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Differential formation of dinitrate metabolites from glyceryl trinitrate in subcellular fractions of rabbit liver

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Glyceryl trinitrate (GTN) was first synthesized in the 19th century. Since then, it has been the most commonly used organic nitrate for the treatment of angina pectoris. The mechanism of action of GTN and other organic nitrates is coupled to the metabolism of the organic nitrates *in vivo*, as defined by the *S*-nitrosothiol hypothesis of Ignarro *et al.* [1]. The first step in the scheme, i.e. formation of glyceryl dinitrate metabolites (1,2-GDN and 1,3-GDN) and an inorganic nitrite ion, is crucial for the production of the pharmacological effect.

It has been widely accepted that the metabolic conversion from GTN to GDNs is mediated by the enzyme glutathione *S*-transferase. Yet, when homogenates of different organs are incubated with GTN, different ratios of the GDNs are formed [2–5]. Previous work from our laboratory [6] has also shown that ratios of GDNs differ following various routes of administration to humans. Although these data may be explained by the presence of isozymes of glutathione *S*-transferases in different organs, it is equally possible that other pathways, both enzymatic and non-enzymatic, can be responsible for GTN metabolism. This study was designed to investigate the patterns of GTN metabolism in cytosolic and microsomal fractions of rabbit livers, to probe whether such alternate pathways may be present.

Methods

New Zealand White rabbits (2–3 kg) were killed by decapitation. The liver of each animal was immediately perfused with ice-cold buffer (0.05 M Tris-Cl–0.15 M KCl, pH 7.4) to clear out the remaining blood in the organ. The organ was then mixed with 2 vol. of buffer, minced, and homogenized. Using differential ultra-centrifugation, the 105,000 *g* supernatant fraction and pellet were obtained.

In each incubation, 2.0 mg/ml of protein from either fraction was used, with protein content determined by the method of Lowry *et al.* [7]. Glutathione (2 mM) was added to the buffered sample solutions to avoid co-factor depletion. GTN, at a starting concentration of 20 ng/ml, was added to the 37° incubation, and 500-μl samples were drawn at 0.5, 5, 10, 15, 30, 60, 90 and 120 min. Further reaction was stopped by immersing the samples into a mixture of dry ice and methanol.

Concentrations of GTN and GDNs were measured simultaneously using our capillary gas-chromatographic assay [8] with one minor change. Instead of a mixture of pentane and methylene chloride, 3 × 10 ml mixtures of pentane and methyl-*t*-butyl ether (80%:20%) were used to extract the samples. 2,6-Dinitrotoluene (10 ng/500 μl sample) was used as the internal standard. A clean baseline was obtained, with the peaks for 1,3-GDN, 1,2-GDN, and GTN clearly separated. Linearity was observed between the range of 0.25 and 20 ng/ml.

Results and discussion

GTN was metabolized in both subcellular fractions. However, the first-order degradation rate of GTN was 2- to 4-fold more rapid in the cytosolic fractions than in the microsomal fractions in each of the preparations from four animals. Moreover, the patterns of metabolite formation were different in the two subcellular fractions.

The formation of GDN metabolites in the cytosolic fraction is shown in Fig. 1a, where preferential formation of

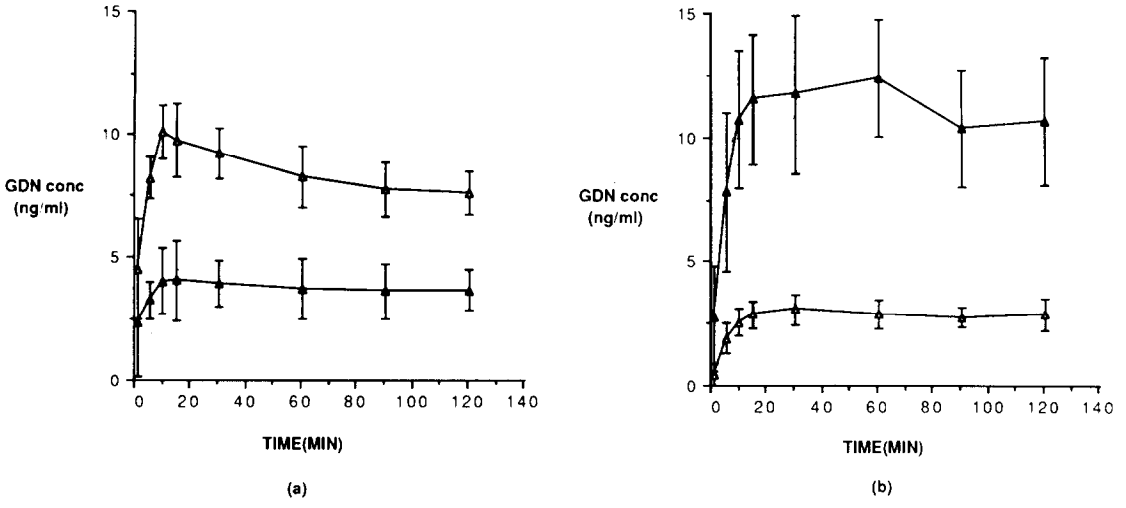

Fig. 1. Formation of 1,2-GDN (—△—) and 1,3-GDN (—▲—) from 20 ng/ml GTN in (a) cytosolic (N = 5) and (b) microsomal (N = 4) rabbit liver fractions. Incubations were performed at pH 7.4 and 37°. 2.0 mg/ml protein was used for each incubation. Values are means ± SD.

1,2-GDN was observed within the 2-hr incubation. Upon addition of sulfobromophthalein (SBP), a classical substrate for glutathione *S*-transferases [9], significant changes in both the GTN and GDN profiles in the cytosolic fraction were observed. The rate of GTN degradation was decreased to 1/2 to 1/3 in all cytosolic preparations (data not shown). Moreover, at 60 min when GTN metabolism was complete, there was a 77.4 ± 9.6% decrease in 1,2-GDN concentrations, accompanied by a 155 ± 26% increase in 1,3-GDN levels when compared to the controls. This indicates that SBP decreased the rate of GTN degradation mainly by blocking the conversion of GTN to 1,2-GDN. Preliminary enzyme kinetic studies in our laboratory show that, in cytosolic fractions, the initial rate of 1,2-GDN formation decreased with addition of SBP, while the rate of 1,3-GDN formation was relatively unaltered. This suggests that cytosolic glutathione *S*-transferase, the enzyme thought to be mainly responsible for GTN metabolism, is primarily specific for 1,2-GDN formation. This specificity could be explained by the steric hindrance encountered when the nitrate group at the carbon-2 position approaches the active site of the enzyme. These data also imply that another pathway for 1,3-GDN formation is present in the cytosolic fraction. The increase in 1,3-GDN concentrations upon addition of SBP is probably due to the fact that more GTN is available to this second pathway when the 1,2-GDN formation pathway is blocked by the inhibitor. As a result, when SBP was added to cytosolic fractions, a decrease in the GDN ratio (1,2-GDN/1,3-GDN) was observed (Fig. 2a). With increasing SBP concentration added to the cytosolic fraction, the GDN ratio decreased further, indicating a greater contribution from the 1,3-GDN formation pathway (Fig. 2c).

In contrast, in the microsomal fraction, 1,3-GDN was preferentially formed at a ratio of 4 to 1 over 1,2-GDN (Fig. 1b). Although glutathione *S*-transferases have been reported to be present in the microsomal subcellular fraction [10], addition of SBP caused no significant changes in either the concentratios or ratio of GDNs (Fig. 2b). The GTN degradation half-life was increased by only 20% with the addition of SBP to the microsomal fraction, compared to 200–300% changes observed in the cytosolic fraction. This suggests that the majority of GTN metabolism in the microsomal fraction may not be mediated by microsomal glutathione *S*-transferases, or it may be carried out with an isozyme which cannot be inhibited under the conditions utilized. However, the nature of this 1,3-GDN predominant microsomal pathway and its relationship with the cytosolic 1,3-GDN formation pathway are still unclear.

When the cytosolic and microsomal fractions were boiled in a water bath before incubation, no significant GTN degradation was found. This indicates that the formation of metabolites under the present experimental condition is enzymic in nature. In 1965, Needleman and Hunter [11] reported that 1,3-GDN is formed in preference to its 1,2-isomer when purified organic nitrate reductase from hog liver is incubated with GTN. However, subsequent investigators [2, 3, 12] found contradictory results with liver homogenate incubations from other species. It is conceivable that these differences are due to inter-species differences in GTN metabolism. Lee [12] compared GDN formation in the 9000 *g* supernatant fractions from livers of several species. It was found that, with the exception of rats, all other animals tested (rabbits, dogs, and cats) produced 1,2-GDN preferably. Preliminary experiments from our laboratory also showed that rat liver cytosolic fraction, unlike that of the rabbit, preferentially formed 1,3-GDN. This species-dependent phenomenon may be explained by the presence of various isozymes of glutathione *S*-transferases, by which the process of GTN degradation might be carried out differently, or it may be due to the presence of other enzyme systems in rats which are capable of producing 1,3-GDN. These results suggest that the rat may not be an appropriate representative animal in which to study GTN metabolism.

An interesting phenomenon relating the preferential formation of GDN metabolites in relation to the pharmacodynamic effects of GTN was reported by Fung and Poliszczuk [13]. A decrease in production of 1,2-GDN in rats was found in homogenates of tolerant aortic tissues when compared to control homogenates. However, the 1,3-GDN concentrations were unchanged. Although it is not clear at this point whether the metabolizing systems present in liver are the same as those in blood vessels, the study of Fung and Poliszczuk [13] also suggests that the GDN metabolites may be formed via different pathways, instead of by glutathione *S*-transferase alone. Brien *et al.* [14] reported that GDN formation in rabbit aortic tissues is related to cyclic GMP elevation, which precedes GTN-induced vasodilation. Theoretically, formation of either

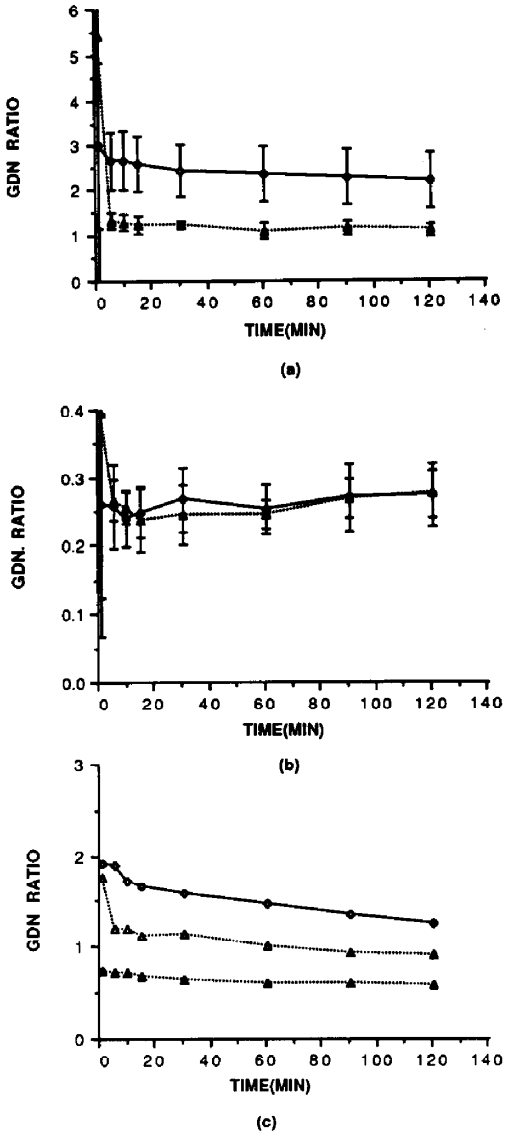

Fig. 2. Change of GDN metabolite ratio (i.e. 1,2-GDN/1,3-GDN) upon addition of sulfobromophthalein (SBP) at a concentration of 17.6 μM (200 times higher than GTN concentration) in (a) cytosolic (N = 4) and (b) microsomal (N = 3) fractions. (c) Effect of increasing SBP concentration in cytosolic fraction of rabbit No. 3. Values are means ± SD for panels (a) and (b). Key: (—◊—) GDN ratio without SBP, (· · · △ · · ·) GDN ratio with 17.6 μM SBP, and (· · · ▲ · · ·) GDN ratio with 88 μM SBP.

GDN from GTN should be accompanied by the formation of an inorganic nitrite ion, which would lead to formation of the active intermediate—*S*-nitrosothiol. The study by Fung and Poliszczuk suggests that the contribution of different pathways to the two dinitrate metabolites may lead to different pharmacological effects, with the pharmacologically "productive" pathway being formation of 1,2-GDN, the pathway apparently mediated by cytosolic glutathione *S*-transferase in our study.

In summary, this study showed differential formation of dinitrate metabolites in the two subcellular fractions in rabbit livers upon GTN administration. A glutathione *S*-transferase pathway, primarily responsible for 1,2-GDN formation and capable of being inhibited with the addition of sulfobromophthalein, was found in the cytosolic fraction. Another pathway, which preferentially forms 1,3-GDN was also present in the cytosolic fraction. Significant degradation of GTN was observed in microsomal fractions, with 1,3-GDN preferentially formed. GDN ratios (1,2-GDN/1,3-GDN) were decreased markedly by sulfobromophthalein in the cytosolic fraction, whereas the GDN ratio in the microsomal fraction was unaffected. Further kinetic and dose-dependent studies of these pathways are ongoing in our laboratory.

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Lack of effect by prostaglandin $F_{2\alpha}$ and verapamil on calcium uptake by isolated corpora lutea from pseudopregnant rats

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Prostaglandin $F_{2\alpha}$ ($PGE_{2\alpha}$)* is of prime importance in initiating luteal regression in many species [1]. In isolated corpora lutea (CL) from pregnant and pseudopregnant rats [2–5], and in rat luteal cell suspensions [6, 7], $PGF_{2\alpha}$ rapidly abrogates the luteinizing hormone (LH)-induced cyclic AMP (cAMP) accumulation via an unclear mechanism. Behrman and his coworkers [7, 8] proposed that calcium ions mediate this action of $PGF_{2\alpha}$, based on the ability of ionophores and ouabain to inhibit cAMP accumulation in luteal cells. However, interference with extracellular [4, 5, 7] and intracellular [5] calcium did not prevent the $PGF_{2\alpha}$-induced suppression of cAMP accumulation. Furthermore, we found that calcium uptake is not affected by $PGF_{2\alpha}$ [4]; the latter study is extended here, using different schedules and CL ages.

Verapamil, a blocker of voltage-dependent calcium channels, has been applied to $PGF_{2\alpha}$-treated CL with the purpose of preventing extracellular calcium influx [4, 7]. However, verapamil-sensitive channels have not been demonstrated in rat CL. Therefore, the effect of verapamil on the uptake of $^{45}Ca^{2+}$ was also examined. For comparison, we tested the effect of lanthanum ions, which inhibit general calcium uptake as well as calcium loss from the cells [9, 10].

Materials and methods

$PGF_{2\alpha}$ (tromethamine salt) was a gift of the Upjohn Co., Kalamazoo, MI, U.S.A., and verapamil was donated by Knoll AG, Ludwigschafen am Rhein, Federal Republic of Germany. $^{45}CaCl_2$ (10–40 mCi/mg calcium) was a product of the Radiochemical Center, Amersham, England. The pregnant-mare-serum gonadotropin (PMSG) preparation was Gestyl (Organon, Oss, Holland). Lanthanum chloride was obtained from Sigma, and amino-acid and vitamin stock solutions were from Bio-Lab, Jerusalem, Israel.

The animal model and experimental procedures were described previously [4, 5]. Briefly, PMSG (15 I.U./rat, s.c.) was administered to 30-day-old female Sprague–Dawley rats; this resulted in the formation of CL which remained functional for 11 days. CL collected on day 10 or day 14 were pooled, and calcium uptake was determined according to published methods [4, 9, 10]. The incubation medium was very similar to Dulbecco's modified Eagle medium [4], and oxygen was bubbled constantly into the medium. CL were distributed in glass vials, or in test tubes, and incubated with $^{45}CaCl_2$ (2×10^5–10^6 cpm/ml) in the presence of various agents. When the effect of lanthanum ions on calcium uptake was examined, a modified medium was used, in which NaH_2PO_4, $NaHCO_3$ and $MgSO_4$ were omitted, and the final pH was 7.0. The calcium-depleted medium contained 0.5 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and $MgSO_4$ at 2.5 rather than 0.81 mM. The concentration of free calcium ions in such a medium is approximately 30 nM [5]. $LaCl_3$ and verapamil were dissolved in medium just before use. To terminate the incubation, CL were transferred into beakers with ice-cold wash solution containing 2 mM $LaCl_3$ [4], and, 50 min later, blotted, weighed in groups of 2–5 CL, and prepared for counting. Radioactivity in the incubation medium was also estimated. In CL held in the calcium-labeled medium for a few seconds, and then subjected to the described rinse procedure, the label was zero.

The methods for determining cAMP [5], 20α-hydroxysteroid dehydrogenase (20α-SDH) [11] and progesterone [12] have been described.

The specific activity of calcium ions taken up by the CL was assumed to be the same as that found in the medium (namely, 2×10^5–10^6 cpm/1.8 μmol). Calcium uptake was thus expressed as mmol/kg tissue (wet weight); sometimes, data from several experiments were combined. Values are presented as means ± SE, and statistical significance was determined by Student's *t*-test.

Results and discussion

The effect of $PGF_{2\alpha}$ on calcium uptake was examined in 14-day-old as well as 10-day-old CL. Day-10 CL were still functional, as indicated by the high concentration of plasma progesterone (284 ± 71 ng/ml, N = 7) and the negligible luteal 20α-SDH activity (0.31 ± 0.08 nmol/min/mg protein, N = 13). These mature CL are very sensitive to $PGF_{2\alpha}$ compared to young CL [3, 13]. On the other hand, 14-day-old CL had already undergone functional luteolysis: they produced very little progesterone (plasma level 9 ± 2 ng/ml, N = 9) and had high 20α-SDH activity (16.4 ± 3.7 nmol/min/mg protein, N = 9). A second generation of CL was not observed in any of the rats included in the study. Day-14 CL were examined because of the indications that $PGF_{2\alpha}$, beside triggering luteolysis in functional CL, also plays a role in later stages of luteal regression [1, 14, 15].

As shown in Table 1, $PGF_{2\alpha}$ added simultaneously with $^{45}CaCl_2$ had no effect on calcium uptake in CL of both ages; basal calcium uptake was similar in the two CL types. Responsiveness of the day-10 CL to $PGF_{2\alpha}$ (in terms of cAMP accumulation) was verified in groups of CL taken from the CL pool of each experiment. Mean cAMP values (pmol/mg protein) were 23.5 ± 5.1 in LH-treated CL, and 5.5 ± 0.8 in CL exposed to LH and $PGF_{2\alpha}$. In another experiment, the effect of $PGF_{2\alpha}$ on calcium uptake was

* Abbreviations: $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; LH, luteinizing hormone; cAMP, cyclic AMP; CL, corpora lutea; PMSG, pregnant-mare-serum gonadotropin; 20α-SDH, 20α-hydroxysteroid dehydrogenase; and EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.