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Norepinephrine Content, Release, and Disposition in Isolated Dog Coronary Artery[1] (40991)

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Abstract. The content of norepinephrine, its release, and subsequent disposition was studied in unlabeled and [^{3}H]norepinephrine-labeled strips of canine coronary artery using standard superfusion techniques. Endogenous norepinephrine was isolated from superfusate and vessel by column chromatography then measured using high-pressure liquid chromatography with electrochemical detection. Release of NE was studied in both unlabeled and radiolabeled preparations. The relative importance of neuronal and extraneuronal dispositions of norepinephrine was determined by quantitation of the efflux of the ^{3}H metabolites of norepinephrine, known to arise by neuronal and extraneuronal metabolism, during basal conditions and during increased efflux of norepinephrine by electric stimulation. Similar amounts of endogenous norepinephrine were present in segments of left (0.36 μg NE/g) and right (0.42 μg NE/g) coronary arteries. Levels were not significantly different after 15 min of continuous electric stimulation (0.37 and 0.41 μg NE/g in right and left arteries, respectively). This was apparent even though the fractional loss with electric stimulation seemed high; approximately three times greater from the coronary artery than from a segment of the peripheral vasculature. Of the quantity of norepinephrine released during electrical stimulation, 37% overflows in the superfusate, 43% is taken up in the sympathetic neurons and 20% is metabolized extraneuronally. Of that entering neurons about one-half is metabolized to 3,4-dihydroxyphenylglycol and the remainder is stored in the vesicles. By comparison with the saphenous vein from the same animal, the amount of NE in the vessels is less and a greater proportion of that released reenters the neurons and presumably helps to maintain the stores of transmitter.

Many studies have established that the metabolic state of the myocardium is of primary importance in the control of coronary blood flow, and there is increasing evidence to suggest that adenosine crosses the myocardial cell membranes to dilate the arterioles and so adjust flow to the metabolic requirements of the heart (1). By contrast, the role of the sympathetic nerves in the regulation of coronary flow continues to be debated (2). Studies on helical strips of dog coronary arteries have shown that the smaller vessels are relaxed by catecholamines, whereas the large vessels either contract, or after a transient contraction, relax. During beta-adrenergic receptor blockade, the small vessels either do not react to catecholamines, or relax slightly, whereas the larger vessels contract strongly (3), presumably because of the unopposed action of the amines on alpha-receptors. Recent evidence suggests that activation of sympathetic fibers to the heart decreases the diameter of a coronary artery and that this response can be blocked by phentolamine (4). Other evidence suggests that in certain circumstances sympathetic alpha-receptor-mediated coronary vasoconstriction can reduce the coronary vasodilatation caused by the local metabolic mechanism (5, 6).

Surprisingly, little information is available of the norepinephrine (NE) content, release, and disposition in coronary vessels. This cannot be deduced from studies on other vessels, because of the difference among vessels in the extent of sympathetic innervation, the width of the synaptic cleft, the relative importance of neuronal to ex-

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traneuronal uptake of released transmitter, and the presence of prejunctional receptors which may modulate transmitter release (7–10).

The purpose of the present study was to obtain this data and to compare it with results obtained previously in the canine saphenous vein (8, 9, 11).

Materials and methods. Preparation and superfusion of coronary arteries. Experiments were performed on coronary arteries and saphenous veins of dogs. The dogs (20–30 kg) were anesthetized with pentobarbital (50 mg/kg, intravenously), the chest was opened, and the heart was removed. The anterior interventricular branch of the left coronary artery and the right coronary artery were dissected from their origin to a diameter of 1 mm, cleaned of perivascular tissue, and cut into helical strips (average weight 70 ± 20 mg). Some strips were placed directly in acetic acid for extraction of endogenous NE. Others were superfused and endogenous NE overflow with electric stimulation was studied. Helical strips from lateral saphenous veins were prepared as described previously (11).

The strips were suspended for superfusion with Krebs–Ringer's solution at 3 ml/min (9, 11).

For electrical stimulation of adrenergic nerves two platinum wires (0.5 mm in diameter) were placed parallel to and in contact with the tissue; both the strips and the electrodes were superfused continuously. Electrical stimulation consisted of rectangular impulses (10 V, 2-msec duration, 5 Hz) provided by a direct current power supply and a switching transistor triggered by a Grass stimulator (Model S-44). In experiments designed to quantitate the overflow of endogenous NE, the superfusate was collected for 15 min before and during electrical stimulation. Superfusion was then discontinued and the endogenous NE in the artery was extracted.

In other experiments arterial strips were incubated before superfusion for 2 hr in Krebs–Ringer's solution containing 6.7×10^{-7} *M* L-[7-^{3}H]NE (specific activity 3.8 Ci/mmole; New England Nuclear), EDTA (0.01 mg/ml), and ascorbic acid (0.2 mg/ml). To remove the extraneuronal [^{3}H]NE from the tissues, the strips were superfused for 90 min and the superfusate was discarded. In some experiments cocaine (10^{-5} *M*) was added to the superfusion fluid during the last 30 min of this period. A sample next was collected over a 6-min interval prior to electrical stimulation, for three successive 6-min intervals during stimulation, and for three successive identical intervals after stimulation. The superfusates from each time period were analyzed for [^{3}H]NE and metabolites of NE.

At the end of the experiment, the arteries were blotted dry, weighed, and immersed immediately in acetic acid solution for extraction of [^{3}H]NE and its metabolites.

Measurement of norepinephrine. Endogenous NE was extracted by immersing the strips for two 30-min periods in separate 5-ml aliquots of 1 *N* acetic acid containing 0.03 m*M* EDTA and 0.2 mg/ml ascorbic acid (12). NE was isolated from the extracts of the artery (10 ml) and from superfusate (45 ml, which had been collected in cooled flasks containing 0.2 ml of 2 *N* HCl) by the method of Valori *et al.* (13). This method involves adsorption of catechols on alumina at pH 8.4, followed by elution with dilute (0.05 *N*) perchloric acid. Catecholamines are separated from catechol acids in the perchloric acid eluate by chromatography on small columns (0.3 cm diameter, 3 cm height) of the cation-exchange resin Amberlite CG 50 (9). Elution from the resin is accomplished with 1.0 ml of 2/3 *M* boric acid. The concentration of NE in this extract was determined by high-pressure liquid chromatography (hplc) with electrochemical detection, using an 18 μBondapak column. A mobile phase solvent system consisted of 0.07 *M* NaH_2PO_4, 0.2 m*M* EDTA, 2 m*M* heptane sulfonate, and 6% methanol at pH 4.8. The electrochemical detector was set at 0.7 V against a silver/silver chloride reference electrode.

The average recovery of 10 ng of NE added to extracts of superfusate or to arteries was 70.6 ± 6.0% ($\bar{m}$ ± SEM); $n = 5$.

Separation of [^{3}H]NE and radiolabeled metabolites. Carrier substances 0.1 mg of each of NE, normetanephrine (NMN), 3,4-

dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylglycol (DOPEG), and 3-methoxy-4-hydroxy-mandelic acid (MOPEG), 3.0 mg of sodium metabisulfite, and 0.6 ml of 2 *N* HCl were added to each sample of superfusate and to each extract of veins. [^{3}H]NE was then separated from its ^{3}H metabolites by the method of Graefe *et al.* (14). The average recovery of 20 μg [^{3}H]NE added to extracts of superfusate or to arteries was 84.5 ± 0.3%; $n = 4$.

Radioactivity measurements. Aliquots (1 ml) of superfusate, of acetic acid extracts of artery, and of the effluents and eluates from columns were added to 10 ml of Insta-Gel (Packard Instrument Company, Inc.) and the radioactivity was measured in a liquid scintillation counter. Corrections for quenching were made with an external standard. The counting efficiency was 35%. Most of the samples were counted for 10 min or until 10,000 counts were reached.

Calculations. The fractional rate of loss of radioactivity from the artery per time period was calculated by dividing the radioactivity in each superfusate by that present in the tissue. The amount of tritium in the tissue at any given time was calculated by adding to the radioactivity in the artery at the end of the study all the tritium collected in samples of superfusate from that time to the end of the sample collection.

Analysis of data. For statistical evaluation of the data, Student's *t* test was used. *P* values <0.05 were considered significant.

Results. Norepinephrine content and its release. Similar amounts of NE were present in unstimulated strips of left and right coronary arteries (Table I). No NE could be detected in the superfusate from unstimulated arteries, but small amounts were detected during electrical stimulation. After 15 min of stimulation the content of NE in the strips was still equal to that in unstimulated strips. The identity of the NE was established by comparison of its retention time with that of authentic NE in two solvent systems of the hplc.

Fractional loss of total radioactivity. The fractional loss of total radioactivity from strips of tissue prelabeled with [^{3}H]NE was two to three times greater in the coronary artery than in the saphenous vein, both under basal conditions and during electrical stimulation (Fig. 1). The maximum loss during stimulation occurred during the first collection period (6–12 min) in the artery but the loss was maintained at a relatively constant rate in the vein.

Basal efflux of [^{3}H]NE and its ^{3}H metabolites. In unstimulated coronary artery strips the most prevalent compound in the superfusate was [^{3}H]DOPEG (Fig. 2). Lesser amounts of [^{3}H]*O*-methylated, deaminated metabolites (OMDA) and only small amounts of [^{3}H]NE were present. When cocaine, which blocks neuronal uptake of released NE, was present in the superfusate there were no effects on the basal efflux of [^{3}H]NE or of its metabolites (Fig. 2).

TABLE I. NOREPINEPHRINE (NE) IN UNSTIMULATED STRIPS OF CORONARY ARTERY, IN SUPERFUSATE COLLECTED DURING 15 min OF CONTINUOUS ELECTRICAL STIMULATION (5 Hz) AND THAT REMAINING IN THE STRIPS AFTER STIMULATION

NE (μg/g in tissue)[a]	Left coronary		Right coronary	
Unstimulated	0.36 ± 0.07		0.42 ± 0.06	
Stimulated	0.37 ± 0.07	(36.3 ± 10.9)[b]	0.41 ± 0.09	(32.8 ± 8.1)[b]
NE overflow in superfusate[a]				
ng/min	0.25 ± 0.05		0.21 ± 0.06	
amole/mg tissue/pulse	49.4 ± 8.8		53.3 ± 6.5	
Overflow/min as a percentage of total NE in tissue after stimulation	0.67 ± 0.15		0.65 ± 0.23	

[a] Means ± SEM, $n = 5$.

[b] The values in parentheses are the mean total content (ng) in the segment of artery.

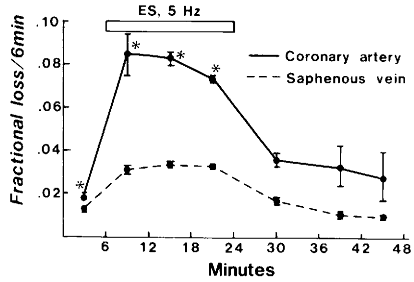

FIG. 1. Fractional loss of total radioactivity with time from strips of dog coronary artery and saphenous vein prelabeled with [^{3}H]NE. Electrical stimulation (ES) was applied between the 6th and 24th min. Means (±SEM); $n = 5$; * = $P < 0.05$ for differences from saphenous veins.

Metabolites of [^{3}H]NE during electrical stimulation. With electrical stimulation for 18 min at 5 Hz there was a release of [^{3}H]NE from the coronary strips which was maximal during the first collection period of 6 min and which decreased progressively during the subsequent two collections. The increase in metabolites was more gradual, and [^{3}H]DOPEG, [^{3}H]OMDA, and [^{3}H]NMN peaked during the second collection period; [^{3}H]DOMA changed little. On cessation of stimulation, the [^{3}H]NE decreased rapidly, and its metabolites more slowly, and in 12–18 min reached levels similar to those observed prior to stimulation (Fig. 2).

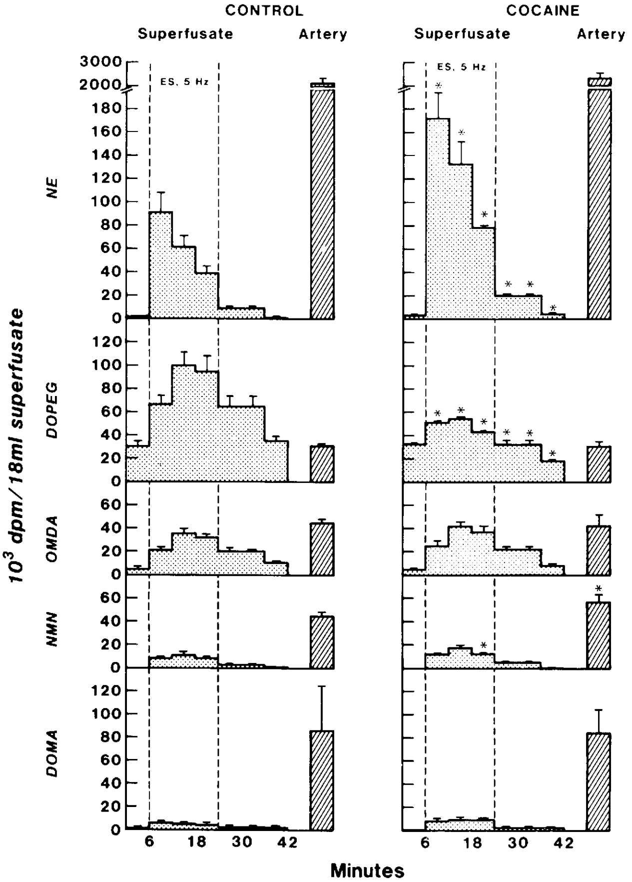

FIG. 2. Changes in the efflux of [^{3}H]NE and its metabolites before, during and after electrical stimulation (ES), at 5 Hz in coronary artery strips superfused with Krebs–Ringer's solution with and without cocaine (10^{-5} M). ES was applied between the 6th and 24th min, $n = 5$. Means (±SEM); * = $P < 0.05$ for differences from control arteries.

When coronary strips were treated with cocaine, the electrical stimulation caused more [^{3}H]NE and [^{3}H]NMN to enter the superfusate, and lesser amount of [^{3}H]-DOPEG, than in untreated strips (Fig. 2 and Table II). At the termination of these experiments, the arteries treated with cocaine had a greater [^{3}H]NMN content but there was no difference in the content of [^{3}H]NE on any other metabolite.

Discussion. These studies demonstrate that endogenous NE is present in the coronary arteries of the dog. Compared with the saphenous vein in the same species (9, 15), the amounts are small, with our average values being 0.4 μg/g and 2.9 μg/g of tissue, respectively. Few detailed morphological studies are available of the innervation of the cardiac blood vessels. Examination of the vessels of the guinea pig atria by electron microscopy showed a paucity of nerve terminals closely associated with the tunica media of the larger arteries (16). It was suggested that the arterioles might be better innervated, but no close junctions between nerve terminals and the muscle cells of the arterioles were observed. In fact, the opposite was found in dog coronary artery (17), where the distribution of terminals related to size of artery; in large arteries the terminals were regularly distributed around the entire circumference whereas in small arteries and arterioles fewer terminals were present. In comparison with other arteries of the dog, the density of the nerve terminals in the coronary wall was described as "middle." From these studies we conclude that the epicardial vessels are innervated by sympathetic fibers, but not richly so.

After 15 min of electrical stimulation, the content of endogenous NE was unchanged indicating that even prolonged activation of sympathetic nerve endings will not deplete NE stores. The maintenance of constant levels of NE cannot be explained by increased neuronal uptake since the tissue content was not decreased in the presence of cocaine. This suggests that in this tissue NE synthesis keeps pace with its release. Of interest is the fact that a greater fraction of the endogenous NE content was lost from the coronary artery during electrical

TABLE II. DISPOSITION OF [^{3}H]NOREPINEPHRINE RELEASED DURING STIMULATION OF SYMPATHETIC NERVES TO CORONARY ARTERIES[a]

Fraction	Control arteries ($n = 5$)	Cocaine-treated arteries ($n = 5$)	
NE overflow	196.06 ± 29.96	382.25 ± 47.30	[186.19 ± 17.34][b,c]
Extraneuronal uptake (DOMA + NMN + OMDA)	103.78 ± 19.99	148.78 ± 23.47	[45.00 ± 3.48][b,c]
Neuronal metabolite (DOPEG)	163.62 ± 27.52	51.64 ± 7.42	[111.98 ± 20.10][b,c]
Total neuronal uptake[d]	231.19 ± 20.82		
Neuronal uptake and metabolism[e]	111.98 ± 20.10		
Neuronal uptake and reuse[f]	119.21 ± 0.72		

[a] Data are presented as 10^3 dpm (means ± SEM) present in each compound or fraction in the superfusate collected during three successive 6-min periods of electrical stimulation and the following two periods after stopping stimulation. Corrections were made for efflux under resting conditions by subtracting the mean amount of each radiolabeled compound present in the superfusate collected before and at 12 to 18 min after stopping stimulation from the amount of the compound present in each of the five time periods.

[b] Data in brackets show differences between control and cocaine-treated arteries.

[c] Differences significant ($P < 0.05$) from control artery.

[d] "Total" neuronal uptake is calculated from the sum of the increases in norepinephrine overflow (186.19) and in the extraneuronal metabolites of norepinephrine (45.0) in the presence of cocaine.

[e] Neuronal uptake and metabolism are calculated from the differences in amounts of DOPEG overflowing in the presence and absence of cocaine.

[f] Neuronal uptake and reuse are calculated by subtracting neuronal uptake and metabolism from the true neuronal uptake (231.19 − 111.98).

stimulation than that found previously to be lost from the saphenous vein (9). The reason for the greater fractional loss is not clear but might reflect poorer vesicular retention of NE in the coronary artery. Although the loss of radioactivity from the coronary artery seemed to decrease during electric stimulation, the decreases were not significant. Mean values for the overflow of attomoles of NE per milligram of tissue per pulse were 50 and 115 in the coronary artery and saphenous vein, respectively, and for the overflow as a percentage of the total tissue content of NE were 0.66 and 0.21, respectively. Thus compared with the saphenous vein the coronary artery contains less NE, has less NE overflow per pulse of electrical stimulation, but a greater percentage of the total tissue content of NE overflows during the period of stimulation.

Concerning the metabolites of NE, in unstimulated strips of coronary vessels prelabeled with [^{3}H]NE these were comprised almost entirely of [^{3}H]DOPEG (Fig. 2), whereas previous studies have shown the overflow from saphenous veins to contain almost equal quantities of DOPEG and *O*-methylated metabolites (11). In basal conditions the amount of [^{3}H]DOPEG in the coronary strip was unaffected by cocaine, which suggests that it was not formed subsequently to the neuronal uptake of some of the released NE, but by the action of monoamine oxidase on the NE which continuously escapes from the vesicles and enters the neuroplasm. By contrast, the increases in the [^{3}H]DOPEG levels in the superfusate during electrical stimulation are largely prevented by cocaine, indicating that the increase in this metabolite above basal levels comes from that portion of the NE taken back up from the synaptic cleft into the nerve endings, and there exposed to monoamine oxidase (18).

While DOPEG is formed by the action of monoamine oxidase and aldehyde reductase, enzymes which in other tissues have been shown to be present in neurons, the formation of the metabolites NMN and OMDA involves the enzyme catechol methyltransferase (COMT). Several investigations suggest that this enzyme is absent from the peripheral neurons, so it is likely that the increase in the level of *O*-methylated metabolites in the superfusate during nerve stimulation is due to their formation from NE in the extraneuronal tissues (19). Thus when the neuronal uptake pump is blocked by cocaine, more NE is available to enter the extraneuronal tissues and more NMN can be detected in the superfusate. The formation of the metabolite DOMA from NE does not involve the action of COMT, and it might be suspected that this metabolite would be formed in the neuron. However, the increase in DOMA during electrical stimulation is not attenuated in the presence of cocaine so it would appear likely that DOMA is formed in extraneuronal sites (11).

An approximation of the relative importance of neuronal and extraneuronal uptake in the disposition of NE released from the nerve endings in the coronary arteries can be obtained by comparing the magnitude of the increases during electrical stimulation of the neuronal (DOPEG) with those of the extraneuronal (NMN + OMDA + DOMA) metabolites (Fig. 2). The increases in DOPEG were greater than the sum of the increases of the other metabolites. This finding emphasizes that neuronal uptake is much more important than extraneuronal for the disposition of the transmitter released from the nerve endings into the synaptic cleft. Further, some of the NE taken up into the neurons enters the vesicles and is not deaminated, so that the increase in DOPEG underestimates the magnitude of the neuronal uptake.

The analysis of [^{3}H]NE and metabolites in the superfusate during electrical stimulation, both before and after blockade of neuronal uptake by cocaine, provides an estimate of the relative importance of neuronal versus extraneuronal uptake in the disposition of the released NE. To make these calculations the data shown in Table II were used. About 37% of the released NE overflows from the synaptic cleft, 43% is taken up into the neuron and 20% is metabolized extraneuronally. Of the amount taken up, about half is converted to

DOPEG and the remainder enters the storage vesicles. These values probably underestimate the true neuronal uptake, since the cocaine in the concentration used may not have completely inhibited the uptake pump; this is suggested by the fact that after cocaine there was still some increase in DOPEG in the superfusate.

Similar calculation based on identical experiments in cutaneous vein strips demonstrated that overflow, neuronal uptake, and extraneuronal uptake made about equal contributions to the disposition of transmitter (8). Of the NE removed by neuronal uptake, approximately two-thirds was metabolized to DOPEG.

These experiments emphasize that the neuronal uptake system is more avid in the coronary artery than in the cutaneous vein. Consequently, blockade of this system in the coronary artery with cocaine resulted in a marked overflow of [^{3}H]NE.

It is concluded that in the coronary arteries lower stores of NE are present than in the saphenous vein, but these stores are maintained during prolonged periods of nerve activity. Because of the lower stores, the greater neuronal uptake and reuse of the released NE may help to maintain the stores of transmitter.

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