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Absence of Metabolite Formation during Nitroglycerin-Induced Relaxation of Isolated Blood Vessels

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SUMMARY

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Nitroglycerin dose-response curves were performed on phenylephrine-contracted helical strips from canine dorsal pedal arteries and medial saphenous veins. A 50-fold difference was observed in the threshold dose required for relaxation in the saphenous vein (1×10^{-9} M) and dorsal pedal arteries (5×10^{-8} M). A 20-fold difference in the ED_{50} for nitroglycerin was observed (5×10^{-8} M in the saphenous veins and 1×10^{-6} M in the dorsal pedal arteries). Although both tissues exhibited a similar maximum inhibition of tone, a 10-fold greater concentration of nitroglycerin (2×10^{-5} M) was required in dorsal pedal arteries than in saphenous veins (2×10^{-6} M) to produce this effect. During maximal relaxation, the incubation medium was analyzed for nitroglycerin and dinitrate metabolite concentration. A concentration of dinitrate metabolite as low as 0.8% of the GTN concentration was accurately measured by the methods used. In all instances maximal relaxation occurred without the release of detectable amounts of the dinitrate metabolite into the incubation medium. These data are at variance with the generally accepted hypothesis that the metabolism of nitroglycerin occurs concomitantly with relaxation. Therefore the intact nitroglycerin molecule is essential for the initiation of relaxation in vascular smooth muscle.

INTRODUCTION

It has been reported in a series of clinical studies that the predominant peripheral effect of the organic nitrates including nitroglycerin is venodilatation rather than arteriolar dilatation (1-5). The possibility of preferential dilator effects of nitroglycerin in veins was explored in a recent experimental study in isolated canine veins and arteries (6). The threshold dose for the relaxation of veins with nitroglycerin was found to be significantly lower than that of arteries (6). In addition, a difference in the maximum relaxation achieved following nitroglycerin administration was observed. While 100% relaxation was induced in strips of femoral vein, the maximum relaxation induced with nitroglycerin in strips of femoral artery was only 50%. The basis for the difference in sensitivity and maximum response to nitroglycerin is unknown.

The mechanism by which nitroglycerin and other organic nitrates initiate relaxation in vascular smooth muscle is also unclear. One hypothesis, advanced by Needleman and Johnson in 1973 (7), has received general acceptance in the literature (8). According to this hypothesis, the active site in the organic nitrate receptor of vascular smooth muscle contains a key sulfhydryl group with which the organic nitrate molecule reacts. During this reaction (Fig. 1), the sulfhydryl group is oxidized to the disulfide form, the organic nitrate molecule is reduced to the denitrated metabolite, nitrite ion is released, and, as a consequence of the interaction, relaxation ensues. Such a mechanism is unprecedented in theoretical pharmacology. The recent development of a sensitive chromatographic assay for nitroglycerin (9) and the dinitrate metabolites permitted us to explore the hypothesis that the denitration of nitroglycerin to the major metabolites glyceryl 1,2-dinitrate and glyceryl 1,3-dinitrate occurs concomitantly with the relaxation of vascular smooth muscle.

The objectives of the current study were to determine: (i) the relative sensitivity of isolated canine dorsal pedal artery and medial saphenous vein to nitroglycerin and to compare the maximum relaxation responses attainable in these tissues; (ii) whether a relationship exists between the extent of relaxation induced with nitroglycerin in isolated veins and arteries and the amount of metabolite detected in the incubation medium; and (iii) whether any observed differences in sensitivity to nitroglycerin in

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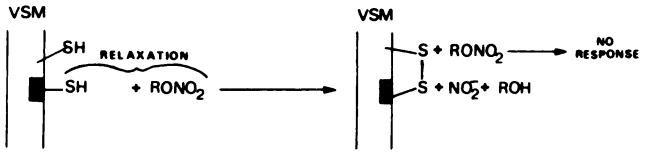

FIG. 1. *Schematic diagram of the reaction of organic nitrates with sulfhydryl groups in the vascular receptor as hypothesized by Needleman and Johnson (7)*

VSM, vascular smooth muscle; $RONO_2$, organic nitrate; ROH, denitrated metabolite; SH, sulfhydryl group.

veins and arteries were reflected in differences in metabolite formation during relaxation.

MATERIALS AND METHODS

Isolated dorsal pedal artery and medial saphenous vein of the dog. Mongrel dogs (10–20 kg) were sacrificed by the administration of an overdose of pentobarbital. Lengths of dorsal pedal artery and medial saphenous vein were removed and placed in Krebs solution at 0°C. Sections (10 mm) were cut spirally and suspended in an organ bath containing Krebs solution (composition: NaCl, 120 mM; KCl, 5.6 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM; NaH_2PO_4, 1.2 mM; dextrose, 10 mM; $NaHCO_3$, 25 mM) at 37°C (10). The solution was gassed with a mixture of 5% CO_2 in O_2. Contractions of the helical strips were measured isometrically using a Grass FT03C force displacement transducer connected to a Narco strain guage coupler amplifier and physiograph. Strips were placed under 0.5 g resting tension and washed every 15 min during a 2-h equilibration period. Both the dorsal pedal artery and the saphenous vein had little resting tone and were contracted with phenylephrine. The maximum tension generated with phenylephrine (1×10^{-4} M) was 1.54 ± 0.7 g (SEM) in the dorsal pedal arteries and 0.73 ± 0.15 g (SEM) in the strips from saphenous veins. Submaximal contractions (60–80%) induced by phenylephrine (1–10 μM) were maintained for at least 30 min and were reproducible. Nitroglycerin (GTN) was added to the baths at increasing concentrations (10^{-9} to 10^{-4} M) without washing and the degree of inhibition of the phenylephrine-induced tone was assessed. When tone was maximally inhibited, the incubation medium was frozen until assayed for GTN and dinitrate metabolite concentration. As a control for any metabolism of GTN unrelated to relaxation, the experiment was repeated with the modification that the tissue was omitted from the bath.

Extraction of GTN. Samples of incubation medium were thawed and 2-ml aliquots were transferred to 50-ml centrifuge tubes. *m*-Dinitrobenzene (DNB) (10 μg/ml) in ethanol was added to serve as the internal standard. The extraction procedure for GTN was a modification of the method of Yap *et al.* (11). Following the addition of 5 ml hexane, the samples were shaken for 10 min, then centrifuged at 194*g* for 2 min, and the hexane supernatant was decanted into 50-ml centrifuge tubes. The extraction procedure was repeated seven times and the pooled hexane supernatant (35 ml) was stored at 4°C until chromatographed. The hexane-extracted incubation medium was reserved for ether extraction. A standard curve for GTN was prepared by adding GTN (1 to 7.5 μg/ml) and DNB (10 μg/ml) to 2 ml Krebs solution, and the hexane extraction was performed as described previously. The recovery of GTN by this procedure was 91.3%, with a coefficient of variation of 8.7%. The recovery of DNB was 96.3%, with a coefficient of variation of 5.9%.

Extraction of glyceryl 1,2-dinitrate. m-Dinitrobenzene (0.4 μg/ml) was added to the hexane-extracted incubation medium. This second addition of DNB was necessitated by the quantitative removal of the internal standard during the hexane extraction. Ethyl ether (10 ml), freshly distilled over potassium hydroxide pellets, was added and the samples were shaken for 10 min and centrifuged at 194*g* for 3 min. The supernatants were decanted into clean 50-ml centrifuge tubes and the ether extraction was repeated. Magnesium sulfate was added to the ether solutions, and following a 20-min period of shaking and 2 min of centrifugation at 194*g*, the solutions were decanted into 50-ml centrifuge tubes. The ether solutions were evaporated to dryness on ice under a stream of dry nitrogen gas, and the residue in each tube was dissolved in 200 μl absolute ethanol. The samples were stored at −20°C until chromatographed. A standard curve was prepared in the following manner: GTN (2500 ng/ml), glyceryl 1,2-dinitrate [GDN_{1-2} (0 to 40 ng/ml)], and DNB (10 μg/ml) were added to 2 ml Krebs solution and the samples were extracted with hexane. Chromatography of the hexane extracts revealed no peak at the GDN_{1-2} retention time, and these solutions were discarded. Although there may have been some loss of the dinitrates during the hexane extractions, as has been suggested by Yap *et al.* (11), since the GDN_{1-2} standard curve was also extracted seven times with hexane, any loss during this manipulation would decrease only the sensitivity of the method and would not alter the accuracy of the measurement of the dinitrate content in the samples. *m*-Dinitrobenzene (0.4 μg/ml) was added to the hexane-extracted incubation medium, which was then extracted with ether by the procedure described previously. The recovery of GDN_{1-2} by this procedure was 83.7%, with a coefficient of variation of 23%.

Chromatography. The chromatographic procedure was a slight modification of a method previously described (9). At the time of chromatography 2 μl of each sample was injected into a Hewlett Packard 5700 series gas–liquid chromatograph with a ^{63}Ni radioactive source in the electron capture detector. The 6-ft coiled glass column, 2-mm i.d. and 0.25-in. o.d. with a 4-in. precolumn, was packed with 10% OV-101 on Chromasorb 750 (100–120 mesh). Operating conditions were as follows: IP, 150°C; oven, 130°C, detector, 200°C. The argon:methane (95:5%) carrier gas flow rate was 55 ml/min. Under these conditions the retention times for GDN_{1-2}, GTN, and DNB were 2.4, 2.8, and 5 min, respectively (Fig. 2). The two dinitrate isomers, GDN_{1-2} and glyceryl 1,3-dinitrate (GDN_{1-3}), had the same retention times in this system, and the sensitivity to the two metabolites was the same. Since GDN_{1-2} is the major metabolite of GTN in dog liver and blood (10), a standard curve was prepared using this chemical. The incubation medium was assayed for total dinitrate metabolite concentration using the GDN_{1-2} standard curve.

Preliminary data revealed that no metabolite could be

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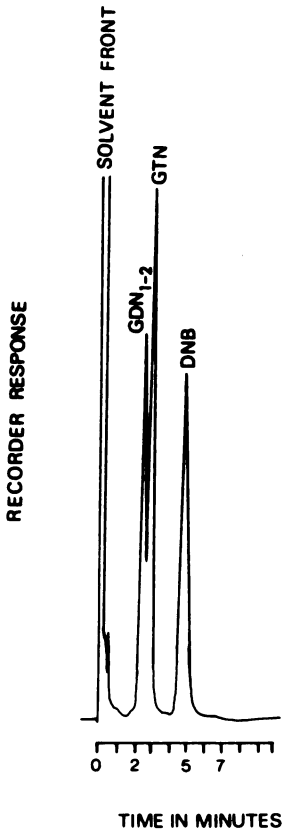

FIG. 2. *Sample chromatogram following the injection of 2 μl of a solution in ethanol containing nitroglycerin (GTN; 0.4 μg/ml), glyceryl 1,2-dinitrate (GDN_{1-2}; 0.4 μg/ml), and m-dinitrobenzene (DNB; 0.4 μg/ml)*

detected when the threshold concentration of GTN for relaxation was administered to tissue strips and the incubation medium assayed. To maximize the possibility of detecting the dinitrate metabolites, the concentration of GTN was increased until maximum relaxation was observed before samples of incubation medium were removed for the determination of GTN and dinitrate content. The similar retention times of GTN and the dinitrate metabolites (2.4 and 2.8 min) and the expectation of measuring microgram per milliliter quantities of GTN in the presence of nanogram per milliliter quantities of dinitrate necessitated the quantitative removal of GTN from the incubation medium prior to the extraction of the dinitrate metabolites. By performing seven hexane extractions, using a hexane:Krebs solution ratio of 2.5:1, a recovery of GTN from Krebs solution of 91.3% was observed. This value is similar to the recovery predicted by Yap *et al.* (11) for the extraction of GTN from water using a hexane:water ratio of 1:1. Although the recovery of GTN in the hexane extractions was 91.3%, no peak appeared at the GTN retention time during chromatography of the ether extracts of samples containing only GTN. The 8.7% unaccounted for represents loss due to evaporation or loss during extraction (e.g., in pipet tips). Therefore GTN was quantitatively removed from the incubation medium and did not interfere with the chromatography of the dinitrates.

In initial control experiments, Krebs solution containing GTN and DNB was extracted seven times with hexane and the hexane was discarded. When the Krebs solution was then extracted with ether and chromatographed, a peak was observed at the dinitrate retention time, but no peaks were seen at the GTNor DNB retention times. A good correlation ($r = 0.99$) was determined between the height of this peak and the concentration of GTN added to the Krebs solution. It appeared likely that this represented a dinitrate contaminant in the GTN stock solution at a concentration ranging from 0.3 to 0.6% of the GTN concentration. In the absence of GTN, GDN_{1-2} at a concentration of 10 ng/ml (signal to noise ratio of 30:1) was readily detected. However, as a result of the GDN_{1-2} contaminant present in the GTN stock solution, a signal to noise ratio of 3.2:1 was determined at a GDN_{1-2} concentration which represented 0.8% of the GTN concentration (GDN_{1-2}, 20 ng/ml; GTN, 2500 ng/ml). Therefore, the method accurately measures GDN_{1-2} when present at concentrations as low as 0.8% of the GTN concentration in the incubation medium.

Materials. GTN was available as a 0.53% solution in alcohol from the pharmacy at the Kingston General Hospital. GDN_{1-2} and GDN_{1-3} were synthesized by the Carbohydrate Research Institute, Queen's University, Kingston, Ontario. Chemicals used in the extraction procedure were pesticide grade. Chromatographic supplies were purchased from Chromatographic Specialities Ltd., Brockville, Ontario.

RESULTS AND DISCUSSION

Sensitivity of isolated blood vessels to GTN. The first objective of this study was to compare the sensitivity and maximum response to GTN in isolated peripheral veins and arteries. Phenylephrine-induced tone in helical strips from both dorsal pedal arteries and medial saphenous veins was inhibited by GTN. However, as shown in Fig. 3, there was a marked difference in the sensitivity to GTN in these tissues. The threshold dose of relaxation by GTN in arteries (5×10^{-8} M) was 50 times greater than that required in veins for a similar inhibition of tone (1×10^{-9} M). Similar results were reported in a study by Mackenzie and Parratt (6) which demonstrated that the threshold dose of GTN required for relaxation was 10 times greater in femoral artery than in femoral vein.

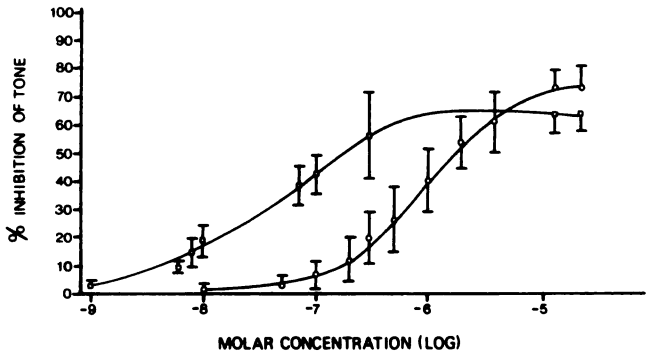

FIG. 3. *Comparative effects of nitroglycerin on phenylephrine-induced tone in canine medial saphenous vein* (□) *and dorsal pedal artery* (○)

Each point represents a mean of three to five determinations. Vertical lines denote the standard error of the mean.

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It is of interest to examine the dose required for threshold relaxation in saphenous vein (1×10^{-9} M or 0.22 ng/ml) and dorsal pedal artery (5×10^{-8} M or 11.4 ng/ml) in view of a recent clinical report from this laboratory (9). In 10 studies conducted in normal volunteers, the average maximum concentration achieved in venous blood following the sublingual administration of 0.6 mg GTN was only 2.3 ± 0.36 ng/ml. This concentration is 10 times the threshold for the relaxation of saphenous vein, but less than one-fifth the threshold dose for the relaxation of dorsal pedal artery. It is possible that an arteriovenous gradient for GTN exists following the sublingual administration of this agent (12). In addition, human veins, arteries, and arterioles may be more sensitive to GTN than the isolated blood vessels studied here. Nevertheless, it is tempting to speculate that if the relative sensitivity of isolated canine saphenous veins and dorsal pedal arteries to GTN is representative of the relative sensitivity of human peripheral veins and arteries to GTN, then the threshold dose for relaxation may be achieved only in veins following the sublingual administration of this agent.

In the current study, the ED_{50} for GTN in saphenous vein was 5×10^{-8} M, while in dorsal pedal artery the ED_{50} was 1×10^{-6} M. The maximum inhibition of phenylephrine-induced tone was similar in saphenous vein (65 ± 7.3% (SEM)) and dorsal pedal artery (74 ± 8.2%), although the concentrations of GTN required to produce maximum relaxation differed in the two preparations (Fig. 3). These data are at variance with the report of MacKenzie and Parratt (6) which demonstrated that tone in femoral vein was 100% inhibited, while femoral arterial tone was inhibited maximally with GTN by only 50%. The differences in maximum response to nitroglycerin in the two studies may be due at least in part to regional differences in the responsiveness of blood vessels to GTN. Such regional differences have been observed in the responsiveness of isolated arteries to other pharmacological agents (13).

Metabolite formation during GTN-induced relaxation. The second objective of the current study was to determine whether a relationship exists between the relaxation induced by GTN in isolated veins and arteries and the amount of metabolite detected in the incubation medium. The incubation medium from helical strips from five dorsal pedal arteries and six saphenous veins was assayed for GTN and GDN_{1-2}. The GTN concentration ranged from 800 to 11600 ng/ml in the medium. The GDN_{1-2} concentration was found to be below the limit of detection of the method (less than 0.8% of the GTN concentration) in all instances. It is possible that some dinitrate may have been produced, remained bound to the tissue, and therefore not been measured by our procedure. However, in other tissues such as liver and blood where GTN is rapidly metabolized (14), the dinitrate is readily detected. Therefore, it is likely that GTN undergoes minimal, if any, metabolism in vascular smooth muscle during maximal relaxation.

Since no metabolite was found, it proved unnecessary to pursue the third objective, i.e., to examine whether the observed differences in sensitivity to nitroglycerin were reflected in quantitative differences in metabolite formation.

In view of these findings it is of interest to reexamine the hypothesis of Needleman and Johnson (7) that organic nitrates are denitrated during the drug–receptor interaction resulting in the relaxation of vascular smooth muscle. This hypothesis was based on two key observations.

(i) A relationship was observed between the potency of organic nitrates as blood pressure depressants and the rate of hydrolysis of these agents when incubated for 1 h at 37°C with rat liver homogenate and glutathione (15). Potent organic nitrates were readily denitrated, while those with no vasodepressor activity were not denitrated in the incubation system. These observations were advanced as evidence to suggest that denitration might be intimately involved in the relaxation process. However, our results show that, unlike liver tissues, where denitration of GTN occurs rapidly (15), this process does not occur to a measurable extent in vascular smooth muscle.

(ii) Observations made in rat aortic strips incubated at pH 9.4 and 7.4 were also cited as evidence in support of this hypothesis (7). It was noted that tissue incubated at the more alkaline pH contained a higher concentration of titratable sulfhydryl groups and converted more GTN to nitrite ion than did tissue incubated at pH 7.4. Although these data are difficult to interpret in view of the alkaline pH employed, the data of Needleman and Johnson (7) suggest that a correlation may exist between the tissue sulfhydryl content (presumably receptors) and the nitrite production from GTN.

In contrast to the work of Needleman and Johnson (7), in the present study at pH 7.4 there was no evidence to suggest that GTN is metabolized during the drug–receptor interaction which leads to a pharmacological response. This observation is in accord with accepted receptor theory (16). All known drug–receptor interactions either are reversible, with the intact drug molecule leaving the receptor following the interaction, or are irreversible, with the intact drug molecule remaining covalently bound to the receptor. There is no drug–receptor interaction known in which the drug is metabolized by its receptor.

Controversy has surrounded the issue concerning the active moiety in the organic nitrate molecule, whether the intact molecule or nitrite ion, for many years. Early workers (17, 18) observed that nitrite ion was present in blood following the administration of the organic nitrates and conjectured that the nitrite ion rather than the intact molecule may initiate the pharmacological responses. In contrast, Krantz *et al.* (19) demonstrated that following alkaline treatment and hydrolysis of a depressor dose of organic nitrate, the drug no longer produced a fall in blood pressure when injected into a dog. Krantz *et al.* (19) concluded that the intact molecule was essential for activity. The current study, in which no evidence was seen for the metabolism of GTN during maximal relaxation of vascular smooth muscle, supports the contention of Krantz *et al.* (19).

Subsequent to the drug–receptor interaction, the mechanism by which GTN induces the relaxation of

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vascular smooth muscle remains unclear. Recent evidence has demonstrated that the incubation of tracheal (20) and arterial (21) smooth muscle with GTN results in an increase in the cyclic GMP content of the muscle. In the latter study, a correlation was seen between the onset of the relaxation and the initial increase in the cGMP content of bovine mesenteric artery, although the time courses of the elevation of cGMP levels and relaxation differed (21). In tracheal muscle, the GTN-induced increase in cyclic GMP content was independent of the calcium content of the incubation medium (20). It may be that the relaxation induced by GTN is a result of an increase in cyclic GMP levels and the activation of an intracellular cGMP-dependent protein kinase involved in the relaxation process. However, before this conclusion can be drawn, the role of both cyclic GMP and calcium in the relaxation of vascular smooth muscle must be clarified.

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