Effects of gemfibrozil on the oxygen transport properties of erythrocytes

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- 1 In the present study we have investigated the effects of the relatively low plasma concentrations of gemfibrozil (GFZ) found in clinical practice on the oxygen dissociation curve (ODC) of erythrocytes.
- 2 ODCs were measured at 30° C and 37° C and at pH 7.4: a) both on HbA solution and erythrocytes incubated *in vitro* with gemfibrozil and clofibric acid; b) on erythrocytes from healthy volunteers treated with a single oral dose of gemfibrozil.
- 3 These experiments showed a significant drug-induced shift of the ODC towards lower O₂ affinity values without any significant modification of metabolic parameters of erythrocytes such as intracellular pH and intraerythrocytic levels of ATP and DPG.
- 4 In our experimental conditions gemfibrozil appears to lower both *in vitro* and *in vivo*, the partial pressure of oxygen required to give 50% of the haemes saturated with oxygen (P₅₀) of erythrocytes from the control value of 24 ± 0.5 mm Hg to 29 ± 0.5 mm Hg (mean ± s.d.; *P* < 0.02 by ANOVA).
- 5 These data clearly indicate that therapeutic doses of gemfibrozil may influence the oxygen transport properties of red cells. This effect could have relevant pharmacological and toxicological implications.

Keywords erythrocytes haemoglobin gemfibrozil fibrates oxygen toxicity
side effects coronary heart disease peroxisome proliferators

Introduction

It has been reported that lipid regulating drugs like fibrates (clofibric acid, bezafibrate, gemfibrozil) may significantly reduce the incidence of coronary heart disease in men with dyslipidaemia [1, 2].

However their mechanism of action is only partially established and remains controversial. Related to the hypotriglyceridaemic effect are an increase in the activity of lipoprotein lipase and a decreased synthesis and secretion of VLDL (very-low-density lipoprotein) at the level of the liver. The mechanism by which fibrates lower LDL (low-density lipoprotein) might involve enhanced hepatic clearance of VLDL and IDL (intermediate-density lipoprotein), which in turn reduce the production of LDL [3].

Moreover studies performed in the search for possible drugs against sickle cell anaemia showed that fibrates *in vitro* do shift the oxygen equilibrium curve of intraerythrocytic human haemoglobin to the

right [4, 5]. It has been demonstrated they permeate freely the erythrocyte membrane and lower the oxygen affinity of human adult haemoglobin (HbA) more strongly than does the natural allosteric effector 2,3 biphosphoglycerate (DPG) by combining with different and multiple binding sites in the haemoglobin molecule [6–7]. In this respect, X-ray crystallographic analysis has shown that three pairs of molecules of bezafibrate bind to walls of the central cavity between the α subunits of the HbA molecule [6–8].

At the functional level it has been reported that when human HbA is incubated with 5 mmol l⁻¹ bezafibrate, pH 7.2, at 25° C, in the presence of 10 mmol l⁻¹ DPG, the P₅₀ rises from 14 to 38 mm Hg [4]. Hence, this drug binds preferentially to deoxy-haemoglobin displaying a synergistic effect with DPG. *In vivo* such an effect could facilitate the

conformational transition oxy $\rightarrow$ deoxy at the level of peripheral tissues causing a greater unloading of oxygen.

In the light of such considerations we have postulated that in treated hyperlipoproteinaemic patients the relatively low plasma concentration of these drugs could modify, though to a small extent, the oxygen dissociation curve of the erythrocytes, thereby influencing the oxygen transport properties of the blood. Even a slight change in oxygen transport, might induce, in the long run, new pharmacodynamic aspects which have to be considered when looking at the pharmacological effects of fibrates such as those observed in the treatment of coronary heart disease.

On the whole, the possibility of iatrogenic modifications of the O₂ transport properties of erythrocytes should be carefully investigated to evaluate all the possible clinical implications of a given pharmacological treatment.

Methods

Subjects

To carry out *in vitro* and *in vivo* experiments blood samples were obtained from healthy donors, ranging in age from 27 to 47 years (mean: 36 ± 7.1). All volunteers were fully informed on the modalities and end points of the study, and all signed consent forms. The study was approved by the University Internal Review Board.

Measurement of oxygen dissociation curves (ODC)

Erythrocytes and haemoglobin solutions were prepared as previously described [9]. Oxygen binding to HbA was followed by absorption spectroscopy. Values of P_{50} and n (Hill coefficient; an empirical index of cooperativity) for oxygen association to HbA, in the presence of the physiological allosteric effector 2,3 DPG, as well as in the presence of clofibric acid, gemfibrozil and pravastatin were determined at 37° C from absorbance changes accompanying the dioxygen molecule binding by the tonometric method as previously described [9]. The high turbidity of the red cells was compensated for by using a scattering opaque glass in the reference beam. The methaemoglobin content was checked after each experiment and never exceeded 2%.

All the data were obtained at pH 7.4 in 5.0×10^{-2} M Tris/HCl buffer plus 1×10^{-1} M NaCl and 3.0×10^{-3} M DPG, temperature = 30° C or 37° C.

Laboratory methods

Analysis of the intraerythrocytic concentration of ATP and DPG was performed by enzymatic determination according to the methods described previously [10, 11]. All enzymes, coenzymes and substrates for metabolite analyses were purchased from Sigma. Gemfibrozil, clofibric acid and 2,3 diphosphoglycerate were from Sigma (St Louis, MO, USA).

Measurement of intracellular pH has been performed by phosphorus-31 nuclear magnetic resonance (³¹P-NMR) as reported [12, 13]. ³¹P-NMR measurements were performed at 121.47 MHz using a Varian Gemini 300 spectrometer.

In vitro study

For *in vitro* studies HbA solutions and citrated whole blood were incubated at 37° C with gemfibrozil and clofibric acid, solubilized in dimethylsulphoxide (DMSO). Citrated whole blood and HbA solutions were incubated with and without DMSO and with pravastatin solubilized in phosphate buffered solution, as controls at the same conditions of pH and temperature.

A number of O₂ equilibrium experiments were performed on erythrocytes and purified haemoglobin A in order to obtain quantitative data on the oxygen affinity and the haem-haem interactions.

The oxygen binding properties of human haemoglobin solutions were investigated at pH 7.4 both in the absence and in the presence of 5 mmol l⁻¹ clofibric acid, 5 mmol l⁻¹ pravastatin, 5 and 10 mmol l⁻¹ gemfibrozil.

The same experiments were carried out using concentrations of drugs corresponding to usual plasma concentrations reported during therapeutic treatments.

In vivo study

For *in vivo* study experiments, therapeutic doses of granular gemfibrozil were administered to healthy volunteers. The subjects were treated after an overnight fast. They abstained from any medication from 1 week before the study day. The volunteers received a single therapeutic dose of gemfibrozil (1200 mg in granular formation); blood was withdrawn at various time intervals (basal, 1 h, 2 h and 3 h after administration) and the oxygen binding curves of erythrocytes for each sample were determined.

Statistical analysis

Where appropriate, data are expressed as mean $\pm$ s.d. For group comparisons, the significance of differences between the means of the groups of data was determined by a one-way analysis of variance.

Results

In vitro studies

Hill plots for the binding of oxygen to human adult haemoglobin in the absence and presence of gemfibrozil 5 mmol l⁻¹ or 10 mmol l⁻¹, clofibric acid 5 mmol l⁻¹, pravastatin 5 mmol l⁻¹, and DMSO alone show a dramatic dose dependent effect of fibrates on P_{50} of haemoprotein (Figure 1). This effect was not accomplished by a significant modification of the n

Figure 1 Hill plot for the binding of oxygen to human adult haemoglobin in the absence (●) and in presence of gemfibrozil 5 mmol l⁻¹ (▽) or 10 mmol l⁻¹ (□), clofibrilic acid 5 mmol l⁻¹ (▽), pravastatin 5 mmol l⁻¹ (○), and DMSO alone (■). Conditions: 1 × 10⁻¹ M Hepes buffer plus 1 × 10⁻¹ M NaCl and 5 × 10⁻³ M DPG at pH 7.4, 37° C (see method section).

value (Hill coefficient, i.e. the slope of the binding curve), reckoned between 20 and 80%. The *n* value did not change significantly under the different conditions and was about 2.5 that of normal human haemoglobin.

In particular, fibrates shifted the *P*₅₀ of human haemoglobin, in terms of log *P*₅₀, from 1.32 (control value) to 1.52 for 5 mmol l⁻¹ gemfibrozil or clofibrilic acid and 1.82 for 10 mmol l⁻¹ gemfibrozil respectively.

A similar set of experiments carried out with concentrations of drugs corresponding to therapeutic plasma concentrations has clearly shown a small but significant effect of fibrates on the O₂ affinity of both haemoglobin solution and erythrocytes. Such an effect appears quantitatively and qualitatively similar and it determines a *P*₅₀ increment of about 12% with respect to the control values (Tables 1 and 2).

Pravastatin, an HMGCoA reductase inhibitor, used as control, has never shown effects on the functional properties of human haemoglobin or of the erythrocytes (Tables 1 and 2). It may be worthwhile to note that, since gemfibrozil and clofibrilic acid have been solubilized in DMSO, a number of control experiments have been performed incubating haemoglobin solutions and erythrocytes with DMSO alone. On the basis of these experiments DMSO, like pravastatin, has no effect on the functional properties of both HbA and erythrocytes (Tables 1 and 2).

Table 1 *In vitro* effects of therapeutic concentrations of clofibrilic acid (1 × 10⁻³ M), gemfibrozil (8 × 10⁻⁴ M) and pravastatin (1 × 10⁻⁴ M) on the oxygen affinity (in terms of *P*₅₀) of human haemoglobin solution. Conditions: 1 × 10⁻¹ M Hepes buffer plus 1 × 10⁻¹ M NaCl and 5 × 10⁻³ M DPG at pH 7.4, 37° C (see method section)

Conditions	<i>P</i> ₅₀ (mm Hg)
Control	25 ± 1.1
Pravastatin	25 ± 1.0
DMSO	25 ± 1.0
Clofibrilic acid	28 ± 0.9*
Gemfibrozil	29 ± 1.2*

Values are mean ± s.d. of five experiments; **P* < 0.05.

Table 2 *In vitro* effects of therapeutic concentrations of clofibrilic acid (1 × 10⁻³ M), gemfibrozil (8 × 10⁻⁴ M) and pravastatin (1 × 10⁻⁴ M) on the oxygen affinity (in terms of *P*₅₀) of human whole blood. Conditions: 5 × 10⁻² M Tris HCl buffer plus 1 × 10⁻¹ M NaCl at pH 7.4, 37° C (see method section)

Conditions	Incubation time (h)	<i>P</i> ₅₀ (mm Hg)	% increment of oxygen release [§]
Control	1	24 ± 0.4	0
	3	24 ± 0.5	0
DMSO	1	24 ± 0.4	0
	3	24 ± 0.4	0
Pravastatin	1	24 ± 0.4	0
	3	24 ± 0.5	0
Clofibrilic acid	1	28 ± 0.5*	10 ± 1
	3	27 ± 0.5*	10 ± 1
Gemfibrozil	1	29 ± 0.5**	12 ± 1
	3	28 ± 0.6*	10 ± 1

[§]Increment of oxygen release has been calculated assuming a Hill coefficient of 2.5.

Values are mean ± s.d. of six experiments; **P* < 0.05;

***P* < 0.02.

Finally under these experimental conditions *in vitro* no significant differences appear between clofibrilic acid and gemfibrozil as far as the effect on haemoglobin is concerned.

In vivo studies

Administration of a single oral dose of gemfibrozil (1200 mg in granular formulation) in healthy volunteers confirmed the *in vitro* data showing a little but significant right shift of the oxygen dissociation curve of erythrocytes. In particular, the *P*₅₀ values significantly changed from the basal level of 25.1 ± 0.4 mm Hg to values of 28.9 ± 0.6 mm Hg at 2 h from administration (mean ± s.d.; *P* < 0.02 by ANOVA). At 3 h the iatrogenic modification of oxygen transport properties of erythrocytes was no longer present. (Table 3).

This *in vivo* modification of ODC was not associated with significant alterations of the intraerythrocytic levels of adenosine triphosphate (ATP) and/or DPG. In fact, the intraerythrocytic concentrations of

such phosphates remain unchanged during the *in vivo* experiment (Figure 2).

Furthermore, no significant modifications of the intraerythrocytic pH were recorded by measuring the separation between the chemical shift values of α and γ peaks of ATP ($\Delta_{\alpha-\gamma}$) utilizing ^{31}P -NMR. In fact, the results obtained have clearly indicated that the change in intraerythrocytic pH, if present, is of the order of ± 0.05 pH unit since the change in the separation of the two peaks (α and γ) was always lower than 3 Hz in the different samples considered (data not shown).

Discussion

In vitro study

The results confirm previous observations and indicate that addition of these fibrates results in a marked effect on the oxygen affinity of HbA. Hence, in the presence of clofibrac acid or gemfibrozil, at concentrations of 5×10^{-3} M, the oxygen affinity is decreased by a factor of about 1.5–2 indicating a

preferential stabilization of the low affinity state of the haemoglobin molecule.

It should be observed that upon addition of increasing concentrations of clofibrac acid or gemfibrozil the oxygen binding curve shifts in an almost parallel manner, indicating that cooperativity between the oxygen binding sites is not affected by the presence of the drugs. In fact, the value of n did not change significantly under the different conditions and was about 2.5 that of normal human haemoglobin A.

In contrast, pravastatin, a different hypolipidaemic agent used as further control, had no effect on oxygen affinity and cooperativity of both purified human haemoglobin and erythrocytes.

The effect of gemfibrozil and clofibrac acid concentrations corresponding to usual therapeutic plasma concentrations was studied *in vitro* on human haemoglobin solution and on erythrocytes. The data reported in Table 1 clearly show a small but significant shift to the right of the ODC. On the basis of a Hill coefficient of 2.5 and of the experimental conditions (see Methods), the observed shift should be associated with a calculated mean increase of oxygen release in peripheral tissues of about 12%.

In vivo study

The *in vivo* experiments confirmed the *in vitro* data and allowed the evaluation of some possible pharmacokinetic influences.

At 1 and 2 h after administration, the P_{50} values showed a small but significant shift of the ODC towards lower oxygen affinity. This decrease was of the same order of magnitude as that previously determined *in vitro* on erythrocytes and appears to correlate with the gemfibrozil plasma concentration

Table 3 Effect of the administration of a single oral dose of gemfibrozil (1200 mg) on the oxygen dissociation curve of whole blood from healthy volunteers: Conditions: 5×10^{-2} M Tris HCl buffer plus 1×10^{-1} M NaCl at pH 7.4, 37° C (see method section)

Basal	After 1 h	After 2 h	After 3 h
25.1 ± 0.4	$28.1 \pm 0.5^*$	$28.9 \pm 0.6^{**}$	$25.5 \pm 0.5^{\S}$

Values are mean $\pm$ s.d. of six experiments; * $P < 0.05$;

** $P < 0.02$; § = NS.

Figure 2 Effect of the administration of a single oral dose of gemfibrozil (1200 mg) on the intraerythrocytic levels of ATP and DPG. Values are mean of five experiments. P: NS for both organic phosphate esters.

observed at the various time intervals when the drug is administered for the usual clinical indications [14].

To confirm the above shift, we checked possible acute alteration in both intraerythrocytic phosphate esters (DPG, ATP), since changes in their cellular concentrations may significantly alter the overall oxygen affinity of the Hb molecule. The results clearly show no modification of the levels of such phosphates during the experiments *in vivo*.

Moreover, the possibility that these drugs may in some way alter the delta pH across the red cell membrane has been verified by ^{31}P -NMR. This was performed by measuring the separation between the chemical shift values of α and γ peaks of ATP ($\Delta_{\alpha-\gamma}$). In fact, $\Delta_{\alpha-\gamma}$ has been proved to be a good indicator of the intracellular pH of intact erythrocytes without any pre-treatment of the cells [12, 13]. The results obtained have clearly indicated that the change in intraerythrocytic pH, if present, is in the order of ± 0.05 pH unit which cannot be responsible for the above mentioned ODC shift.

In conclusion, iatrogenic modifications of the functional properties of red blood cells have to be considered as a real possibility. This may also allow new therapeutic approaches in different diseases. In some cases this possibility has been recently verified; for example, the functional effect of fibrates, like clofibric acid and bezafibrate, has been studied: a) as a possible tool for hindering *in vivo* polymerisation of HbS; b) to improve oxygenation of the neoplastic mass thereby sensitizing it towards the action of radiotherapy [11, 12]. However in both cases the concentration necessary to have the specific activity was greater than that usually allowed by the drug toxicity.

However the possibility that drugs interacting with haemoglobin may modify the functional properties of the red blood cells has been underestimated. In fact, the experiments reported here clearly indicate, both *in vivo* and *in vitro*, that therapeutic doses of drugs can influence the oxygen transport properties of the red cells. A right shifted ODC could result in a greater oxygen release at the level of tissues and in particular of those tissues characterised, because of physiologi-

cal and/or pathological conditions, by a lower partial pressure of oxygen due to a high metabolic activity or specific circulatory aspects (i.e. liver, heart).

Considering that the physiopathological (concomitant ischaemic disease) and pharmacokinetic (slow detoxification activity) aspects of the patients usually treated with such drugs may surely enhance the above reported pharmacodynamic influences (which may, in turn, depend on the pharmacological properties of the specific fibric acid derivative), we think that *in vivo* modification of HbA secondary to administration of fibric acid derivatives should be carefully investigated to evaluate all the possible implications.

Hence we are faced with the following possibilities:

- i) the role of modified ODC in the mechanism of action of fibrates via an oxygen mediated stimulation of the peroxisomal β oxidation at the level of the liver;
- ii) the possible toxic effects, mainly at the level of the liver, related to an increased production of oxygen free radical species;
- iii) the influence, direct and/or mediated by compensatory mechanisms, on some of the physiopathological aspects of the coronary heart disease; in fact the effect outlined in this paper could modify the evolution of the disease independently of the hypolipoproteinaemic effect of the drugs;
- iv) the possibility that some typical acute side effects of fibrates (i.e. arrhythmias, crises of angina and abrupt variations of haematocrit) may arise from a small but significant modification of the oxygen transport capacity of the blood.

On the whole the data indicate that in the pharmacological approach to the new and old therapeutic agents the fundamental role of haemoglobin, as well as of other proteins, as carriers of exogenous compounds should be carefully examined.

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