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NITRITES XIX. STUDIES OF THE MECHANISM OF ACTION OF GLYCERYL TRINITRATE

JOHN C. KRANTZ, JR., GORDON G. LU, FREDERICK K. BELL AND HELMUT F. CASCORBI

Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md., U.S.A.

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Abstract—Further evidence has been presented to indicate that the dilation of the coronary arteries evoked by glyceryl trinitrate is due to the intact molecule. 1-Chloro-2,3-propanediol dinitrate (CPD) containing only two nitrate groups, equipotent to glyceryl trinitrate in dilating the perfused coronary vessels of the rabbit, is more refractory to nitrite formation by the vascular bed. The coronary vessels and the vascular bed of the ear have been shown to reduce a fraction of glyceryl trinitrate perfused through them to nitrite. Isosorbide dinitrate evokes increased coronary flow in the rabbit's heart and does not undergo hydrolysis and reduction. The resistance to alkaline hydrolysis has been studied with regard to glyceryl trinitrate and isosorbide and isomannide dinitrates. The latter two dinitrate esters are more resistant to alteration than is glyceryl trinitrate.

IN PREVIOUS studies, Krantz *et al.*[1] showed that the depressor response to glyceryl trinitrate was dependent upon the intact molecule. Upon alkaline hydrolysis and partial reduction to nitrite, occurring simultaneously, the depressor activity was nullified. This does not prevail with the same alkali treatment of isomannide dinitrate, owing to the fact that this organic nitrate is relatively refractory to hydrolysis. Our former studies showed that the number of nitrate groups in the molecule was not a salient factor in determining potency for vasodilation. However, the oil/water coefficient of the ester was found to be of prime importance in predicting vasodilation activity. Our attention was again directed to this problem in our studies with 1-chloro-2,3-propanediol dinitrate as a coronary dilator and with isosorbide dinitrate (isomer of isomannide dinitrate) that is now available for the treatment of coronary insufficiency.

These studies describe the retention of glyceryl trinitrate by the isolated rabbit's heart and nitrite formation along with a comparison with more stable nitrate esters.

MATERIALS AND METHODS

The glyceryl trinitrate met the standards of the U.S.P. CPD, synthetisized in this laboratory, assayed 99·2 per cent. The nitrogen content is 13·88 per cent (theoretical 13·97). The isosorbide dinitrate was synthesized in this laboratory (m.p. 51 °C) as were other nitrite and nitrate esters. They were analyzed and met the requirements for identity and purity set forth in the literature describing their synthesis.

The rabbit's heart perfusion for studies of coronary flow was the Anderson and Craver[2] modification of Langendorff's procedure. Perfusates circulated through the coronary vessels only once; they were not recirculated.

Comparative hydrolysis rates of nitrate esters were determined *in vitro*. Each compound was dissolved in acetone at concentrations of 0·1 M. One volume of the respective solutions and two volumes of 0·2 N NaOH solution were mixed and incubated at 37 °C. Volumes of 10 ml were removed at intervals of 1, 2, 3, 4, and 5 min and titrated with 0·2 N H_2SO_4.

The determination of the amount of nitrate ester in the various perfusates was carried out as follows. A portion of the perfusate was rendered 0·2 N in alkali by the addition of NaOH and heated for 10 min in boiling water. After acidifying this hydrolysate with hydrochloric acid, the liberated nitrous acid was allowed to diazotize sulfanilic acid which was added in excess. This diazonium salt was coupled with added N (1-naphthyl) ethylenediamine yielding the corresponding azo dye, the concentration of which was then determined spectrophotometrically by measuring the absorbance at a wave length of 548 mμ. Standard curves were prepared for each ester by carrying out the foregoing procedure on known amounts of ester.

Upon alkaline hydrolysis a portion of the nitrate in each ester is reduced to nitrite. This was determined by diazotization of sulfanilic acid and subsequent coupling with N(1-naphthyl) ethylenediamine and determining the color intensity by spectrophotometry.

Studies were conducted on the various nitrate esters upon passage through the heart in a modified Langendorff[2] preparation omitting the dextrose from the perfusion fluid; the presence of dextrose interfered with the nitrite determination. The respective esters were added to the perfusion fluid, and the total ester and nitrite content of the perfusate was determined at definite time periods after the drugs had passed once through the vessels of the rabbit's heart.

The perfusion of the vessels of the rabbit's ear was conducted by the method of Burn.[3] The hydrolysis studies of the various esters and their effect upon blood pressure were carried out according to the procedure described by Krantz *et al.*[1] The nitroprusside test was used to detect the presence of glutathione.

RESULTS

The effect of coronary perfusion on nitrate esters

In previous studies (Burgison *et al.*[4]) it was shown that glyceryl trinitrate and CPD were equipotent as coronary dilators by the Langendorff procedure. The quantities of the respective esters fixed by the rabbit's heart and the capacity of the tissue to reduce nitrate to nitrite are shown in Table 1. It is clear from the data that, although the total ester recovered in the perfusate in 2 min is approximately the same for each ester, the amount of nitrite reduced from the glyceryl trinitrate far exceeds that reduced from CPD. If the nitrite formed is calculated on the basis of the nitrate present in the respective esters, the difference remains statistically significant.

It has been previously shown (Krantz *et al.*[1]) that isomannide dinitrate is refractory to alkaline hydrolysis *in vitro*. Accordingly, isomannide dinitrate and isosorbide reduce nitrate to nitrite. It is observed from the data in Table 2 that neither isomannide dinitrate nor isosorbide dinitrate is appreciably reduced by cardiac tissue to nitrite.

To determine whether or not glyceryl trinitrate owed its activity to the nitrite formed by reduction in the cardiac tissue, sodium nitrite and amyl nitrite were perfused through five hearts each at levels comparable to the nitrite found in the glyceryl trinitrate perfusate. Neither sodium nitrite nor amyl nitrite evoked any measurable degree of coronary dilatation in 10 to 12 μg per 100 ml of perfusion fluid.

TABLE 1. CPD AND GLYCERYL TRINITRATE (GT) RECOVERED FROM PERFUSED HEART AND REDUCED TO NITRITE*

	GT			CPD		
	Volume† (ml)	Total drug (μg)	Total NO_2^- (μg)	Volume† (ml)	Total drug (μg)	Total NO_2^- (μg)
Mean ± s.d.	54·9 ± 16·3	429·0 ± 33·9	4·95‡ ± 1·5	55·3 ± 10·9	420·5 ± 37·9	1·84 ± 0·81

* Eight experiments; 500 μg of each ester added. After 2 min 85% of the ester was in the perfusate.

† All data were obtained by collecting the perfusate for 2 min immediately after the ester administration.

‡ The difference between the nitrite formed from each ester by the heart, calculated by Student's *t* test of paired differences, is significant ($P < 0{\cdot}01$ s.d.).

TABLE 2. NITRITE REDUCED FROM ISOSORBIDE DINITRATE (ISD) AND ISOMANNIDE DINITRATE (IMD) BY PERFUSED HEART*

	ISD		IMD	
	Volume† (ml)	Total NO_2^- (μg)	Volume† (ml)	Total NO_2^- (μg)
Mean	70·4	0·40	74·0	0·08
Range	48–87	0–1·5	62–105	0–0·27

* Five experiments; 500 μg of each ester added. After 2 min 85% of the ester was in the perfusate.

† All data were obtained by collecting the perfusate for 2 min immediately after the drug.

Effect of glyceryl trinitrate on the ear vessels

We were interested to determine whether or not another vascular system would reduce glyceryl trinitrate as does the perfusion through coronary arteries of the rabbit's heart. Perfusion studies were conducted through the vascular bed of the rabbit's ear. The results are shown in Table 3. Glyceryl trinitrate evoked marked dilatation of the vascular bed of the rabbit's ear. It is apparent that nitrite also was formed. Although the amount of nitrite was only approximately 20 per cent of that formed by coronary perfusion, the perfusate volume was also relatively smaller.

Hydrolysis studies of nitrate esters

To determine whether or not resistance to alkaline hydrolysis and nitrate reduction paralleled the effects observed on the rabbit's coronaries, each ester was subjected to this hydrolytic procedure. Confirmatory results are shown in Table 4.

Blood pressure studies

Krantz *et al.*[1] showed that isomannide dinitrate was resistant to alkaline hydrolysis, and glyceryl trinitrate was readily hydrolyzed under the same conditions. The criterion

was the depressor response in the anesthetized dog upon intravenous injection. The same experiment was conducted with isosorbide dinitrate. Our results show that the depressor response to glyceryl trinitrate is nullified by the alkali treatment; the per cent decrease in blood pressure was 35·4 prior to and +0·8 after alkaline hydrolysis (five experiments).

TABLE 3. GLYCERYL TRINITRATE REDUCED TO NITRITE BY RABBIT'S EAR PERFUSION*

	0 min		5 min	
	Volume† (ml)	Total NO_2^- (μg)	Volume (ml)	Total NO_2^- (μg)
Mean ± s.d.	19·6 ± 8·68	0·65 ± 0·63	17·8 ± 7·92	1·04 ± 0·56

* Five experiments; 500 μg of ester added.

† All data were obtained by collecting the perfusate for 2 min, either immediately or 5 min after the addition of the ester,

TABLE 4. EFFECT OF ALKALINE HYDROLYSIS ON NITRATE ESTERS*

Time (min)	Hydrolysis (%)		NO_3 nitrogen reduced to NO_2^-† (%)			
	CPD	GT	CPD	GT	IMD	ISD
1	27	68	11	20	0	0
2	39	89	14	19	0	0
3	51	98	17	23	0	0
4	59	100	18	27	0	0
5	65	100	21	30	0	0

* GT = glyceryl trinitrate; IMD = isomannide dinitrate; ISD = isosorbide dinitrate.

† Hydrolysis does not occur without nitrite formation.

On the other hand, alkali treatment failed to modify the depressor response to either isomannide or isosorbide dinitrate: 30·9 and 28·4 per cent fall in blood pressure prior to and 32·7 and 27·7 per cent fall in blood pressure after alkaline hydrolyiss (five experiments each). These blood pressure differences are not statistically significant.

DISCUSSION

These studies show that only a small fraction of the organic nitrates, glyceryl trinitrate and CPD, are converted to nitrite by passage through the coronary vessels. In addition, they demonstrate that only a small portion of the nitrate ester is adsorbed by the tissue receptor sites. Evidence indicates that the reduction to nitrite is not essential for coronary dilatation, since isosorbide dinitrate is not reduced by coronary perfusion. It was shown that amyl nitrite and sodium nitrite exert no coronary dilatation at levels of 10 to 12 μg, comparable to the quantities of glyceryl trinitrate reduced by the coronary vessels.

The depressor response of isosorbide dinitrate, like that of isomannide dinitrate, is not nullified by boiling with alkali. This is due to their relatively higher resistance to hydrolytic cleavage and substantiates the proposition that their action is dependent upon the intact ester. Glyceryl trinitrate, on the other hand, that readily hydrolyzes and undergoes reduction, loses its capacity to reduce the blood pressure under the same conditions. Heppel and Hilmoe[5] showed that, in a buffered medium, pH 8·5 at 37°C glutathione was incapable of reducing sodium nitrate. Under the same conditions, however, glyceryl trinitrate yielded nitrite in an orderly time reaction. Perfusion of the vessels of the rabbit's ear with glyceryl trinitrate solution also showed hydrolysis and reduction of the ester comparable to coronary perfusion. Glutathione could not be identified in the blood-free perfusates from either vascular bed, but this does not preclude the possibility that glutathione plays a role in the reduction of the esters.

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