difference spectrum typical for a nitrogen ligand binding to cytochrome P450 [7]. The observed spectral change with a peak at 438 nm and a trough at 417 nm is similar to the difference spectrum produced by *trans*-2-phenylcyclo-propylamine (tranylcypromine, 435 vs 415 nm with a K_s-value of 120 μM [6]), the most well-known inhibitor of prostacyclin synthase. From the corresponding titration curve (Fig. 1) a spectral dissociation constant of 2.4 μM can be calculated which, with respect to the enzyme concentration employed (1.6 μM), reflects an almost stoichiometric binding of minoxidil to the heme active site of prostacyclin synthase (therefore the term "K_s" and not K_s should be used).

In order to clarify which nitrogen atom of the minoxidil molecule is involved in the binding process, difference spectra were recorded also with piperidine and pyrimidine. None of these compounds caused a spectral change similar to that of minoxidil in concentrations of up to 1 mM, suggesting that the primary amino group attached to position 2 of the pyrimidine ring interacts with the heme iron (data not shown). Neither minoxidil nor tranylcypromine produced difference spectra with solubilized thromboxane synthase from human platelets in concentrations of up to 1 mM (data not shown). Since the small imidazole molecule selectively interacts with thromboxane synthase [7] but not with prostacyclin synthase (data no shown), this also indicates that there exists a characteristic difference in the topology of the active site of both enzymes.

Thus, only the primary amino groups of minoxidil or tranylcypromine seem to have access to the heme iron of prostacyclin synthase whereas aromatic nitrogen derivatives seem to be sterically hindered. This is just opposite for thromboxane synthase.

* Abbreviations used: BHT, butylated hydroxytoluene (2,6-di-t-butyl-*p*-cresol); DTT, DL-dithiothreitol; PG, prostaglandin; PGI_2, prostacyclin. *Enzymes:* Guanylate cyclase (EC 4.6.1.2); prostacyclin synthase (EC 5.3.99.4); thromboxane synthase (EC 5.3.99.5).

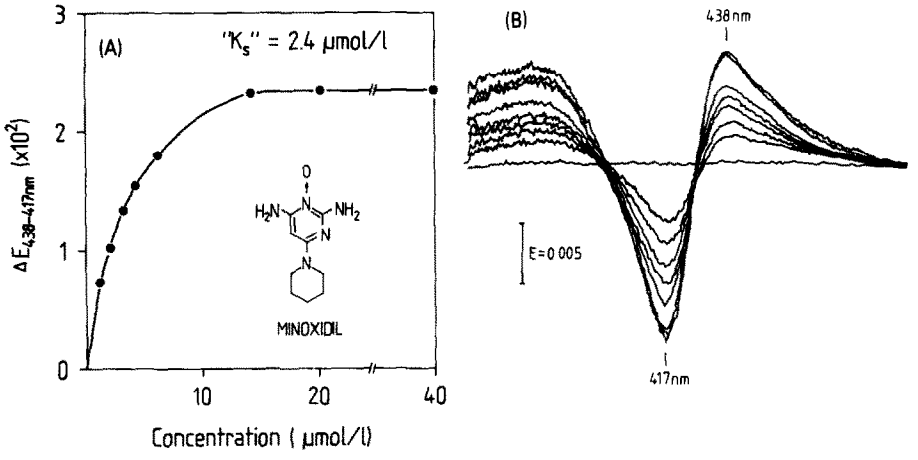

Fig. 1. Spectral interaction of minoxidil with solubilized prostacyclin synthase. Difference spectrum (B) and concentration dependence (A). Solubilized bovine aortic microsomes (0.5 ml corresponding to 0.8 nmol cytochrome P450) were titrated with increasing concentrations of minoxidil (1–40 μM, dissolved in KP_i buffer) at 10°. The spectral dissociation constant was calculated from the height of the resulting difference spectra plotted *vs* minoxidil concentration in a Lineweaver–Burk diagram (N = 2).

Minoxidil also inhibited the enzymatic isomerization of PGH_2 to PGI_2 catalyzed by solubilized prostacyclin synthase yielding 50% inhibition at 90 μM (Fig. 2). By comparing the K_s and IC_{50}-value of minoxidil on the basis of the different enzyme concentrations used in both assay systems, it turned out that the binding affinity of minoxidil is approximately 1500-times greater than its inhibitory potency, suggesting a competitive interaction with PGH_2 at the active site. Apart from this consideration, the IC_{50}-value of 90 μM is well in line with the results of Kvedar *et al.* which reported IC_{50}-values of 10–700 μM depending on their various cells and stimuli employed (cf. [3]).

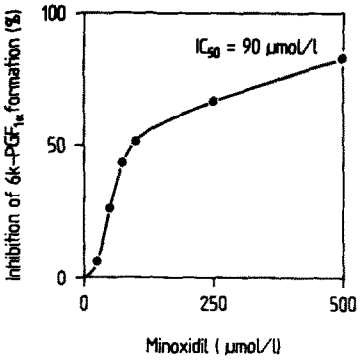

Fig. 2. Inhibition of prostacyclin synthase activity by minoxidil. Aliquots of [1-^{14}C]PGH_2 (20 μM, 0.45 Ci/mol, dissolved in 2-propranol) were pipetted into glass tubes, the solvent was removed under nitrogen and subsequently 20 pmol of solubilized prostacyclin synthase (dissolved in 0.2 ml of 50 mM KP_i buffer, pH 7.4) were added (control experiments). Minoxidil (25–500 μM) was preincubated with the enzyme for 2 min at 0–4°. The incubations were carried out for 5 min at 25° followed by acidification to pH 3.0 with 2 M citric acid and extraction with ethyl acetate (2 ml). The extracted products were separated by reversed-phase HPLC on Nucleosil C_{18} (5 μm-material from Macherey & Nagel, Düren, F.R.G.). The column (250 × 4.6 (ID)mm) was isocratically eluted with acetonitrile/water/acetic acid 35:65:0.1 (v/v, pH 3.5) at 1 ml/min. The amount of [1-^{14}C]-labelled 6-keto-$PGF_{1\alpha}$ formed enzymatically was calculated from the radioactivity co-eluting with authentic 6-keto-$PGF_{1\alpha}$ (retention time 7.2 min, UV-detection at 200 nm) (N = 2).

Conclusions

With respect to the present results it can be concluded that the recently observed inhibition of PGI_2 synthesis in bovine endothelial and smooth muscle cells caused by minoxidil is based on the direct interaction of this drug with the heme active site of prostacyclin synthase. In view of the well-known vasodilator properties of PGI_2 [8], it seems puzzling that an antihypertensive agent inhibits PGI_2 synthesis in the vessel wall. However, since Böhme *et al.* demonstrated that minoxidil can stimulate soluble guanylate cyclase *in vitro* [9], it seems likely that the antihypertensive effect of minoxidil is due to an increase in intracellular cGMP which in turn leads to smooth muscle relaxation. PGI_2 has been also implicated to modulate the proliferation and differentiation state of endothelial cells and preadipocytes in culture, probably by raising intracellular cAMP [8]. Whether the inhibition of PGI_2 formation by minoxidil is therefore related to some of the side effects of this drug, e.g. the increased growth of vellus hair (cf. [3] and literature cited therein), is unclear at present but merits further elucidation.

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Status of reduced glutathione in the human hepatoma cell line, HEP G2

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There have been a number of reports recently suggesting that human hepatoma cell lines which retain differentiated parenchymal functions may provide an *in vitro* system for studying drug metabolism and cytotoxicity directly in man. Certain human hepatoma cell lines can carry out both cytochrome P-450-dependent mixed-function oxidase (MFO) reactions and conjugations with glucuronic acid and sulphate [1–4]. One cell line, Hep G2, has been used to activate chemicals for studies on mutagenesis and sister chromatid exchange [5, 6] and, furthermore, it has been shown to form similar adducts with benzo(*a*)pyrene as are formed in explants from normal human tissue [2]. In addition, the MFO activities and the glucuronic acid conjugation in these cells respond to *in vitro* exposure to the inducing agents phenobarbitone (PB)†, 1,2-benzanthracene (BA) and 3-methylcholanthrene [3, 7].

Intracellular reduced glutathione (GSH) plays an important role in the detoxification of a variety of chemicals by conjugating with electrophilic drug and carcinogen metabolites or by acting as a reductant in the metabolism of hydroperoxides and free radicals. Thus, the intracellular GSH content of cells controls the extent of carcinogenicity and cytotoxicity of many chemicals [8–10] and it is important therefore to investigate the status of GSH in the Hep G2 cells. This manuscript reports on the GSH levels in cultured Hep G2 cells, the ability of the cells to synthesise GSH, the effect of GSH depletion of cell viability and growth and compares the activity of glutathione-*S*-transferase (GST) in Hep G2 cells with that in freshly-isolated human adult hepatocytes. Two methods for depletion of GSH were used in this study. For measurement of GSH synthesis, the intracellular GSH was depleted by treatment with diethylmaleate (DEM), which acts by conjugating with the GSH [11]. The effect of GSH depletion on cell growth in culture was investigated using DL-buthionine-*SR*-sulfoximine (BSO), a potent irreversible inhibitor of γ-glutamylcysteine synthetase [12].

† Abbreviations used: GSH, reduced glutathione; DEM, diethylmaleate; BSO. DL-buthionine-*SR*-sulfoximine; CDNB, 1-chloro-2,4-dinitrobenzene; LDH, lactate dehydrogenase; PB, phenobarbitone; BA, 1,2-benzanthracene; GST, glutathione-*S*-transferase.

Materials

Reduced glutathione, diethylmaleate, 1-chloro-2,4-dinitrobenzene, Trypan Blue, 1,2-benzanthracene, and DL-buthionine-*SR*-sulfoximine were obtained from Sigma. Flow Laboratories supplied the Dulbecco's medium and foetal calf serum was purchased from Gibco. Hep G2 cells were obtained by Dr W. T. Melvin, Department of Biochemistry, Aberdeen University from Professor C. N. Hales, Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge.

Methods

1. *Cell culture.* Hep G2 cells were routinely grown in monolayer or multilayer culture in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) foetal calf serum. They were grown in a humidified atmosphere of 5% CO_2 in air and subcultured every 7 days at a split ratio of 1 to 3. For experiments on enzyme induction, confluent cells (day 7 after subculture) were treated with 2 mM PB or 25 μM BA for 3 days before GSH content and GST activity was measured on day 10. The medium was changed every day during these experiments.

2. *Isolation and incubation of Hep G2 cells.* Cell suspensions were prepared by treating confluent cultures with a 1:5 solution of 0.25% (w/v) trypsin: 0.02% (w/v) versene in phosphate buffered saline, pH 7.4. The viability of the suspensions was assessed by Trypan Blue exclusion (typically >90%). Cells were incubated in 50 ml round bottomed flasks in Krebs–Henseleit buffer, pH 7.4, containing 10 mM HEPES at 37° under an atmopshere of 95%O_2:5%CO_2.

3. *GSH resynthesis.* To deplete intracellular GSH, 10^6 cells/ml were incubated for 60 min at 37° with 0.02% (v/v) diethylmaleate (DEM). After this period the cells were sedimented by centrifugation at 600 *g* for 4 min and viability determined by Trypan Blue exclusion. For measurement of GSH resynthesis the cells were resuspended (10^6 viable cells/ml) in Krebs–Henseleit buffer supplemented with Dulbecco's amino acid solution deficient in sulphur containing amino acids.This amino acid solution was supplemented with 0.5 mM L-methionine, and/or 0.5 mM L-cysteine and in certain experiments 5 mM BSO was present as an inhibitor of GSH synthesis. Samples (1 ml) were