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Relationship between *in vivo* nitroglycerin metabolism and *in vitro* organic nitrate reductase activity in rats

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Nitroglycerin has been an important drug in the treatment of angina pectoris for several decades [1]. Despite the recent introduction of numerous synthetic organic nitrates, nitroglycerin continues to be the drug of choice for angina because of its prompt and reliable pharmacological actions. Although the drug has had extended use in the clinic, a number of important therapeutic and toxicological questions remain unresolved. One of the most controversial subjects of debate is the therapeutic effectiveness, or lack thereof, of orally administered nitroglycerin for prophylaxis of angina. While metabolic studies [2] clearly indicate almost complete first-pass metabolism [3] when the drug is administered orally to several animal species in single doses, a number of investigators have demonstrated [4, 5] the clinical usefulness of oral nitroglycerin.

With the recent development of a sensitive analytical technique capable of determining subnanogram per ml concentrations of nitroglycerin in plasma [6], it was possible to initiate detailed pharmacokinetic studies on the oral absorption of nitroglycerin in man and in animals. Measurable plasma concentrations were obtained following a therapeutic oral dose of nitroglycerin in man [7]. In rats, a preliminary study [8] showed that an average of only about 2 per cent of an oral dose (7 mg/kg) reached the systemic circulation intact, but the inter-animal variability in this absorption parameter was large (0.6 to 3.8 per cent in five animals). If similar large differences in the *in vivo* availability of nitroglycerin occur also in humans, then there may be corresponding differences in response to oral nitroglycerin therapy. A better understanding of the factors which contribute to the inter-subject variability in nitroglycerin absorption and metabolism may be useful in defining the clinical effectiveness of oral nitroglycerin in man. We, therefore, undertook an investigation to examine the biochemical basis for the variation in the systemic availability of orally administered nitroglycerin in rats.

Nitroglycerin is metabolized primarily by the enzyme organic nitrate reductase (glutathione: polyolnitrate oxidoreductase, EC 1.8.6.1) [1]. The activity of this enzyme in various organs and tissues was determined in order to assess the relative contribution of these systems to nitroglycerin metabolism. Male Sprague–Dawley rats, 250–300 g, were fasted overnight and killed by decapitation without anesthesia. Each organ or tissue which was studied was rapidly excised and flushed with cold 0.15 M NaCl. Abdominal skin [9], liver, lung, heart, small intestine and kidney were then homogenized separately with 4 vol. of 0.25 M sucrose, and the 100,000 *g* supernatant fluid was assayed for organic nitrate reductase activity at 37°, using a procedure described previously by Needleman and Hunter [10]. The reaction sequence upon which this assay is based involves two coupled enzymatic reactions: nitroglycerin is denitrated by organic nitrate reductase using reduced glutathione (GSH) as a co-factor which is, in turn, regenerated by glutathione reductase (GR) with NADPH serving as a source of hydrogen. The initial rate of disappearance of NADPH was shown to be equivalent to that of production of nitrite ions as a consequence of nitroglycerin metabolism [10]. In the assay, GSH, GR and NADPH and other co-factors were added in excess, so that over a 10-fold range of total protein in the reaction mixture (0.5 to 5.0 mg) the activity in μmoles/min was directly proportional to the amount of added protein. The total protein used in the assay ranged between 1 and 3 mg. When the concentrations of all cofactors were doubled, the specific activity was only altered by 10 per cent, which was approximately the precision of this assay.

Pharmacokinetic and biochemical experiments were conducted on eleven animals. Male, Sprague–Dawley rats, 250–300 g, were fasted overnight and given 100 mg nitroglycerin/kg of body weight by gastric intubation. This dose was selected because a preliminary experiment had indicated an even greater degree of inter-animal variability than had been demonstrated previously at a lower dose (7 mg/kg). The dosing vehicle (1 ml) was 10% ethanol in propylene glycol. Use of a non-aqueous solvent was necessitated by the poor aqueous solubility of nitroglycerin.

Following oral dosing, the animals were restrained, and blood (*ca.* 0.3 ml) was collected from the tail vein at 0, 5, 10, 20, 40, 60, 90, 180, 240, 300 and 360 min. Plasma from these samples was then assayed using the gas chromatographic procedure of Yap *et al.* [6].

Immediately after the pharmacokinetic experiment, the liver of each animal was excised and the 100,000 *g* supernatant fluid was assayed for organic nitrate reductase activity. Protein concentration in the supernatant fluid was determined by the procedure of Lowry *et al.* [11]. Total activity was obtained by multiplying specific activity (μmoles/min/mg protein) by total protein. Every determination of reductase activity was corrected for concurrent enzymatic or chemical oxidation of NADPH [10].

The effect of the administered nitroglycerin dose on the systolic blood pressure of rats was studied. Animals were given either drug-free vehicle (two rats) or the organic nitrate (five rats), and the systolic blood pressure was monitored with an electrosphygmomanometer via a tail cuff (Narco Bio-Systems, model PE-300).

It was found that homogenized liver possessed the highest glutathione-dependent enzyme activity of all the organs and tissues studied (Table 1). This is consistent with

Table 1. Organic nitrate reductase activity in various rat tissues

Tissue	Total activity* (μmoles/min)	
Kidney	1.3 ± 0.3	(7)†
Liver	10.6 ± 1.9	(7)
Small intestine‡	NA§	(4)
Lung	NA	(4)
Skin‖	NA	(2)
Heart	NA	(2)

* Mean ± S.E.

† Numbers in parentheses indicate numbers of animals tested. Samples were assayed at least in duplicate.

‡ The excised small intestine was 10–15 cm in length distal to the pyloric sphincter.

§ NA = negligible activity (< 0.6 μmole/min).

‖ Shaved abdominal skin, 3 × 3 cm in area, was examined.

numerous *in vivo* and *in situ* data previously reported. For example, Lang *et al.* [12] showed that eviscerated animals exhibited much slower elimination of nitroglycerin than control animals. Johnson *et al.* [13], using a recirculating liver perfusion system, obtained a degradation half-life of 2 min for nitroglycerin in this organ. The only other organ with measurable glutathione-dependent organic nitrate reductase activity was the kidney, which gave an apparent enzymatic activity slighty above the detection limit. The kidney has been shown to be capable of drug metabolism. For example, Reach *et al.* [14] showed that the isolated perfused rat kidney is capable of metabolizing cortisol. However, in this case, it appears that kidney metabolism of nitroglycerin is relatively unimportant.

The lack of detectable glutathione-dependent organic nitrate reductase activity in the intestine may be of importance since it suggests that gut metabolism of nitroglycerin is insignificant and that pre-systemic elimination of nitroglycerin after oral administration is primarily hepatic. The lack of glutathione-dependent organic nitrate reductase activity in homogenized lung and skin is also of interest. Munitions workers, chronically exposed to nitroglycerin, have been known to develop tolerance and withdrawal symptoms to this compound. In these subjects, absorption of nitroglycerin most likely occurs via inhalation and skin contact. Since pre-systemic elimination in the lung and skin appears insignificant, these routes of drug absorption apparently do not act as metabolic barriers for nitroglycerin. The absence of skin metabolism is also consistent with clinical observations which showed that topically applied nitroglycerin produced significant and sustained plasma drug concentrations [7] and hemodynamic effects [15] in man.

Figure 1 shows the plasma concentrations of nitroglycerin as a function of time. There was considerable inter-animal variability in drug plasma concentrations. For example, the peak concentration range from 36 to 475 ng/ml, with a coefficient of variation of about 68 per cent. The area under the plasma concentration versus time curve (AUC), from time 0 to infinity, ranged from 4.07×10^3 to 51.95×10^3 ng-min/ml, with a coefficient of variation of about 60 per cent. In comparison, the coefficient of variation of the assay was less than 10 per cent.

The AUC is a function of the cumulative amount of intact drug which has been absorbed systemically and of the systemic clearance (Cl_S):

$$\frac{F\,D}{Cl_S} = \text{AUC}, \tag{1}$$

where F is the fraction of the dose, D, reaching the peripheral circulation intact [16]. In a linear system, hepatic clearance (Cl_H) represents a fraction (f_H) of Cl_S. Thus,

$$Cl_H = f_H Cl_S\,. \tag{2}$$

Cl_H can also be expressed in terms of hepatic blood flow, Q, and intrinsic clearance, Cl_I:

$$f_H Cl_S = Cl_H = \frac{Q\,Cl_I}{Q + Cl_I}. \tag{3}$$

Since absorption of nitroglycerin is virtually complete over a wide dose range [17, 18] and gut metabolism appears to be insignificant (Table 1), F can be described by equation:

$$F = \frac{Q}{Q + Cl_I}. \tag{4}$$

Equation 5 can be obtained by substituting equations 3 and 4 into equation 1:

$$\frac{f_H\,D}{Cl_I} = \text{AUC}\,. \tag{5}$$

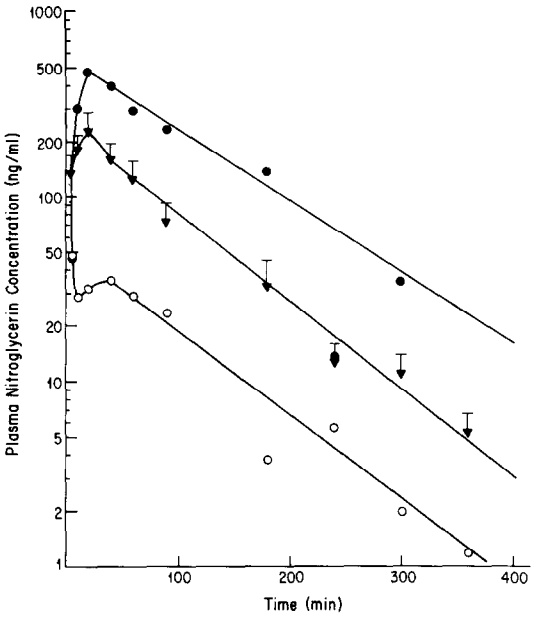

Fig. 1. Plasma concentrations of nitroglycerin in rats following a 100 mg/kg oral dose. Key: (▼) mean values (bars indicate S.E.); and (●, ○) individual rats showing highest and lowest plasma concentrations respectively.

If it can be assumed that Cl_I in each animal is proportional to the total *in vitro* liver organic nitrate reductase activity found for the same animal, then the latter should be inversely proportional to AUC. A statistically significant ($r = 0.84$, $P < 0.001$) inverse correlation between organic nitrate reductase activity and AUC was indeed obtained (Fig. 2).

Because the oral nitroglycerin dose used in this study (100 mg/kg) appeared large compared to that used clinically in man (6.5 mg orally per dose), there may be some concern that the animals could be in complete circulatory shock after drug adminstration. Sphygmomanometric measurements, however, showed that, in five animals tested, nitroglycerin at 100 mg/kg orally gave a drop in systolic blood pressure of no more than 40 mm Hg at maximum change, and baseline values were re-established around 10 min post drug dosing. In two control animals dosed with just the vehicle, the corresponding maximum changes were about 20 mm Hg. It thus appeared that the dose used did not cause such a catastrophic circulatory effect on the test animals as to render them totally unphysiologic.

Results from this study thus provide an example of significant correlation between *in vitro* enzyme activity and *in vivo* first-pass metabolism. The large inter-animal variability in plasma drug concentrations after oral nitroglycerin administration was shown to result from differences in the capacity of individual animals to metabolize nitroglycerin. Inter-animal variability in hepatic blood flow did not affect the AUC value because both Cl_H and F are simultaneously influenced (equations 3 and 4). Liver organic nitrate reductase activity could potentially be altered by factors such as age, sex, diet, smoking habits and administration of other drugs. It is then possible that some of these factors could potentially affect the biological availability of oral nitroglycerin, and hence its clinical efficacy, in humans.

In summary, the inter-animal variability in plasma concentrations after oral nitroglycerin dosing is quite large. This variability is shown to arise mainly from individual variation in liver organic nitrate redutase activity in each animal. A good correlation is obtained between *in vivo*

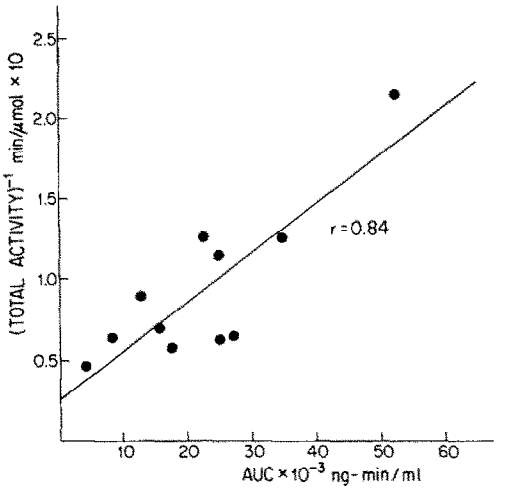

Fig. 2. Relationship between *in vitro* organic nitrate reductase activity and area under the plasma nitroglycerin concentration vs time curve in eleven rats.

nitroglycerin systemic availability and *in vitro* enzyme activity.

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Effects of methotrexate esters and other lipophilic antifolates on methotrexate-resistant human leukemic lymphoblasts

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A major clinical problem in cancer patients treated with methotrexate (MTX) is temporary remission followed by renewed tumor growth and lack of response to further MTX therapy. According to current views [1, 2], this type of 'acquired' resistance, as opposed to 'intrinsic' resistance wherein little or no response occurs even at the start of therapy, is due to the survival of a few initially resistant cells which can repopulate the tumor within a relatively short time. The rate of appearance of clinically resistant disease depends, among other things, on the number of such refractory cells and on their cytokinetic properties.

Although several other factors have been suggested as possible causes of MTX resistance in experimental animal systems [2], the principal relevant phenomena in humans are believed to be (1) the ability of tumor cells to synthesize increased levels of dihydrofolate reductase after exposure to MTX [3, 4] ('enzyme resistance'), and (2) the loss of the ability of tumor cells to take up the drug in quantities sufficient to inhibit the enzyme [5–7] ('transport resistance'). It may be possible for both phenomena to coexist in the same host, though their onset need not necessarily occur at the same time or to the same degree [7, 8]. There is evidence which indicates that in human cell lines transport resistance is more likely to occur than enzyme resistance [7], in contrast to murine systems in which enzyme resistance seems to be favored and stable transport-resistant mutants are difficult to maintain [8].

Uptake of MTX into human tumor cells can occur via either of two pathways. In the presence of relatively low concentrations (i.e. in the micromolar range), uptake in many cells has been demonstrated to occur by way of the same saturable, carrier-mediated and energy-dependent transport route as is utilized by naturally occurring reduced folates [9–11]. At millimolar levels, which more closely approximate conditions prevailing *in vivo* during high-dose MTX therapy, a large part of the intracellular MTX concentration results from passive diffusion [6, 12]. The two highly polar, negatively charged COOH groups in the side-