Microdialysis: Touching the fingertips of the cardiac sympathetic nervous system

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Microdialysis: Touching the fingertips of the cardiac sympathetic nervous system

Microdialyse: Reiken naar de vingertoppen van het sympathische zenuwstelsel in het hart

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Contents

Chapter 1

Introduction

For years sympathetic activity in man and experimental animals has been assessed by measuring circulating catecholamines. As their plasma concentration is not only determined by sympathetic activity but also by spillover and clearance, it is only useful as a screening tool for gross disturbances in general sympathetic tone. The isotope dilution method uses tritiated norepinephrine to differentiate between norepinephrine release into and its removal from the circulation. This technique is not only more precise but can also be applied for the assessment of regional sympathetic tone. Alternatively, local sympathetic activity can be monitored by microneurography, i.e. measuring the electrical activity of postganglionic sympathetic efferents. However, due to its invasive nature, microneurography in humans is restricted to monitoring sympathetic control of skin and muscle vasculature. Microdialysis is a new technique that can monitor local sympathetic activity almost continuously by measuring interstitial norepinephrine concentrations. The technique is based on the diffusion of norepinephrine from the intercellular space through a semi-permeable membrane mounted in a small catheter into a suitable perfusion fluid like Ringer's or Ringer's lactate, which can be collected continuously for later analysis. In conclusion, none of the mentioned techniques for monitoring sympathetic activity is superior to another as each has its own strengths and limitations. The choice for one or more of these methods strongly depends on the question that has to be answered; a combination of the various techniques may provide the investigator with a more powerful tool to monitor the sympathetic nervous system.

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Introduction

For years, sympathetic activity in man and experimental animals has been assessed by measuring circulating catecholamines. As their concentration is not only a reflection of release but is also determined by clearance, other more precise methods for assessing sympathetic activity have been developed. The aim of this review is to discuss the merits and limitations of three established techniques for studying sympathetic tone, i.e. measurement of plasma catecholamine concentrations, the isotope dilution method and microneurography, and of a recently introduced fourth technique, microdialysis.

Plasma Catecholamines

One of the simplest and -therefore- the most widely used techniques for the assessment of sympathetic activity is the measurement of circulating catecholamines and in particular plasma norepinephrine (Table 1, Figure 1). While it is a helpful screening tool for gross disturbances in overall sympathetic tone, e.g. pheochromocytoma and autonomic dysfunction, its value as a measure of sympathetic activity is limited. The plasma norepinephrine concentration is not only determined by (i) sympathetic activity, but also by (ii) spillover, i.e. the amount of norepinephrine that is released from the sympathetic nerve terminals and ultimately reaches the circulation, and (iii) plasma clearance, i.e. the rate of removal of norepinephrine from the circulation.

(i) Sympathetic activity

Sympathetic activity is characterized by a considerable regional differentiation.[1,2] At rest, the bulk of norepinephrine in plasma originates from sympathetic nerve terminals; the nerve terminals of the kidney, skeletal muscles and the lungs provide the largest contribution, whereas approximately 30 percent is derived from the sympathetic neurons of the liver, the gastrointestinal tract, the heart and the skin. In contrast, epinephrine is largely produced in and released from the adrenal medulla as a systemically active hormone. During heavy physical exercise and heart failure a minute amount of epinephrine is co-released with norepinephrine from sympathetic nerve terminals, and contributes in these circumstances to circulating epinephrine concentrations.[3-5] As a precursor of norepinephrine, dopamine is only sparingly released from the nerve terminals and the adrenal medulla. In the kidney, dopamine is synthesized from DOPA that is extracted from the circulation by

the renal tubules. A small proportion of this renal dopamine may reenter the circulation while most is excreted in urine.[4,6]

Upon stimulation, sympathetic nervous system (SNS) responses typically show regional patterns of activation or inhibition depending on the type of the stimulus. For example, mental stress induces a preferential increase in cardiac and renal sympathetic outflow, whereas cardiac failure and ventricular arrhythmias show a preferential cardiac SNS response. Submaximal exercise, however, is one of the very few stimuli resulting in a generalized increase in SNS activity.[7]

Table 1. Strengths and Limitations of Four Different Techniques for Monitoring Sympathetic Activity.

Method	Strengths	Limitations
Plasma norepinephrine concentration	+ Simple, widely used + Good screening tool for gross SNS disturbances + Barely invasive	- Only assesses overall sympathetic activity - Indirect measure of activity, confounded by clearance and spillover
Isotope Dilution Method	+ Monitors regional SNS activity + Distinguishes release and removal	- Assumes negligible gradients between the sites of release and plasma, hence underestimates NE release - Requires arterio-venous sampling - Uses radioactive compounds
Microneurography	+ Monitors regional SNS activity + Distinguishes between central and local neuronal responses + Relatively simple + No withdrawal of blood samples + Online, real-time assessment of SNS activity	- In humans, it can only be used for monitoring skin and muscle sympathetic nerve activity - Only measures nerve firing rate, does not account for factors that affect the proportional relation between nerve firing and norepinephrine release
Microdialysis	+ Measures interstitial concentrations + Monitors regional SNS activity + Distinguishes release and removal + Semi-continuous assesment of SNS activity + No withdrawal of blood samples + Can be used in 'stop-flow' experiments + Allows for the simultaneous measurement of other compounds + Allows for local pharmacological manipulation without systemic effects	- Requires sensitive analysis techniques - In humans, it can only be used for monitoring skin and muscle SNS activity - Only measures mean interstitial concentrations - When investigating other compounds, the size of the analyte is limited by the cut-off value of the membrane

(ii) Spillover

In humans, only 10-20 percent of the norepinephrine that is released from the sympathetic nerve terminals eventually enters the circulation. This spillover is determined by sympathetic activity, neuronal reuptake (U1), and local metabolism, and amounts to 200-600 ng/min in resting healthy individuals.

During strenuous exercise, the spillover rate can increase enormously to 1500-4000 ng/min.[6]

(iii) Clearance

Catecholamines are cleared from the plasma by neuronal uptake, extra-neuronal uptake by various cell types, and local intra- and extra-cellular metabolic processes, e.g. O-methylation (COMT), oxidative deamination, and sulfoconjugation. Under normal conditions, catecholamines are cleared very rapidly from the circulation; norepinephrine, for instance, is cleared at a rate of 1.5 to 4 liters per minute.[6] Like sympathetic activity, the plasma clearance of catecholamines is tissue-dependent. Not only the rate of removal, but also the relative contribution of the processes that comprise this removal, is different for the various tissues and depends on the density of the sympathetic innervation of that particular tissue. For instance, in the densely sympathetically innervated heart, 80 percent of circulating norepinephrine is removed in a single pass, of which 69 percent is cleared by neuronal uptake. In the forearm, with a less dense sympathetic innervation, only 54 percent of norepinephrine that passes through is removed, with neuronal uptake constituting only 14 percent of local clearance.[8,9]

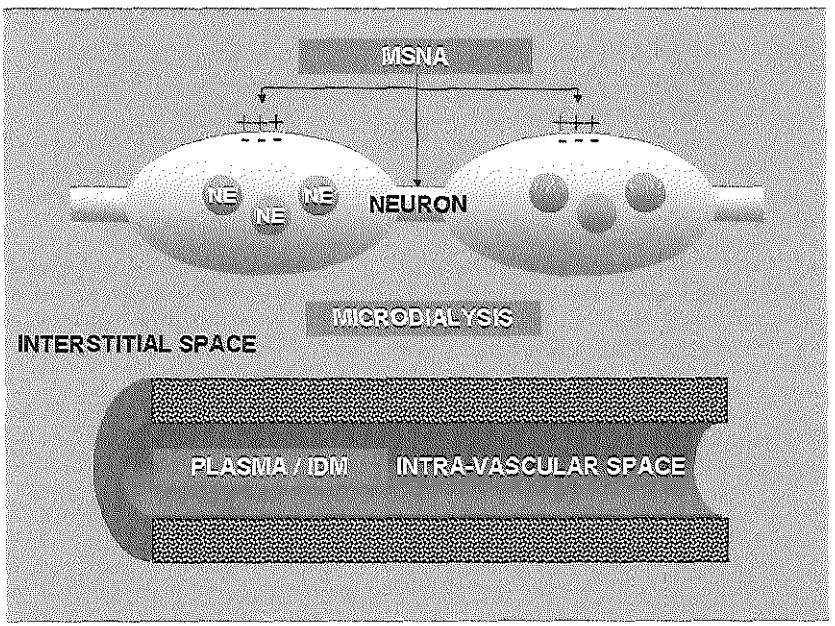

Figure 1. Four techniques to assess sympathetic nerve activity.

- **MSNA:** Muscle Sympathetic Nerve Activity
- **Microdialysis**
- **PLASMA:** measurement of plasma NE concentrations
- **IDM:** Isotope Dilution Method.

Isotope Dilution Method

The use of tritiated norepinephrine as applied in the isotope dilution method provides a useful estimation of the relative contribution of release and removal to the norepinephrine plasma level (Table 1, Figure 1). The technique is based on a one-compartment model, which assumes that all de novo sources of norepinephrine enter the circulation and all losses are irreversible, i.e. any recirculation is negligible. However, only very little norepinephrine that is released from the sympathetic nerve terminals into the synaptic cleft and intercellular space spills over into the circulation. The bulk of norepinephrine is taken up by the neurons and adjoining cells, or is metabolized locally. Thus, the use of a two-compartment model seems more appropriate.[10] Esler et al [11] introduced 'spillover' (SO) which aims to reflect the rate of norepinephrine entering the circulation rather than true production as a more adequate parameter of sympathetic activity. Later, this parameter was refined to account for regional differentiation with the use of arterio-venous organ sampling according to the following formula.[12]

$$\text{Regional norepinephrine Spillover} = Q.(V - A + FxNE.A)$$

Q = organ plasma flow
V = venous norepinephrine concentration
A = arterial norepinephrine concentration
FxNE = fractional extraction of tritiated norepinephrine

One example of the merits of spillover is the association of elevated norepinephrine plasma levels with age. At first glance, elevated norepinephrine plasma levels would suggest an increased sympathetic activity. However, several studies have shown a marked decrease in norepinephrine clearance in relation to age, while there is barely any evidence for such age-related differences in total norepinephrine spillover, either at rest or during exercise. Thus, the elevation of norepinephrine plasma levels with advancing age appears to be primarily due to a diminished ability to remove norepinephrine from the body.[13]

Chang et al [14] agree with the application of the two-compartment model but the investigators introduce an alternative parameter, the plasma appearance rate (PA), which is defined as the total amount of norepinephrine that enters the sampled plasma compartment.

$$PA = \frac{spillover}{1 - FxNE}$$

In contrast to spillover, PA should account for norepinephrine that spills over into the circulation and subsequently is taken up before it leaves the organ. Additionally, it should be less sensitive to changes in organ flow and local clearance.

The compartmental kinetic model for catecholamines is based on the fundamental assumption that transport or diffusion of catecholamines from the circulation to the sites of metabolism in the target organ do not differ for locally released catecholamines, i.e. the existence of an endothelial barrier is ruled out.[14,15] Several studies, however, have provided evidence for such a barrier.[16-20] Accordingly, previous studies from our department [5,21] as a well as others [22,23] showed a marked difference in hemodynamic response to exogenously administered norepinephrine through an intravenous infusion and endogenously released norepinephrine. For instance, an infusion of 30 ng/kg/min norepinephrine increased the norepinephrine plasma level about five fold without any significant pressor response, whereas 20 μgkg/min of tyramine increased norepinephrine plasma levels only two fold while diastolic blood pressure rose by 20 mmHg.[5] Tyramine forces norepinephrine out of the nerve terminals because of its higher affinity for the storage proteins, thus acting as an endogenous source of norepinephrine.[35]

To account for these diffusion barriers Cousineau et al [17] and Rose et al [24] developed the "distributed" model for interstitial-capillary exchange. Based on the same multiple indicator technique, Johnson [25] devised the tissue homogeneity model that, in contrast to the distributed model, assumes a random distribution of capillary entrances and exits throughout the tissue thus providing for a 'well-mixed' interstitium. While accounting for a possible endothelial barrier, both models do underestimate norepinephrine release because of the assumption that the concentration gradients between the neuroeffector junctions and the interstitial-capillary barrier are negligible. In fact, several clinical and experimental studies have demonstrated a considerable interstitial gradient of about threefold between the sites of release and plasma.[15,26,27]

Microneurography

An alternative method for monitoring local sympathetic activity is measuring the electrical activity of the postganglionic sympathetic efferents that innervate the organ of interest (Figure 1).[28] Due to the invasive nature of this technique, microneurography in humans is mainly targeted to monitor sympathetic control of the vasculature of skeletal muscle or skin with small percutaneous tungsten electrodes (Table 1). While skin sympathetic nerve activity (SSNA) is

primarily modulated by thermal, respiratory and emotional responses, muscle sympathetic nerve activity (MSNA) is mainly under baroreflex control and as such is a more appropriate tool for assessing cardiovascular sympathetic activity. Indeed, in several conditions associated with increased sympathetic tone like the early phase of essential hypertension, obesity and congestive heart failure, MSNA is increased whereas SSNA remains unchanged.[29,30]

One of the major benefits of this elegant technique is that it allows for an online, real-time assessment of sympathetic activity. Additional benefits are its relative simplicity, no withdrawal of blood samples, no use of radioactive compounds and its ability to distinguish central from local neuronal responses, like those elicited by mechano- and metabo-receptors.[30,31] On the downside, however, this technique also has some important limitations. Firstly, while its ability to investigate local sympathetic activity is one of its strengths, it is also one of its weaknesses since the alteration of sympathetic tone in some disease states or conditions is limited to a single organ, like salt depletion (kidney), mild to moderate heart failure (heart), coronary insufficiency (heart), and mental stress (heart).[4] Secondly, although sympathetic nerve firing rate is the principle force behind norepinephrine release, it is certainly not the only factor determining norepinephrine concentration at the neuroeffector junction.

Other factors that will affect the relation between nerve firing and norepinephrine release have to be considered, like changes in norepinephrine clearance, e.g. alterations in regional blood flow and impaired neuronal reuptake (heart failure, use of tricyclic antidepressants), modification of the amount of norepinephrine released per nerve impulse by pre-synaptic inhibition or facilitation, the use of sympathetic nerve-blocking drugs like guanethidine (firing without release), and events or drugs that give rise to non-exocytotic release (release without firing), e.g. myocardial ischemia and tyramine [4,32]. Subsequently, microneurography by itself does not allow for the investigation of processes that occur at the fingertips of the SNS, the neuroeffector junction.

Microdialysis

Until recently, the extra-vascular compartment could only be monitored either by estimation through application of mathematic kinetic modeling, or by using in-vitro or semi-in-vivo preparations. Microdialysis is a technique that allows for a semi-continuous measurement of interstitial concentrations in vivo (Table 1). It is based on diffusion of analytes from the intercellular space through a semi-permeable membrane into a suitable perfusion fluid like Ringer's or Ringer's lactate (Figure 2). The microdialysis catheter can be inserted in the

organ of interest, clinically in skin or muscle, and experimentally for example in the heart, the brain, or the kidney, like one would insert a simple percutaneous venous catheter. The perfusion fluid is pumped through the microdialysis catheter via the inlet tube using a microinjection pump at a set perfusion rate. The dialysate is collected at the end of the outlet tube in microvials and stored for later analysis.

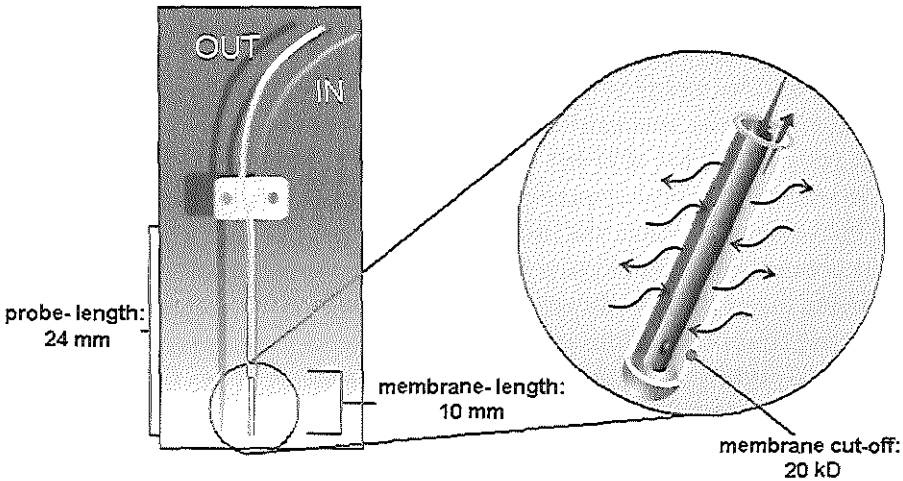

Figure 2. The microdialysis probe, dimensions and principles. **IN**: Inlet tube; **OUT**: Outlet tube. Derived from pictures on the internet site of Carnegie Medicine AB (http://www.microdialysis.se).

The procedure is relatively simple and the technique is now in experimental and clinical use in a number of medical disciplines.[33-37] It should be noted, however, that sample volumes are small (μL's) requiring sensitive analytical techniques. The technique does not involve the withdrawal of circulatory volume so it is possible to take an almost endless amount of "samples", only limited by the length of the experiment and the laboratory's capacity to process these samples. Furthermore, it allows for the measurement of concentrations in stop-flow conditions like myocardial norepinephrine concentrations in the flow area of a clipped coronary artery. The size of the analyte is limited by the cut-off of the membrane. Most probes that are available commercially have a cut-off of 20 kDa, which in reality limits the size of the substance of interest that can be analyzed to a MW of about 2000 Da. In general, this technique cannot be used for measuring the interstitial concentration of larger peptides, proteins or smaller conjugated substances.

Fortunately, catecholamines are quite small (MW < 200 Da) and readily diffuse through the microdialysis membrane, making microdialysis well suited for measuring local SNS activity; dialysate catecholamine concentrations are

about 50 % of actual interstitial catecholamine concentrations when the perfusion of the microdialysis catheter is set at a rate of 2 μL/min.[38,39] Microdialysis can be particularly useful for monitoring sympathetic tone since it allows for the measurement of norepinephrine in the intercellular space, i.e. close to the sites of release and reuptake (Figure 1). In fact interstitial norepinephrine levels are reported to correlate much more closely with regional sympathetic activity and it's subsequent physiologic response than plasma norepinephrine concentrations.[40]

Another advantage of this technique is the possibility of modification of local processes such as norepinephrine reuptake without provoking an undesired systemic response, by adding a reuptake blocker like desipramine to the perfusion fluid.[39,41-43] Thus, the amount of norepinephrine that is removed by neuronal reuptake can be measured. In addition, microdialysis has revealed that the aforementioned concentration gradient of norepinephrine between the interstitial and intra-vascular compartments in the heart is not due to an endothelial barrier but is the result of neuronal as well as extra-neuronal norepinephrine uptake.[14,15,39]

Finally, it is important to realize that while it is a more direct technique to measure interstitial norepinephrine concentrations than the estimates based on the isotope dilution technique, it will only provide information about the mean interstitial norepinephrine concentration and not about the concentration at sites of release.

Summary

None of the described techniques for monitoring sympathetic activity is strictly superior to another as each has its own strengths and limitations (Table 1). The choice for one or more of these methods strongly depends on the question that has to be answered. A large-scale patient study in which overall sympathetic tone is only monitored to rule out gross SNS disturbances at baseline or during the intervention, requires another approach than a smaller scale study in which the assessment of regional sympathetic tone is the principal objective. Whereas measuring plasma catecholamine concentrations is most suited for the former study, a more specific technique like the isotope dilution method would the better option in the latter situation.

In many instances a combination of various techniques would provide the investigator with a more powerful tool to monitor the SNS. For instance, the combination of MSNA with microdialysis would allow for investigating the relationship between nerve-firing and interstitial norepinephrine concentrations. This would be of particular interest in heart failure as the

mechanism underlying the associated increase in sympathetic tone is still unclear.[44,45] Such an approach could distinguish whether the activation of the SNS is due to augmented sympathetic nerve traffic or an increase in interstitial norepinephrine concentrations as a result of diminished clearance, e.g. due to a failing neuronal reuptake.

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Chapter 2

Aim and outline of the thesis

Introduction

This thesis comprises six studies concerning the pharmacokinetics, modulation and pathophysiological role of norepinephrine in the porcine heart. When investigating norepinephrine kinetics, the heart is of particular interest. Although the human and porcine hearts are both para-sympathetically dominant and the contribution to total body norepinephrine levels is rather small, only 3 percent,[1] regional spillover is distinctively high because of the heart's extensive sympathetic innervation. Indeed the sympathetic nervous system plays a major role in maintaining cardiac output and adjusting cardiovascular function to the body's demand.

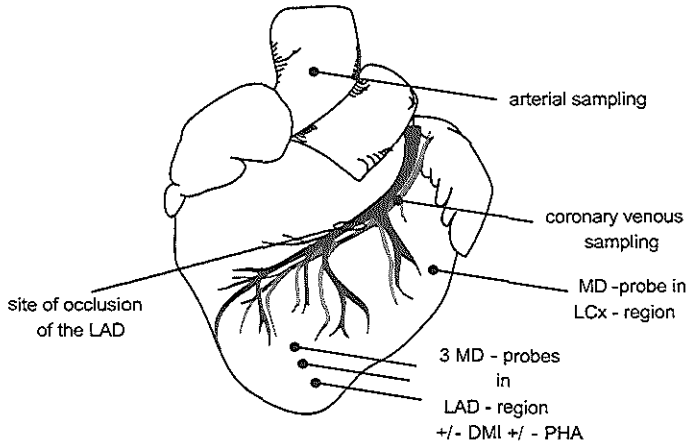

Figure 1. Experimental setup and instrumentation. MD: microdialysis; DMI: desipramine, neuronal reuptake-blocker; PHA: phentolamine, non-selective α-adrenergic receptor-antagonist.

After describing the technical aspects of the measurement of norepinephrine and other catecholamines in chapter 3, we will explore the pharmacokinetics of norepinephrine in chapter 4. In chapters 5 and 6 the modulation of norepinephrine by the vaso-active hormones angiotensine II and epinephrine will be discussed. Finally, the fate and potential role of norepinephrine in pathophysiological processes like ischemia will be described

in chapters 7 and 8. In all studies we use the aforementioned microdialysis technique to measure catecholamines in the myocardial interstitial fluid. To this end, four microdialysis probes are inserted into the left ventricular wall (figure 1), three of which in the region perfused by the left anterior descending coronary artery (LAD) and one in the region perfused by the left circumflex coronary artery (LCx).

As over 80% of neuronally released norepinephrine is taken up by sympathetic nerves through the neuronal reuptake (U1) mechanism (figure 2, the U1-inhibitor desipramine is added to the perfusate of one the microdialysis probes to provide local U1-blockade. In addition, the non-selective α-adrenergic receptor antagonist phentolamine is added to the perfusate of another probe in combination with desipramine (figure 1) to account for a possible inhibition of norepinephrine release through stimulation of presynaptic α_2-adrenergic receptors (figure 2).

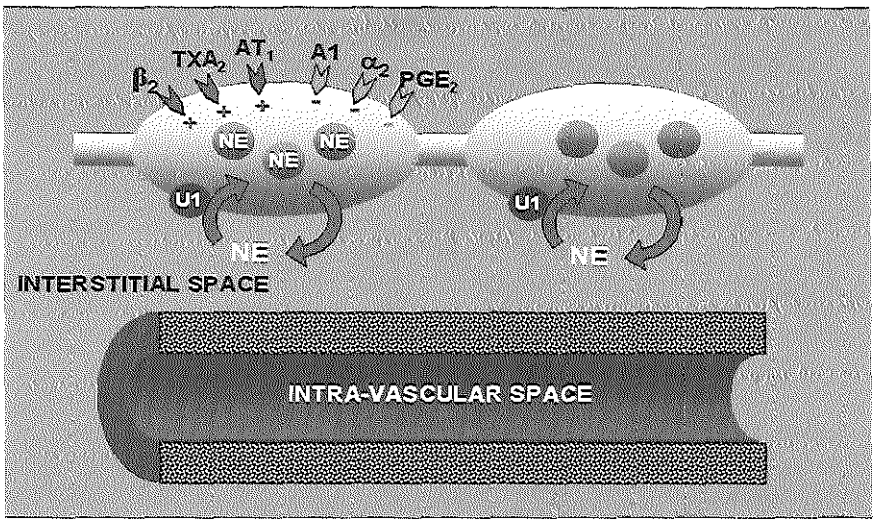

Figure 2. Cardiac sympathetic nerve terminals: presynaptic facilitation and inhibition. Shown are the varicosities of sympathetic nerve terminals containing the norepinephrine (NE) storage vesicles, the neuronal reuptake mechanism (U1), facilitatory presynaptic receptors (+), e.g. β_2-adrenergic receptor (β_2), thromboxane type 2 receptor (TXA_2) and angiotensin II type 1 receptor (AT_1), and inhibitory pre-synaptic receptors (-), e.g. adenosine A1-receptor (A1), α_2-adrenergic receptor (α_2) and prostaglandin PGE_2-receptor (PGE_2).

Chapter 3. *Determination of catecholamines in microdialysis samples*

This chapter concerns the technical aspects of the measurement of catecholamines in microdialysis samples. The slow perfusion rate with microdialysis (in the order of 0.5 to 5 μL/min) on the one hand and the need

for a reasonable time-resolution (10 minutes) on the other, result in small sample volumes (20 μL), and thus call for a highly sensitive assay method with a large through-put. Furthermore, the studies in this thesis require the simultaneous measurement of not only the natural catecholamines (norepinephrine, epinephrine and dopamine), but also some synthetic catecholamines (e.g. isoproterenol, *l*-erythro-α-methyl-norepinephrine and epinine, see also chapter 4). In addition, the *in vitro* and *in vivo* recovery, i.e. the quotient of dialysate catecholamine concentration and the actual catecholamine concentration in the medium that is dialyzed, is assessed for all natural as well as a few synthetic catecholamines.

Chapter 4. *Catecholamine handling in the porcine heart*

This chapter will focus on the pharmacokinetics of norepinephrine in the heart and its modulation by the U1-mechanism. Several experimental findings suggest that in the heart a pronounced concentration gradient for norepinephrine exists between the interstitial and intra-vascular compartments. For instance, Silverberg et al.[2] showed that the coronary sinus norepinephrine concentration induced by a norepinephrine infusion that led to only a small increase in the heart rate was eight times higher than with stellate ganglion stimulation causing a similar increase in heart rate. Cousineau et al.[3] estimated from tracer dilution experiments a ratio between the interstitial and arterial compartments of 15 % in canine hearts. Because of the dense sympathetic innervation, especially in the heart, neuronal reuptake of norepinephrine could be an important determinant for maintaining such a gradient.[1,4] However, other investigators have suggested that the concentration gradient is caused by a physical barrier of the blood vessel wall as well.[3,5-9]

In a series of experiments, in which either the circulatory or the interstitial norepinephrine concentration is increased, we investigate how the norepinephrine concentration in the myocardial interstitial fluid relates to its concentration in the arterial and coronary venous circulation. The importance of the neuronal and extra-neuronal uptake to this relationship is explored by adding desipramine (U1-inhibitor) to the dialysate of one of the microdialysis probes (figure 1) and by performing experiments with isoproterenol, a catecholamine known not to be handled by the U1-mechanism (figure 2). Furthermore, the cardiac spillover, uptake and release of norepinephrine are estimated with the use of the norepinephrine concentrations in myocardial interstitial fluid, arterial plasma and the coronary effluent at baseline and during intravenous norepinephrine infusions.

Chapter 5. *Epinephrine in the heart*

This chapter describes to which extent epinephrine is taken up by and released from cardiac sympathetic nerves and whether it can increase myocardial interstitial norepinephrine concentrations under basal conditions and during sympathetic activation. Several studies have shown that cardiac epinephrine is released into the coronary circulation of the human heart during exercise, at rest with advancing age and in conditions like hypertension, heart failure and panic disorders.[10-12] Whether cardiac epinephrine is released from sympathetic nerve terminals after it has been taken up from the circulation or whether it is released from extraneuronal stores remains unclear.[13,14]

Another point of debate relates to the role of locally released epinephrine. Several *in vitro* as well as *in vivo* studies have suggested that epinephrine enhances neuronal norepinephrine release through stimulation of presynaptic β_2-receptors located at the sympathetic nerve terminals (figure 2).[15-18] This presynaptic facilitation of norepinephrine release by epinephrine is essential to the "epinephrine hypothesis", which proposes that excessive adrenomedullary activation leads to the development of hypertension through increasing sympathoneural norepinephrine release.[18] Other studies, however, have failed to confirm this mechanism.[19-21]

To unravel the source of cardiac epinephrine, the effect of intracoronary tyramine infusions on epinephrine concentrations in myocardial interstitial fluid is investigated before and after loading the heart with epinephrine by means of intracoronary epinephrine infusions. Finally, we explore whether intracoronary infusions of epinephrine can increase myocardial interstitial norepinephrine concentrations under basal conditions and during sympathetic activation induced by electrical stimulation of the left stellate ganglion.

Chapter 6. *Angiotensin II and norepinephrine release*

In this chapter, we determine whether physiological (pM) to pathophysiological (nM) concentrations of angiotensin II modulate interstitial norepinephrine concentrations in the porcine heart under various conditions. Activation of the sympathetic nervous system simultaneously leads to activation of the renin-angiotensin-system via stimulation of β-adrenergic receptors within the kidney resulting in an increased renin release. There is also, albeit conflicting, evidence that the sympathetic nervous system is activated by the renin-angiotensin-system.[22-34] This activation supposedly occurs through stimulation of

angiotensin II receptors within the central nervous system and/or stimulation of presynaptic angiotensin II receptors located at sympathetic nerve terminals.

In those studies that demonstrated interaction between angiotensin II and the sympathetic nervous system, most evidence points towards direct facilitation mediated by presynaptic angiotensin II type 1 (AT_1) receptors (figure 2) resulting in either a 'classic' calcium-dependent augmentation of exocytotic norepinephrine release,[22-24] or in enhanced nonexocytotic release via activation of the Na^+/H^+ exchanger.[35,36] Therefore, the modulation of interstitial norepinephrine concentrations by angiotensin II is not only investigated under basal conditions but also during enhanced exocytotic norepinephrine release evoked by stimulation of the left stellate ganglion as well as during nonexocytotic norepinephrine release induced by ischemia.

Chapter 7. *Cardiac ischemia and local catecholamine release*

In this chapter, we explore the fate and potential role of catecholamines and in particular of norepinephrine in myocardial ischemia. Myocardial ischemia is associated with a marked accumulation of norepinephrine in ischemic tissue.[37-40] Interestingly, *in vitro* studies in the sympathetically dominant rat heart suggest that the ischemia-induced norepinephrine release can be attenuated by blocking neuronal reuptake, indicating that under ischemic conditions the U1-mechanism is reversed, and can operate as a carrier for outward instead of inward norepinephrine transport.[41,42] However, it is not known whether this mechanism is also operative in parasympathetically dominant human and porcine hearts.

Furthermore, there is little information about myocardial release of epinephrine and dopamine in the ischemic heart *in vivo*. Hence, in this chapter we investigate time course and magnitude of changes in myocardial interstitial fluid concentrations of catecholamines during severe myocardial ischemia and reperfusion, as well as the contribution of reversal of the U1-mechanism to ischemia-induced norepinephrine release.

Finally, the functional integrity of the cardiac sympathetic nerve endings is assessed by comparing local norepinephrine response to an intracoronary infusion of tyramine in the post-ischemic myocardium to the response observed in the non-ischemic porcine myocardium of control animals. As tyramine is taken up via U1 into the sympathetic nerve endings where it releases norepinephrine, it provides information on the norepinephrine content as well as U1-function of sympathetic nerve endings.

Chapter 8. *Cerebral ischemia, norepinephrine and cardioprotection*

This chapter describes the role of norepinephrine in local and remote preconditioning. Ischemic preconditioning, originally described for the myocardium,[43] also occurs in kidney,[44] skeletal muscle,[45] lung[46] and brain.[47] Przyklenk et al.[48] showed that brief regional myocardial ischemia protects not only the jeopardized myocardium during a subsequent coronary artery occlusion, but also the adjacent "virgin" myocardium. Furthermore, it has been shown that brief ischemia in remote organs is also capable of limiting myocardial infarct size produced by a prolonged coronary artery occlusion.[49,50]

As norepinephrine is one of the mediators involved in the signaling pathway leading to ischemic preconditioning,[51,52] and because cerebral ischemia causes a profound release of norepinephrine from sympathetic nerve endings in normal myocardium,[53] this raises the question whether transient cerebral ischemia prior to a coronary artery occlusion may also be cardioprotective.

Therefore, we investigate the effect of cerebral ischemia on myocardial infarct size produced by a coronary artery occlusion. Because the cardioprotective effect of norepinephrine has not been established in pigs, we first explore whether intracoronary infusions of norepinephrine are capable of limiting myocardial infarct size. Finally, we quantitate myocardial norepinephrine concentrations during cerebral ischemia and exogenous norepinephrine infusions, and determine whether limitation of infarct size is mediated by attenuation of myocardial interstitial norepinephrine levels during coronary artery occlusion.

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Chapter 3

Sensitive and specific method for the simultaneous determination of natural and synthetic catecholamines and 3,4-dihydroxyphenylglycol in microdialysis samples

The relatively new technique of microdialysis provides new possibilities for investigating in vivo the functioning of the sympathetic nervous system. The small sample volumes obtained, however, are a great challenge for analytical chemists. We report here a HPLC method for measuring in one run both natural and synthetic catecholamines (dopamine, (nor)epinephrine, *l*-erythro-α-methyl-norepinephrine,, isoproterenol and epinine) and the intraneuronal metabolite 3,4-dihydroxyphenylglycol in small microdialysis samples after derivatization with the fluorogenic agent 1,2-diphenylethylenediamine. No prior clean-up step is necessary. N-Ethylmaleimide is necessary for preventing an inhibitory action on derivatization occurring in *in vivo* microdialysis samples. The method can handle large numbers of samples, is sensitive (on-column detection limits 30 to 200 fg) and reproducible (RSD 1 - 7%). Recovery characteristics of the commercial microdialysis probe used (CMA/20) were extensively investigated both in vitro and in vivo at various perfusion rates; for practical purposes a rate of 2 μL/min and sampling at 10-min intervals was found to be workable and to give good and reproducible recoveries (50 - 70%).

Alberts G, Lameris T, van den Meiracker AH, Man in 't Veld AJ, Boomsma F. Sensitive and specific method for the simultaneous determination of natural and synthetic catecholamines and 3,4-dihydroxyphenylglycol in microdialysis samples. *J Chromatogr B Biomed Sci Appl.* 1999;730:213-219.

Introduction

The availability of sensitive methods for measuring the catecholamines norepinephrine (NE), epinephrine (EPI) and dopamine (DA) in plasma has increased our understanding of the sympathetic nervous system. The development of the isotope dilution method made it possible to unravel the contributions from spillover and clearance to a measured concentration, both overall and in various organs and tissues.[1,2] The method, however, also has its drawbacks, both from a theoretical and from a practical viewpoint.[3] With the introduction of the microdialysis technique, in which a small catheter with a semipermeable membrane at its end is introduced into the interstitial space of an organ or tissue, it has become possible to actually measure as well as to influence the concentration of compounds in the interstitial space.[4] The slow perfusion rate with microdialysis (in the order of 0.5 to 5 μL/min) means however that the amount of dialysate which can be collected in a reasonable time is very small, thus leading to a need for highly sensitive assay methods with a large through-put.

It would be advantageous to be able to measure at the same time not only the natural catecholamines NE, EPI and DA, but also some synthetic catecholamines, which are used sometimes in the study of the sympathetic nervous system (e.g., isoproterenol, ISO) or used as internal standards (e.g., *l*-erythro-α-methyl-norepinephrine, AMN, and epinine, EPN). Likewise, simultaneous measurement of 3,4-dihydroxyphenylglycol (DHPG), an important intra-neuronal metabolite of NE, would be desirable.[5] Since dialysates are relatively clean samples, it may be possible to avoid the time-consuming extraction procedures necessary for plasma and urine samples.

So far, catecholamine measurements of microdialysis samples are, apart from some radio-enzymatic methods,[6-8] mostly based on high-performance liquid chromatography (HPLC) with electrochemical detection;[9-13] in most reports a prior clean-up procedure was deemed necessary. An alternative, more specific method based on derivatization with the selective fluorogenic agent 1,2-diphenylethylenediamine (DPE) has been used successfully for measurement of both natural and synthetic catecholamines in plasma and urine.[14-17] Its sensitivity and selectivity would make it ideally suitable for measurements in microdialysis samples, as has already been proposed.[18] In one report, such a method has already been used.[19] For the measurement of DHPG in microdialysis samples, one separate method has been reported employing HPLC with electrochemical detection.[20]

In this study we describe a sensitive and selective method in which natural and synthetic catecholamines as well as DHPG in microdialysate samples are simultaneously measured after direct derivatization, without an

extraction step, with the fluorogenic agent DPE followed by HPLC separation and fluorimetric detection. The method has been optimized and some unexpected and hitherto unnoticed problems have been solved. The simple derivatization procedure and the short chromatographic runs (15 min) allow for a high throughput. We also report here an extensive evaluation of in vitro and in vivo recovery experiments using the new method.

Methods

Reagents

Bicine, NE, EPI, DA, AMN, ISO, EPN and DHPG were obtained from Sigma (St. Louis, MO, USA), ethylenediaminetetraacetic acid (EDTA), N-ethylmaleimide (NEM) and hydrochloric acid from Merck (Darmstadt, Germany), potassium ferricyanide (PFC) from Aldrich (Bornem, Belgium), -glutathione from Fluka (Buchs, Switzerland), acetic acid and acetonitrile from Baker (Deventer, The Netherlands), Ringer's solution from Braun (Melsungen, Germany) and Ringer's lactate from Baxter (Uden, The Netherlands). DPE was prepared as reported previously.[16] All water used had been purified by a Milli Q-Plus system (Millipore, Bedford, MA, USA).

Apparatus

The instrumentation for chromatography consisted of a Spectra System P4000 pump (Thermo Separation, San Jose, CA, USA), a Kontron 460 autosampler (Kontron, Milan, Italy) and an FP920 fluorescence detector (Jasco, Tokyo, Japan) operated at excitation and emission wavelengths of 350 and 480 nm, respectively. Data acquisition was performed using an SP4500 Labnet interface and a PC1000 system v3.0.1 from Thermo Separation. Separations were performed on a 3 m Allsphere ODS-2 (100 mm×2.1 mm I.D.) column (Alltech, Deerfield, IL, USA) at ambient temperature. For some preliminary experiments, an electrochemical detector (Antec, Leiden, The Netherlands; operated at 600 mV) was used.

Microdialysis samples

CMA/20 microdialysis catheters (Carnegie Medicine AB, Stockholm, Sweden; membrane 10 mm×0.5 mm, cut-off: 20 kD) were inserted into left ventricular myocardium, coronary vein and carotid artery of pigs, and in freshly obtained blood samples from pigs and humans. CMA/60 catheters (membrane 30 mm×0.6 mm, cut-off: 20 kD) were inserted into subcutaneous fat and muscle of humans. Catheters were perfused with Ringer's lactate solution at a rate of 2 μL/min using a CMA/100 microperfusion pump; 10-min fractions (20 μL)

were collected into microvials containing 20 µL of 0.08 M acetic acid containing 2% (w/v) of disodium-EDTA (HAc/EDTA) which usually also contained 100 pg of internal standard AMN. Samples were stored at -80°C until assay.

Assay method

To the samples or standards were added subsequently 40 µL of acetonitrile, 10 µL of 20 mM NEM in 1.75 M bicine buffer containing 1% (w/v) of disodium-EDTA (pH 7.50), 20 µL of 0.1 M DPE in 0.1 M HCl, and 4 µL of 20 mM PFC in water. Samples were then incubated for 2 h at 37°C in the dark. After cooling to ambient temperature 6 µL of an 80 mM solution of glutathione in water was added, and the sample was put into the storage compartment of the autosampler, which was kept at 4°C. The autosampler injected 20 µL into the chromatographic system. For elution, 0.05 M sodium acetate buffer (pH 7.0)/methanol/acetonitrile was used in the proportions (60:20:20, v/v/v) (mobile phase A) and (30:10:60, v/v/v) (mobile phase B). A linear gradient was used starting with A/B (70:30) at time 0 to 100% B at 14 min; flow-rate was 200 µL/min.

All assays also contained blank samples and three different standard concentrations in triplicate. Standard mixtures containing (per mL) 5 ng of NE, AMN and EPI each, 10 ng of DA, ISO and DHPG each, and 25 ng of EPN were prepared in HAc/EDTA. Derivatizations of 5 (10,20) µL of standard mixture to which were added 15 (10,0) µL of HAc/EDTA and 20 µL of Ringer's solution were used for constructing a standard line.

Results and discussion

Electrochemical method

Preliminary experiments showed that measurement of catecholamines by direct injection of microdialysis samples into a chromatographic system equipped with an electrochemical detector leads to a large number of peaks completely obscuring the peaks of interest. Thus extensive clean up of microdialysis samples will be necessary in order to reliably measure catecholamines using an electrochemical detector.

Derivatization with DPE

The alternative method of derivatizing samples with the fluorogenic agent DPE without prior clean-up procedures was carried out essentially as described previously for extracts of plasma and urine, except for smaller volumes and a different optimal pH of the bicine buffer.[14-17] To 20 µL of a mixture of

catecholamines and DHPG in HAc/EDTA and 20 μL of Ringer's solution were added subsequently 40 μL of acetonitrile, 20 μL of 0.1 M DPE in 0.1 M HCl, 10 μL of 1.75 M bicine buffer containing 1% (w/v) of disodium-EDTA (pH 7.50), and 4 μL of 20 mM PFC in water. The order of addition of the various reagents has to be strictly adhered to, as has been described previously.[18]

After 1 h at 37°C in the dark the vials were placed into the autosampler, and 20 μL was injected into the chromatographic system. Fluorescence observed was equal to the fluorescence observed when samples were first extracted as described previously and then derivatized, except for DHPG.[16] DHPG can be derivatized with DPE but is lost to a great extent during the extraction procedure necessary for plasma and urine samples.[21] A longer period of derivatization did not result in any difference in fluorescence of the catecholamines, but did increase the fluorescence of DHPG. The optimal incubation time for catecholamines and DHPG was found to be 2 h.

When the thus established derivatization procedure was executed on catecholamines and DHPG standards dissolved not in Ringer's solution or HAc/EDTA, but in real microdialysis samples, we noticed that the fluorescence of DA, NE, AMN and DHPG was greatly inhibited. The fluorescence of EPI, ISO and EPN on the other hand was much more as expected (Table 1). This phenomenon occurred in microdialysis samples from pigs and humans as well as in microdialysis samples from freshly obtained blood, albeit to different extents. Apparently, real microdialysis samples contain (an) unknown compound(s), which inhibit(s) the derivatization reaction. Longer incubation, incubation at higher temperatures or the addition of extra amounts of PFC did not solve the problem.

Table 1. Relative fluorescence of compounds in *in vivo* microdialysis samples, as a percentage of the fluorescence of the same amount of the compounds in Ringer's solution.

Compound	- NEM - GLUT	+ NEM - GLUT	+ NEM + GLUT
3,4-Dihydroxyphenylglycol	8 ± 5	120 ± 10	102 ± 5
Norepinephrine	12 ± 2	108 ± 7	103 ± 4
l-erythro-α-methyl-norepinephrine	47 ± 3	102 ± 5	100 ± 7
Epinephrine	96 ± 3	102 ± 3	103 ± 4
Dopamine	6 ± 1	102 ± 5	102 ± 5
Isoproterenol	84 ± 3	102 ± 1	99 ± 2
Epinine	74 ± 9	95 ± 4	96 ± 6

The apparent inhibition of the DPE derivatization reaction for some, but not all, catecholamines is reminiscent of the report by Nohta et al.[22] who noticed that in erythrocytes and platelets samples compounds are present which interfere with the fluorescence derivatization reaction of NE and DA, but not EPI. They concluded that the sulfhydryl-reagent NEM can prevent this interference, and we found that the same applies for microdialysis samples: including 20 mM of NEM in the bicine buffer was sufficient to overcome the inhibitory action of the unknown compound(s) on the derivatization reaction (Table 1). During microdialysis, these compounds apparently cross the membrane and enter the dialysate.

As can be seen in Table 1 the fluorescence peak of DHPG was now higher than expected. This was caused by a small peak arising from the added NEM, which had nearly the same retention time as DHPG. Adding 6 μL of a 80 mM solution of glutathione in water at the end of the derivatization procedure got rid of this interfering peak, and resulted in optimal and complete fluorescence for all catecholamines and DHPG as compared to derivatization in Ringer's solution or HAc/EDTA (Table 1).

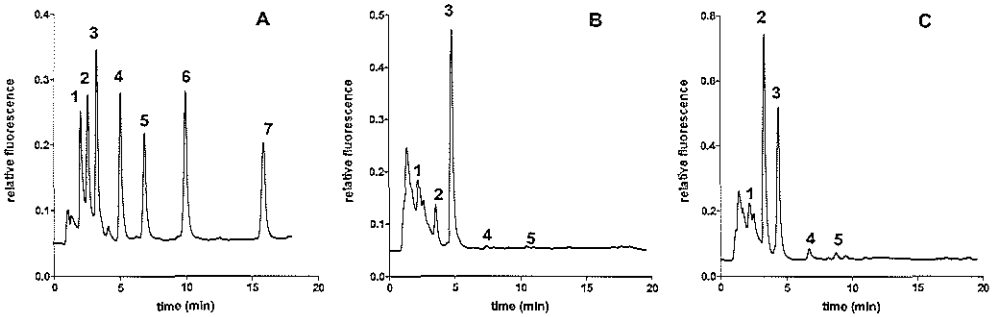

Figure 1. Chromatograms of microdialysis samples. (A) Standard mixture. On-column amounts: 8.3 pg for NE(2), EPI(4) and AMN(3), 16.7 pg for DHPG(1), DA(5) and ISO(6), and 41.7 pg for EPN(7). (B) Porcine myocardium sample. On-column amounts: DHPG 1.7 pg; NE 2.8 pg; EPI 0.2 pg; DA 0.2 pg. (C) Porcine myocardium sample (after occlusion of coronary artery). On-column amounts: DHPG 2.6 pg; NE 33.6 pg; EPI 1.4 pg; DA 2.1 pg.

All peaks of interest are clearly separated in the chromatograms (Figure 1). Although it is possible to change the elution conditions in such a way that the DHPG and NE peaks, which ride on the descending part of the front peak, come completely clear from the front, this will lengthen the runs considerably, with great loss of sensitivity for the later-eluting peaks. At low concentrations of DHPG and NE, automatic quantification of the chromatographic peaks by a computer program is not good enough, and careful delineation by hand is necessary. This can however be done reproducibly, certainly if performed by the same person. Since the

superposition thus does not appreciably interfere with reliable quantification of DHPG and NE, we have left the elution conditions as stated. With small variations in the percentage of acetonitrile, however, the elution times of the peaks can be easily influenced.

Table 2. Intra- and inter-assay variabilities at two different concentrations (C_1-C_2)*

Compound	DHPG	NE	EPI	DA	ISO	AMN	EPN
C_1 Intra-assay, mean±RSD (%)	1673 ± 2.3	325 ± 3.3	969 ± 1.3	1891 ± 3.1	1818 ± 0.8	978 ± 2.5	5019 ± 2.5
C_1 Inter-assay, mean±RSD (%)	1699 ± 4.7	361 ± 6.1	959 ± 2.9	1918 ± 1.3	1830 ± 2.1	976 ± 1.6	5168 ± 2.3
C_2 Intra-assay, mean±RSD (%)	175 ± 7.6	57 ± 3.3	169 ± 0.9	381 ± 1.8	345 ± 2.4	212 ± 2.5	1025 ± 3.3
C_2 Inter-assay, mean±RSD (%)	159 ± 7.3	58 ± 1.5	172 ± 6.3	378 ± 1.6	336 ± 6.5	221 ± 5.8	1036 ± 5.1

* Intra-assay: n=6; inter-assay: n=6. Concentrations: pg/mL.

Reproducibility and detection limit

Two mixtures of microdialysis samples enriched with different amounts of catecholamines and DHPG were used for determining the intra- and inter-assay variabilities (Table 2). Taking an S/N ratio of 3 as the detection limits, the minimal on-column detectable amounts were 30 fg for NE, EPI and AMN, 50 fg for ISO, 80 fg for DHPG and DA, and 200 fg for EPN. With standard assay conditions, this amounts to 10 pg for NE, EPI and AMN, 15 pg for ISO, 25 pg for DHPG and DA, and 60 pg for EPN per mL of dialysate.

In vitro and in vivo recoveries

For relating measured dialysate concentrations to actual interstitial concentrations, one must know the relative recovery, i.e., the ratio between concentrations in dialysate and surrounding medium. Furthermore, from a practical point of view, it is important to know the absolute recovery, i.e., the amount of a compound entering the dialysate in a defined period of time.[11] We have determined relative and absolute recoveries of NE, EPI, DA, ISO, AMN and DHPG in vitro with CMA/20 microdialysis probes in triplicate by inserting them in standard mixtures of the above-mentioned compounds (concentrations 4 to 12 ng/mL) in Ringer's solution and perfusing the probes with Ringer's solution at flow-rates of 0.5, 1, 2, 4 and 8 µL/min. The standard mixtures were kept at 37°C and 1 h after starting perfusion four consecutive 20 µL samples were collected and assayed.

The results (Figure 2) show that both relative and absolute recoveries of all compounds are similar and reproducible. The absolute recoveries are

represented here as a percentage of the amount found in the dialysate per min at the highest flow-rate of 8 μL/min. It can be concluded that a flow-rate of 2 μL/min, which gives good relative (53-66%) and absolute (69-77%) recoveries and allows for sampling at 10 min intervals, is appropriate for experiments. The inter-probe variability is quite small: on average 3±2% (NE, EPI and AMN 2±1%, DA 4±2%, ISO 3±1%, DHPG 7±5%).

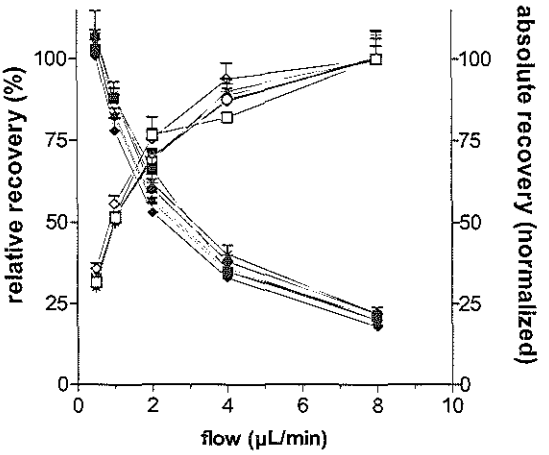

Figure 2. Relative (closed symbols) and absolute (open symbols) recoveries of the compounds using the CMA/20 microdialysis probe at various perfusion flow rates. DHPG(■); NE(●); E(π); DA(✳); ISO(◆); AMN(θ).

In vivo recovery was determined in two different ways. First, since characteristics of AMN are quite similar to those of the other catecholamines, we perfused the microdialysis probes inserted in the porcine heart with Ringer's lactate solution containing a known concentration of AMN and measured the concentration of AMN in the collected dialysate samples. This retrodialysis method, employed in four pig experiments, gave a recovery for AMN of 52±8% (n=286), in close agreement with the results of the in vitro recovery determination. Second, with probes inserted into the carotid artery, we compared the concentrations of NE and EPI in these dialysate samples with the concentrations in the plasma samples obtained at the same time from the same artery (midway between the 10-min microdialysis period) during periods of elevated NE and EPI concentrations. Results showed a recovery of 51±4% for NE (n=19) and somewhat higher recoveries for EPI (68±3%, n=17).

The recovery experiments show that catecholamines and DHPG can reliably be measured in this way and that they can reproducibly give a good indication of the interstitial concentrations. The recovery compares favorably with the scarce results (on NE only) given in the literature with different, laboratory-made microdialysis probes (23–41%).[6,11,23] As a compromise between high relative but low absolute recovery at a perfusion rate of 0.5

μL/min, and low relative but high absolute recovery at 8 μL/min, a perfusion rate of 2 μL/min is satisfactory, with a sufficiently rapid sampling period of 10 min.

Conclusions

The method described is sensitive, simple and rapid. With the use of an autosampler a large number of samples can be processed. For our hospital setting, it was not practical to develop an automated on-line method; we preferred a manual derivatization procedure, which is time-consuming but not labor-intensive. If desired, our method can be adapted to an on-line method, as has been described previously,[18,19] provided NEM is also included in the derivatization mixture. The addition of NEM is essential for obtaining good and reproducible derivatizations, and thus reliable measurements, of the compounds in question. The inhibition of the derivatization seen in the absence of NEM was never seen with plasma and urine samples, probably because the extraction procedures always used with such samples also remove these inhibitory substances.

The fact that the intra-neuronal metabolite DHPG can also be quantitated at the same time is a great advantage, as is the possibility of measuring various unnatural catecholamines which provides flexibility both in choosing internal standards and in determining concentrations of infused synthetic substances like isoproterenol. The O-methylated metabolites cannot be measured with the present method. For measurement of these compounds, HPLC with electrochemical detection is the method of choice, which does not need derivatization, but is not selective enough to be used without extensive pre-purification of microdialysis samples.

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Chapter 4

Catecholamine handling in the porcine heart

Introduction - Experimental findings suggest a pronounced concentration gradient of norepinephrine (NE) between the intra-vascular and interstitial compartments of the heart, compatible with an active neuronal reuptake (U1) and/or an endothelial barrier.
Methods and Results - Using the microdialysis technique in 8 anesthetized pigs, we investigated this NE gradient, both under baseline conditions and during increments in either systemic or myocardial interstitial fluid NE concentration (NE_{MIF}). At steady state, baseline NE_{MIF} (0.9±0.1 nmol/L) was higher than arterial NE (0.3±0.1 nmol/L), but not different from coronary venous NE (1.5±0.3 nmol/L). Local U1-inhibition raised NE_{MIF} concentration to 6.5±0.9 nmol/L. During intravenous NE infusions (0.6 & 1.8 $nmol \cdot kg^{-1} \cdot min^{-1}$), the fractional removal of NE by the myocardium was 79±4% - 69±3% depending on infusion rate. Despite this extensive removal, the change in MIF/arterial concentration ratios ($\Delta MIF/\Delta A$) for NE were only 0.10±0.02 for the lower and 0.11±0.01 for the higher infusion rate, whereas U1-blockade caused the $\Delta MIF/\Delta A$ ratio to rise to respectively 0.21±0.03 and 0.36±0.05. From the differences in $\Delta MIF/\Delta A$ ratios with and without U1-inhibition it could be calculated that 67±5 % of NE_{MIF} is removed by U1. Intracoronary infusion of tyramine (154 $nmol \cdot kg^{-1} \cdot min^{-1}$) caused a 15-fold increase in NE_{MIF} concentration. This pronounced increase was paralleled by a comparable increase of NE in the coronary vein.
Conclusion - We conclude that U1 as well as extra-neuronal uptake and not an endothelial barrier are the principal mechanisms underlying the concentration gradient of NE between the interstitial and intra-vascular compartments in the porcine heart.

Lameris TW, van den Meiracker AH, Boomsma F, Alberts G, de Zeeuw S, Duncker DJ, Verdouw PD, Man in 't Veld AJ. Catecholamine handling in the porcine heart: a microdialysis approach. *Am J Physiol.* 1999;277:H1562-H1569.

Introduction

Several experimental findings suggest that in the heart a pronounced concentration gradient for norepinephrine exists between the interstitial and intra-vascular compartments. Silverberg et al.[1] showed that the coronary sinus norepinephrine concentration induced by a norepinephrine infusion that led to only a small increase in the heart rate was eight times higher than with stellate ganglion stimulation causing a similar increase in heart rate. Cousineau et al.[2] estimated from tracer dilution experiments a ratio between the interstitial and arterial compartments of 15 % in canine hearts. More recently, Obst et al.[3], using ultrafiltration of interstitial fluid from isolated perfused rat hearts, found ratios of interstitial transudate to arterial concentrations of norepinephrine of 0.14 to 0.77 depending on the concentration of norepinephrine administered. These investigators interpreted these findings as to suggest that the neuronal uptake of norepinephrine is the most important determinant for maintaining a concentration gradient between the circulatory and interstitial compartment. Because of the dense sympathetic innervation, especially in the heart, neuronal reuptake (U1) of norepinephrine could be an important determinant for maintaining such a gradient.[4,5] However, other investigators have suggested that the concentration gradient is caused by a physical barrier of the blood vessel wall as well.[2,3,6-9]

A profound understanding of catecholamine kinetics is invaluable when interpreting catecholamine data and their representation of sympathetic activity. Using the isotope dilution method and arterio-venous sampling, Esler et al.[10] introduced regional spillover (SO) as a kinetic parameter that aims to reflect the rate of norepinephrine entering the circulation rather than true production. In a recent study, Kopin et al.[11] have modified this technique and introduced a new method to estimate neuronal release, which is based on the measurements of the specific activities of radiolabeled norepinephrine and its extra-neuronal metabolite normetanephrine in plasma. Neuronal release of norepinephrine into the interstitial space is estimated as the sum of the spillover (SO) of norepinephrine from the interstitium to the circulation and uptake of released norepinephrine (Ur) from the interstitium. Nonetheless, the interstitial compartment can only be monitored either by estimation through application of mathematic kinetic modeling, or by using *in vitro* or semi-*in vivo* preparations. The microdialysis technique, however, allows for the accurate estimation of the concentration of catecholamines in the myocardial interstitial fluid (MIF) *in vivo*.[12-15] The technique should allow for measuring the hitherto unquantifiable amount of norepinephrine that is released but taken up before reaching the vascular compartment, thus filling the gaps in existing kinetic models.

In a series of experiments, in which either the circulatory or the interstitial norepinephrine concentration was increased, it was investigated how the concentration of norepinephrine in the MIF relates to its concentration in the arterial and coronary venous circulation. The importance of U1 and extra-neuronal uptake to this relationship was explored by adding desipramine (DMI), a well known U1-inhibitor, to the dialysate of one of the microdialysis probes and by performing experiments with isoproterenol, a catecholamine known not to be handled by U1. Furthermore, norepinephrine concentrations in MIF (NE_{MIF}), arterial plasma and the coronary effluent at baseline and during infusion of norepinephrine provided an estimate of spillover, uptake and release of norepinephrine.

Methods

Animal care

All experiments were performed in accordance with the "Guiding Principles for Research Involving Animals and Human Beings" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam (The Netherlands).

Surgical procedure

After an overnight fast, crossbred Landrace x Yorkshire pigs of either sex (30-35 kg, n=8) were sedated with ketamine (20-25 mg/kg i.m., Ketalin®) and anesthetized with sodium pentobarbital (20 mg/kg i.v., Narcovet®). The animals were intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture of oxygen and nitrogen. Respiratory rate and tidal volume were set to keep arterial blood gases within the normal range: pH between 7.35 and 7.45, pCO2 between 35 and 45 mmHg, and pO2 between 100 and 150 mmHg.

Catheters were positioned in the superior caval vein for continuous administration of sodium pentobarbital (10-15 mg/kg per hour) and Haemaccel® for replacing blood withdrawn during sampling. In the descending aorta, a fluid-filled catheter was placed for monitoring aortic blood pressure and for withdrawal of blood samples. Through the left carotid artery, a micromanometer-tipped catheter (B. Braun Medical BV, Uden, The Netherlands) was inserted into the left ventricle for measurement of left ventricular pressure and, by electrical differentiation, the maximum of its first derivative (LV dP/dt_{max}). After administration of pancuronium bromide (4 mg, Pavulon®), a midsternal thoracotomy was performed, and the heart was

suspended in a pericardial cradle. An electromagnetic flow-probe (Skalar, Delft, The Netherlands) was then placed around the ascending aorta for measurement of aortic blood flow (cardiac output). A proximal segment of the left anterior descending coronary artery (LAD) was dissected free for placement of a Doppler flow-probe. Distal to this site a small cannula (1.3 mm outer diameter) was inserted into the LAD for the administration of tyramine.

The microdialysis catheters were implanted in the tissue with the help of a steel guiding needle and split plastic tubing. Three microdialysis probes were inserted in the left ventricular myocardium: one in the region of the left circumflex coronary artery (LCx) and two in the area perfused by the LAD. In order to achieve local U1-inhibition, one of the LAD probes was co-perfused with DMI (100 μM).[16] Additionally, microdialysis probes were placed in the right carotid artery and the anterior interventricular coronary vein that drains the territory perfused by the LAD.

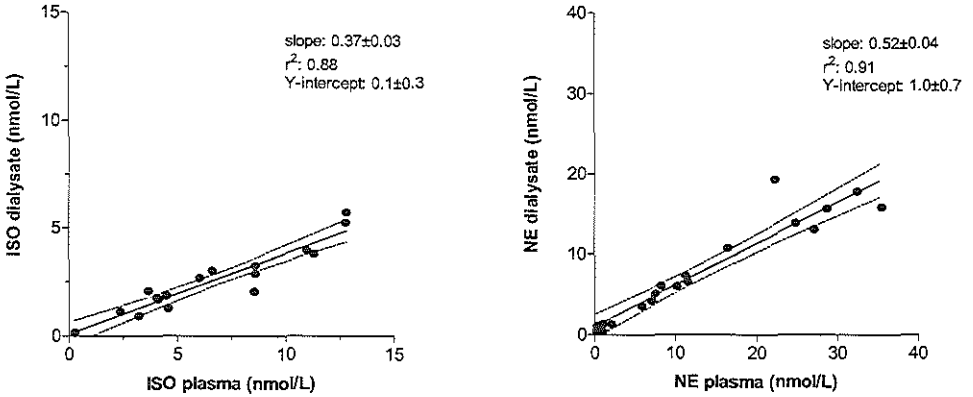


Figure 1. Determination of *in vivo* recovery for isoproterenol (ISO) and norepinephrine (NE): arterial microdialysis vs. arterial sampling. 95 % confidence limits are indicated by the dotted lines. 7 animals, 17 data points

Dialysis methodology

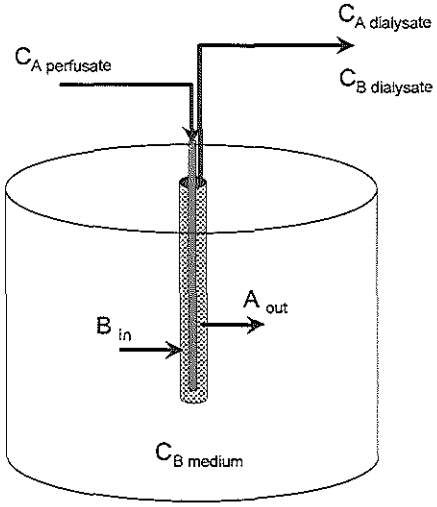
For microdialysis CMA/20 probes (Carnegie Medicine AB, Sweden) were used. The polycarbonate dialysis membrane of these probes has a cut-off value of 20 kD, a length of 10 and a diameter of 0.5 mm. The probes were perfused with an isotonic Ringer's solution at a rate of 2 μL/min using a CMA/100 microinjection pump. Dialysate volumes of 20 μL (sampling time 10 minutes) were collected in microvials containing 20 μL of a solution of 2 % (w/v) EDTA and 150 nM epinine as internal standard in 0.08 N acetic acid. Sampling was started immediately after inserting the catheters. The plasma samples were drawn into chilled heparinized tubes containing 12 mg glutathione, and, like

the microdialysis samples, stored at –80 °C, and analyzed within the next five days.[17]

Determination of in vivo probe recovery

Probe recovery is defined as the quotient of dialysate catecholamine concentration and the actual catecholamine concentration in the medium that is dialyzed. In vivo probe recovery was determined separately for blood as well as myocardial intercellular fluid. Probe recovery for blood was estimated by comparing catecholamine concentrations in plasma to catecholamine concentrations in the corresponding dialysate of the microdialysis probe in the carotid artery (Figure 1).

Recovery of norepinephrine for the probes in the myocardium was estimated by applying the retrodialysis method. This method relies on the assumptions that diffusion of a substance into the microdialysis probe equals outward diffusion and that the diffusion characteristics of the calibrator (substance A, Figure 2) match those of the analyte (substance B, Figure 2).[18,19] Furthermore, this method will only provide an estimation of the concentration of the analyte at the outer membrane of the microdialysis probe rather than the mean interstitial concentration. Ideally, while the calibrator should not have any pharmacodynamic effects, both substances should have the same pharmacokinetic properties. Consequently, the concentration of the calibrator in the perfusate should be as low as possible to prevent any significant influence on the metabolism of the analyte, e.g. saturation of active uptake mechanisms like U1.



$$Fx\,A_{out} = \frac{C_{A\,perfusate} - C_{A\,dialysate}}{C_{A\,perfusate}}$$

$$Fx\,B_{in} = \frac{C_{B\,dialysate}}{C_{B\,medium}}$$

$$\text{Retrodialysis}: Fx\,A_{out} = Fx\,B_{in}$$

$$\Rightarrow \text{Recovery}\,B = Fx\,A_{out}$$

Figure 2. The retrodialysis method. C_A: concentration of calibrator A; C_B: concentration of analyte B; Fx A_{out}: Fraction of calibrator A that has diffused out of the perfusate; Fx B_{in}: Fraction of analyte B that has diffused out of the medium in to the dialysate, i.e. the recovery of analyte B

In this study *l*-erythro-α-methyl-norepinephrine (AMN) was chosen as the calibrator for probe recovery for norepinephrine. This AMN isomer is considered to be a "false transmitter"; it is handled like norepinephrine, while it does not share its pharmacodynamic effects. Retrodialysis was performed in four animals for the length of the whole experiment. The average percentage of loss of AMN, i.e. in vivo probe recovery for norepinephrine in MIF, was based on the data derived from the LAD and LCx probes of all four animals. This method revealed a probe recovery of 52±1% (2 probes in 4 animals). Comparison of the concentration of norepinephrine in arterial plasma with the norepinephrine concentration in the dialysate obtained from the probe positioned in the carotid artery showed a similar value for probe recovery of norepinephrine, (52±4%, Figure 1). For isoproterenol, using a similar approach, the *in vivo* probe recovery was 37±3% (Figure 1).

Protocol

After 120-150 minutes, steady state conditions were reached at baseline. Thereafter, norepinephrine and isoproterenol were infused consecutively intravenously for 30 minutes for each dose, followed by a 30-min intracoronary infusion of tyramine. After each infusion, a 30-min stabilization period was introduced, allowing for a complete washout of the infused substances and return to baseline conditions. The infusion rates of isoproterenol (0.16 & 0.48 nmol/kg/min) and tyramine (154 nmol/kg/min) were chosen to correspond with the hemodynamic response of the norepinephrine infusions (0.6 & 1.8 nmol/kg/min).[20] At the end of the experiments, the pigs were killed with an overdose of pentobarbital.

Analytical procedure

Plasma catecholamines were determined by HPLC with fluorimetric detection after liquid-liquid extraction and derivatization with the fluorogenic agent 1,2-diphenyl-ethylenediamine (DPE).[21] For microdialysis samples, the catecholamines are not extracted prior to fluorimetric detection with HPLC, but directly derivatized according to the procedure described by Alberts et al.[22] This method suppresses the interference of *in vivo* factors on derivatization, thus improving sensitivity.

Reagents and pharmaceuticals

Ketalin® and Narcovet® were obtained from Apharmo BV (Arnhem, The Netherlands), Pavulon® from Organon Teknica BV (Boxtel, The Netherlands), Ringer's solution from Baxter (Uden, The Netherlands), Haemaccel® from Behringwerke A.G. (Marburg, Germany), and epinine from Zambon (Milan,

Italy). Tyramine, norepinephrine and isoproterenol for infusions were obtained from the department of pharmacy of the University Hospital Rotterdam. DMI, bicine, norepinephrine, and AMN were purchased from Sigma (St. Louis, MO, USA), EDTA, N-ethylmaleimide and hydrochloric acid from Merck (Darmstadt, Germany), potassium ferricyanide from Aldrich (Bornem, Belgium), L-glutathione from Fluka (Buchs, Switzerland), and acetic acid and acetonitrile from Baker (Deventer, The Netherlands). DPE was prepared as reported previously.[21]

Statistics and calculations

Results are expressed as mean±SEM. Norepinephrine and isoproterenol concentrations obtained with microdialysis were corrected for probe recovery to yield NE_{MIF} and ISO_{MIF}. Baseline values were determined by averaging the data of the steady state prior to the infusions. During the infusions of norepinephrine and isoproterenol, the cardiac extraction (E(%)) was calculated as

$$E(\%) = \frac{\Delta A - \Delta V}{\Delta A} \cdot 100\% \qquad (I)$$

where ΔA and ΔV are the change from baseline of respectively arterial and coronary venous concentrations. The ratio of interstitial norepinephrine to arterial plasma norepinephrine is presented as

$$\Delta MIF/\Delta A \text{ ratio} = \frac{\Delta MIF}{\Delta A} \qquad (II)$$

where ΔMIF is the change from baseline of the myocardial interstitial fluid concentration. The percentage of norepinephrine that can be recovered from the MIF and that is taken up by U1 (FxU1%) can be calculated from the difference between the $\Delta MIF/\Delta A$ ratio with and without U1-inhibition

$$FxU1\% = \frac{\Delta MIF/\Delta A_{DMI} - \Delta MIF/\Delta A}{\Delta MIF/\Delta A_{DMI}} \cdot 100\% \qquad (III)$$

in which $\Delta MIF/\Delta A_{DMI}$ is the $\Delta MIF/\Delta A$ ratio with U1-inhibition. The percentage of NE_{MIF} that is originating from the circulation (MIF NE_A%) at baseline can be estimated as follows

$$\text{MIF } NE_A\% = \frac{\Delta MIF/\Delta A \text{ ratio} \cdot NE_A}{NE_{MIF}} \cdot 100\% \qquad \text{(IV)}$$

where NE_A and NE_{MIF} are the baseline norepinephrine concentration in arterial plasma and MIF.

Although no radiolabeled material was used in the present study, the method introduced by Kopin and colleagues[11] can still be applied to estimate neuronal release by replacing specific activity of radiolabeled norepinephrine with the ratio of change to baseline of the norepinephrine concentration in plasma (R_A, R_V) and MIF (R_{MIF}) during systemic infusion of norepinephrine. The use of this ratio is based on the assumptions that endogenous norepinephrine is reflected by the baseline norepinephrine concentration, and that the change in its release is negligible compared to the change in norepinephrine concentrations induced by infused norepinephrine. According to these revised equations, spillover (SO) can be calculated as

$$SO = Q \cdot NE_A \cdot (1 - E) \cdot \left[\left(\frac{R_A}{R_V} \right) - 1 \right] \qquad \text{(V)}$$

where Q is the coronary plasma flow (CPF) that is calculated from the coronary blood flow and the hematocrit which was estimated using the plasma hemoglobin concentration, NE_A is the arterial plasma norepinephrine concentration at baseline, E is the extraction fraction, and R_A and R_V are the ratios of change to baseline of the norepinephrine concentrations in arterial plasma and coronary effluent. The uptake of released norepinephrine (Ur) from the interstitium can be estimated using the ratio of change for MIF (R_{MIF}) to baseline NE_{MIF} in an analogous equation

$$Ur = Q \cdot NE_A \cdot E \cdot \left[\left(\frac{R_A}{R_{MIF}} \right) - 1 \right] \qquad \text{(VI)}$$

As mentioned above, the sum of equations V and VI provides estimation of the neuronal release rate (Rr)

$$Rr = SO + Ur \qquad \text{(VII)}$$

Subsequently, the ratio of uptake of released norepinephrine and neuronal release rate can be used as a measure for the efficiency of total uptake ($Eff_U\%$)

$$Eff_U\% = \frac{Ur}{Rr} \cdot 100\% \qquad \text{(VIII)}$$

For statistical analysis two-way analysis of variance, one-way analysis of variance for repeated measures with Dunnet's multiple comparison test as post-hoc test, Student's *t* test and linear regression analysis were used as appropriate.

Results

Norepinephrine infusions

Steady state concentrations of NE_{MIF} were observed 120-150 minutes after placement of the probes and start of microdialysis. Baseline arterial plasma norepinephrine concentration was about three times lower than NE_{MIF} ($P<0.001$, Figure 3). NE_{MIF} concentrations in the LAD and LCx region were similar and values did not differ from those in the coronary vein. During U1-blockade, NE_{MIF} increased over six-fold ($P<0.01$, Figure 3).

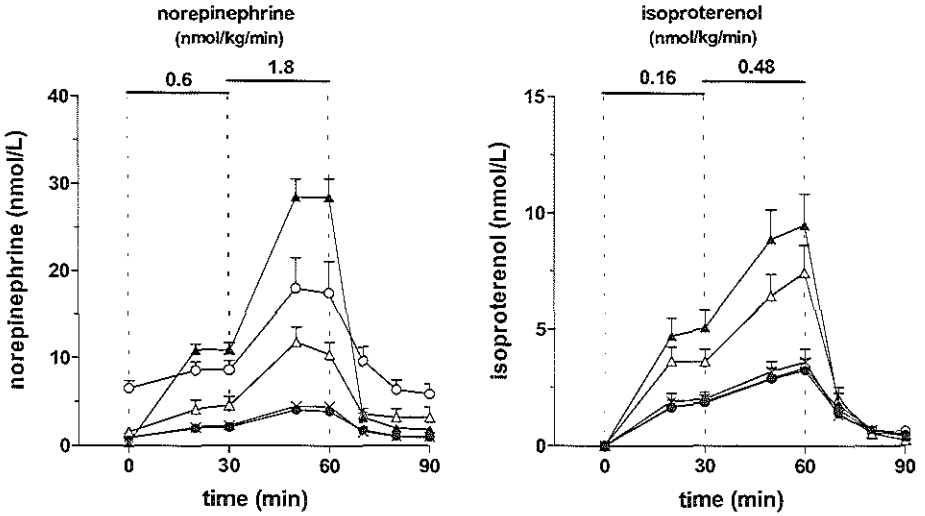


Figure 3. Norepinephrine (left) and isoproterenol concentrations (right) during successive intravenous infusions of norepinephrine and isoproterenol. Data are shown for MIF in LAD region in absence (●) and presence of U1-blockade (○), MIF in LCx region in U1 blockade (x), and concentrations in arterial plasma (▲) and in the coronary vein (ρ). Data presented as mean ± SEM, 8 animals.

Circulatory and MIF norepinephrine concentrations at 20 and 30 minutes after the start of both systemic norepinephrine infusions did not

differ, suggesting steady state was reached within 20 minutes (Figure 3). The extraction of arterially delivered norepinephrine was 79±4 % and 69±3 % for the low and high infusion rates respectively. Without U1-blockade, NE_{MIF} (LAD and LCx region) remained considerably lower than arterial and coronary venous norepinephrine concentration. During U1-blockade however, NE_{MIF} was between arterial plasma and coronary venous concentration (Table 1, Figure 3). Without U1-blockade, the $\Delta MIF/\Delta A$ ratio (*eq. II*) was 0.10±0.01 for the lower and 0.11±0.01 for the higher norepinephrine infusion rate, whereas DMI caused the $\Delta MIF/\Delta A$ ratio to rise to respectively 0.21±0.02 and 0.36±0.05 ($P<0.05$).

Table 1. Comparison of the increments of circulatory and interstitial concentrations of norepinephrine and isoproterenol during systemic intravenous infusions of norepinephrine or isoproterenol.

Location		Norepinephrine infusion nmol/kg/min		Isoproterenol infusion nmol/kg/min	
		0.6	1.8	0.16	0.48
Carotid artery, nmol/L		10.6 ± 0.8	28.2 ± 2.1	4.9 ± 0.8	9.2 ± 2.1
Coronary vein, nmol/L		2.7 ± 0.8	9.6 ± 1.6	3.6 ± 0.6	6.9 ± 1.1
E (%)		79 ± 4	69 ± 3	24 ± 5	23 ± 4
MIF LAD, nmol/L		1.1 ± 0.2	3.1 ± 0.3	1.8 ± 0.3	3.1 ± 0.5
MIF LAD + DMI, nmol/L		2.1 ± 0.5	11.2 ± 2.9†	1.8 ± 0.2	3.1 ± 0.4
MIF LCx, nmol/L		1.3 ± 0.2	3.6 ± 0.3	1.9 ± 0.3	3.4 ± 0.5
$\Delta MIF/\Delta A$ ratio	- DMI	0.10 ± 0.01	0.11 ± 0.01	0.37 ± 0.02	0.34 ± 0.02
	+ DMI	0.21 ± 0.03*	0.36 ± 0.05†	0.37 ± 0.03	0.35 ± 0.02

Values are mean±SEM, n=8. * $P<0.05$, † $P<0.01$ DMI (-) vs. DMI (+)

Spillover (SO), rate of uptake (Ur), rate of neuronal release (Rr) and the efficiency of uptake (Eff_U%) at baseline were calculated from the data of both norepinephrine infusions using *eqs. V-VIII*. Despite the large increment in circulatory norepinephrine and NE_{MIF} concentrations represented by the ratios of ΔNE values to baseline between the lower and the higher norepinephrine dose, the kinetic parameters for norepinephrine remained unchanged (Table 2).

Systemic infusions of norepinephrine caused a marked dose-dependent increase in heart rate, blood pressure, coronary blood flow and LV dP/dt_{max} (Table 3). The relationship between changes in LV dP/dt_{max} and NE_{MIF} was much steeper than the relationship between changes in LV dP/dt_{max} and arterial norepinephrine concentration ($P<0.001$, Figure 4).

Table 2. Spillover, uptake and release of norepinephrine at baseline derived from the data during systemic intravenous infusions of norepinephrine.

	Norepinephrine (nmol/kg/min)	
	0.6	1.8
R_A	59.1 ± 8.2	155.3 ± 22
R_V	1.8 ± 0.3	6.9 ± 0.6
R_{MIF}	1.3 ± 0.2	3.8 ± 0.5
SO, pmol/min	35.1 ± 5.6	38.8 ± 5.4
Ur, pmol/min	194.1 ± 33.4	204.0 ± 50.8
Rr, pmol/min	229.3 ± 36.8	242.5 ± 53.4
EffU%	84 ± 2	79 ± 2

Values are mean±SEM, n=8

Isoproterenol infusions

Systemic isoproterenol infusions caused dose-dependent increments in arterial and venous isoproterenol concentrations (Figure 3, Table 1). Circulatory and MIF isoproterenol concentrations at 20 and 30 minutes after the start of both systemic isoproterenol infusions did not differ significantly, suggesting steady state was reached within 20 minutes (Figure 3). The extraction of arterially delivered isoproterenol in the coronary circulation was 24±5%, with both the low and the high infusion rate. ISO_{MIF} concentrations were lower than arterial and coronary venous concentrations and values did not alter in the presence of U1-blockade. The ΔMIF/ΔA ratio for isoproterenol was 0.37±0.02 with the low and 0.34±0.02 with the high infusion rate.

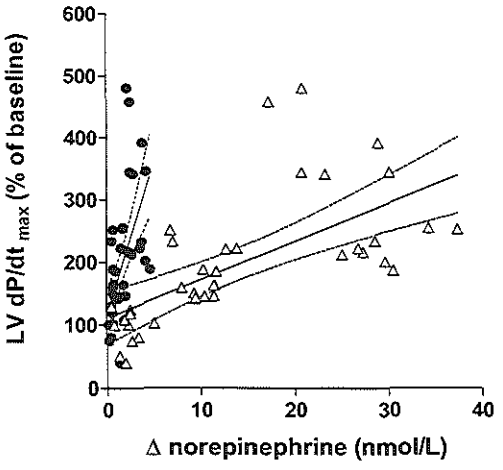


Figure 4. Regression analysis of the responses of LV dP/dt_{max} and norepinephrine concentrations in arterial blood and MIF to infusion of norepinephrine. LV dP/dt_{max} data in graph presented as percentage of baseline. Carotid artery (ρ), slope: 6.1±1.2, r^2: 0.42. MIF LAD region (π), slope: 48.5±10.0, r^2: 0.38. 95 % confidence limits are indicated by the dotted lines. 8 animals

Isoproterenol infusions caused dose-dependent increments in heart rate, systolic blood pressure, coronary blood flow and LV dP/dt_{max} and a dose-dependent decrease in diastolic blood pressure (Table 3). The slope of the regression line of the relationship between LV dP/dt_{max} (%change) and isoproterenol concentration was 46±21 for ISO_{MIF} and 13±8 for arterial isoproterenol concentrations.

Table 3. Comparison of cardiac and systemic hemodynamics during systemic intravenous infusions of norepinephrine and isoproterenol

	Norepinephrine infusion nmol/kg/min			Isoproterenol infusion nmol/kg/min		
	Baseline	0.6	1.8	Baseline	0.16	0.48
Heart rate, bpm	120 ± 8	127 ± 7	141 ± 5 *	125 ± 6	182 ± 4 *	197 ± 7 *
Systolic arterial pressure, mmHg	103 ± 4	121 ± 4 *	137 ± 5 *	97 ± 5	106 ± 5 *	112 ± 5 *
Diastolic arterial pressure, mmHg	72 ± 5	85 ± 4 *	93 ± 5 *	66 ± 5	58 ± 3 *	54 ± 4 *
LV dP/dt_{max}, mmHg/s	1734 ± 175	2991 ± 202 *	4832 ± 377 *	1621 ± 325	3810 ± 355 *	5327 ± 550 *
LAD flow, mL/min	21 ± 3	25 ± 3 *	28 ± 3 *	20 ± 3	29 ± 3 *	29 ± 4 *
Cardiac output, L/min	2.3 ± 0.1	2.6 ± 0.2	2.9 ± 0.3 *	2.2 ± 0.2	2.8 ± 0.2	3.3 ± 0.3 *

Values are mean±SEM. * $P < 0.05$ vs. Baseline

Tyramine infusion:

Tyramine, like norepinephrine, is taken up by neurons through U1 and it subsequently displaces norepinephrine from the nerve terminals because of its higher affinity for the neuronal storage proteins than norepinephrine.[15] Consequently, the degree of attenuation of tyramine-induced norepinephrine release is a measure of the degree of U1-blockade. Without U1-blockade, infusion of tyramine in the LAD caused a 15-fold rise in NE_{MIF} in the LAD-region, accompanied by a similar increase of the norepinephrine concentration in the coronary vein (Figure 5). Under U1-blockade, this response was almost completely abolished; indicating that with the dose of DMI used the blockade of U1 was virtually complete.

Tyramine infusion in the LAD was also associated with a 5-fold increase of NE_{MIF} in the LCx region. Because the systemic arterial norepinephrine concentration also slightly increased, this increase was most likely caused by overflow of tyramine from the coronary into the systemic circulation. The hemodynamic response to tyramine was mainly confined to the heart, LV dP/dt_{max} increased almost three fold to 3933±465 mmHg/s,

comparable to the increase as seen during the infusions of norepinephrine and isoproterenol.

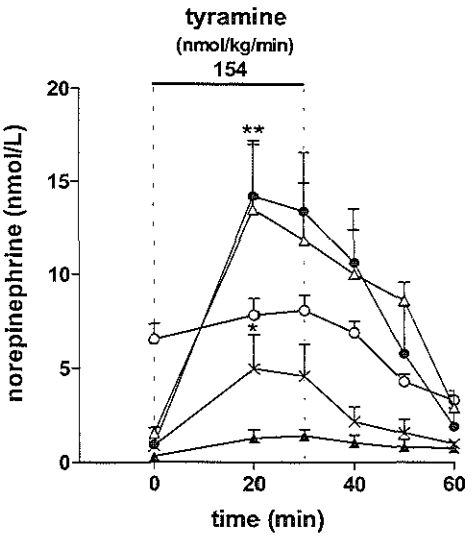


Figure 5. Norepinephrine response to an intracoronary infusion of tyramine. Data are shown for MIF in LAD region in absence (●) and presence of U1-blockade (○), MIF in LCx region in U1 blockade (x), and concentrations in arterial plasma (▲) and in the coronary vein (ρ). * $P<0.05$, ** $P<0.01$, compared to baseline. Data presented as mean ± SEM, 4 animals.

Discussion

We investigated to what extent the concentration of norepinephrine in the myocardial intercellular space relates to its concentration in the arterial and coronary venous circulation at baseline and after increments in plasma or interstitial norepinephrine concentration induced either by systemic infusions of norepinephrine or by an intracoronary infusion of tyramine. In addition, the importance of the U1-mechanism for the NE_{MIF} concentration was assessed by perfusing one of the probes with the U1-inhibitor desipramine and by performing studies with isoproterenol, a catecholamine that is not handled by U1. Finally, adaptation of the method introduced by Kopin et al.[11] provided an estimate of spillover, uptake and consequently, release of norepinephrine.

In agreement with the results of other studies using the microdialysis technique, steady state NE_{MIF} concentrations were observed 120-150 minutes after insertion of the probes and start of microdialysis. Basal NE_{MIF} concentrations measured in this study were similar to those as reported by Akiyama et al. who performed microdialysis in feline hearts.[23,24] At baseline, NE_{MIF} concentrations in the LCx and LAD region were similar, suggesting no important regional differences in myocardial sympathetic activity. In contrast to various other microdialysis studies that reported arterial plasma levels at baseline to be similar to or even higher than interstitial norepinephrine levels,[23,25,26] NE_{MIF} concentrations in the present study were three times

higher than arterial plasma concentrations. These results are in keeping with estimates made in other studies that report that the concentration of norepinephrine at sites of release is about 3 to 5-fold higher than in plasma.[27-29] This concentration gradient is the driving force behind the exchange of norepinephrine from the interstitial compartment to the circulation. Accordingly, one would expect this gradient to be reflected in somewhat higher norepinephrine concentrations in MIF than in the coronary vein. In contrast, norepinephrine concentrations in the coronary vein were similar to those in MIF, both under baseline conditions and during intracoronary infusion of tyramine, which induced a 15-fold increase in the NE_{MIF} concentration. A possible explanation for this unexpected finding is that the norepinephrine concentration that is measured around the membrane of the microdialysis probe to some extent underestimated the concentration of norepinephrine at sites of release.

The observed absence of a norepinephrine gradient between the MIF and coronary vein does not support previous suggestions of the existence of an endothelial barrier for the diffusion of norepinephrine from the interstitial to the intra-vascular compartment.[3,6-9] The presence of such an endothelial barrier could provide an explanation for the well-known difference in the relation of blood pressure response and plasma norepinephrine concentration observed for exogenously administered norepinephrine or for tyramine-induced endogenously released norepinephrine.[30,31] If an endothelial barrier for the diffusion of norepinephrine is present, NE_{MIF} and the norepinephrine concentration in the coronary vein, as a reflection of the norepinephrine concentration in the myocardial capillaries, should be different. However, both at baseline and during infusion of tyramine through the LAD, NE_{MIF} concentrations in the LAD region and coronary vein were similar, indicating an unhindered exchange of endogenous norepinephrine from the interstitial to the vascular compartments. The considerable gradient between norepinephrine concentrations in the coronary vein and MIF as seen during systemic infusion of norepinephrine was absent under U1-blockade and therefore is attributable to U1. Thus, no endothelial barrier to the diffusion of norepinephrine appears to be present in the porcine heart.

Experimental studies and studies in man using labeled infusions of norepinephrine have shown that 60 to 80% of arterially delivered norepinephrine is removed by the myocardium.[4,32,33] Using unlabeled norepinephrine in the present study the extraction of norepinephrine ranged from 79 to 69% depending on the infused dose (Table 1). Notwithstanding this high fractional removal, NE_{MIF} remained extremely low as reflected by $\Delta MIF/\Delta A$ ratio of about 0.10. This value is close to the MIF/A ratio of 0.15 reported for the canine myocardium by Cousineau et al.[2] using the capillary-

interstitium concentration model developed by Ziegler and Goreski.[34] The important role of U1 in the removal of norepinephrine from the MIF was confirmed by comparing the NE_{MIF} concentrations in the microdialysis probes in the LAD region with and without the U1-inhibitor desipramine. Although the two probes were placed no more than 1 cm apart, no inter-probe interference was observed. Basal NE_{MIF} increased over six-fold during U1-inhibition, while the increase of NE_{MIF} due to infusion of norepinephrine was also markedly augmented. Local U1-inhibition in the LAD probe with desipramine increased the $\Delta MIF/\Delta A$ ratio to 0.21 - 0.36. Especially under U1-blockade, the $\Delta MIF/\Delta A$ ratio for unlabeled norepinephrine is likely to be affected by norepinephrine that is released by or has leaked from the neurons. Considering this artifact, our results with DMI are quite comparable to the MIF/A ratio under U1-blockade as estimated by Cousineau.[2] From the differences in $\Delta MIF/\Delta A$ ratio measured in the probes with and without U1-inhibition it can be calculated that 67±5 % of NE_{MIF} is removed by U1 (*eq. III*). This value is in close agreement with values reported for the rabbit [29] but lower than those observed in the human myocardium.[4,35]

As expected, since isoproterenol is not taken up by U1, similar ISO_{MIF} concentrations were measured in the probes with and without the U1-inhibitor DMI. The $\Delta MIF/\Delta A$ ratios for isoproterenol were very similar to the $\Delta MIF/\Delta A$ ratio for norepinephrine during the high infusion rate of norepinephrine and local inhibition of U1 by DMI. As isoproterenol is not taken up by U1, the difference in removal of norepinephrine and isoproterenol over a certain vascular bed has been proposed to be a useful measure for U1 activity.[4,36] Although there is some debate about the validity of the comparison of the pharmacokinetics of isoproterenol and norepinephrine during U1-blockade,[2,37] the present findings suggest such an approach will indeed provide a reliable estimation of U1 activity. Despite similar extractions of norepinephrine, the extraction of isoproterenol in the porcine heart (24%) was considerably higher than that reported for the human heart (14%). This difference in extraction suggests that the cardiac extra-neuronal uptake of norepinephrine is more important in the porcine than in the human heart. As proposed by Goldstein et al.[4], the proportionate fractional tissue removal of norepinephrine by U1 can be calculated by subtracting the percent removal of isoproterenol from the percent removal of norepinephrine and dividing this by the percent removal of norepinephrine. Applying this equation in the present study, it appears that about 66% of norepinephrine in the porcine myocardium is removed by U1. Although considerably lower than the value reported for the human heart (82%), this value agrees well with the proportionate fractional tissue removal of norepinephrine by U1 derived from the differences in the $\Delta MIF/\Delta A$ ratios with and without local U1 inhibition.

Because of the active U1 of norepinephrine in the myocardium, NE_{MIF} during systemic norepinephrine infusions remained relatively low as compared to the arterial norepinephrine concentration. This explains why the relationship between LV dP/dt_{max} and changes in NE_{MIF} was much steeper than the relationship between LV dP/dt_{max} and the changes in arterial norepinephrine concentration. Accordingly, since isoproterenol is not taken up by U1, the difference between relationships between LV dP/dt_{max} and interstitial or arterial isoproterenol concentrations during isoproterenol infusions was less distinct.

Studies performed in humans and dogs have shown that less than 5% of norepinephrine that is released in to the myocardial interstitium spills over to the circulation.[11] In the present study, the calculated proportional spillover (about 15%) was considerably higher. As cardiac spillover in our experiments is similar to the value measured in dogs, [38] it seems likely that proportional spillover was relatively high because the calculated uptake of norepinephrine was relatively low. As shown in *eq. VI*, the calculated uptake of norepinephrine strongly depends on the ratio of R_A and R_{MIF}. Although microdialysis is a more direct technique to measure interstitial norepinephrine concentrations than the estimates based on the isotope dilution technique, it will only provide information about the mean interstitial norepinephrine concentration and not about the concentration at sites of release. Because of the downward concentration gradient of norepinephrine from the site of entry in the interstitial fluid to the sites of uptake, the R_{MIF} based on the mean interstitial norepinephrine concentration during systemic infusion of norepinephrine will be higher than R_{MIF} at the sites where uptake of norepinephrine takes place. Considering *eq. VI*, overestimation of R_{MIF} will lead to the underestimation of the calculated uptake of norepinephrine.

In conclusion, microdialysis is a valuable tool for measuring as well as modifying local sympathetic activity. The present *in vivo* experiments largely confirm the information about norepinephrine kinetics obtained by more indirect methods. We conclude that U1 as well as extra-neuronal uptake and not an endothelial barrier are the principal mechanisms underlying the concentration gradient of norepinephrine between the interstitial and intra-vascular compartments in the porcine heart.

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Chapter 5

Epinephrine in the heart

Introduction - Several studies have suggested that epinephrine augments the release of norepinephrine from sympathetic nerve terminals through stimulation of presynaptic receptors but evidence pertaining to this mechanism in the heart is scarce and conflicting. Using the microdialysis technique in the porcine heart, we investigated to which extent epinephrine is taken up by and released from cardiac sympathetic nerves and whether it can increase norepinephrine concentrations in myocardial interstitial fluid (NE_{MIF}) under basal conditions and during sympathetic activation.
Methods and Results - Before intracoronary epinephrine infusions, intracoronary infusion of tyramine increased NE_{MIF} by 12.8±2.9 nmol/L, while the increase in epinephrine concentration in MIF (EPI_{MIF}) was negligible. After infusion of epinephrine, however, tyramine induced an increase in EPI_{MIF} of 9.5 nmol/L. While 68-78% of epinephrine was extracted over the heart during intracoronary epinephrine infusion of 10, 50 and 100 $ng \cdot kg^{-1} \cdot min^{-1}$, the ratio of interstitial to arterial epinephrine concentrations was ~20%. This ratio increased to 29% when neuronal reuptake was inhibited. Despite large increments in interstitial and coronary venous epinephrine concentrations, NE_{MIF} did not change during the infusion of epinephrine. Finally, left stellate ganglion stimulation increased NE_{MIF} from 3.4±0.5 to 8.2±1.5 nmol/L. Again, this increase was not enhanced by intracoronary epinephrine infusion.
Conclusion - The present findings demonstrate that epinephrine is taken up by and released from cardiac sympathetic neurons. However, we did not find any evidence of a stimulatory effect of epinephrine on norepinephrine release from sympathetic nerves in the heart either under basal conditions or during sympathetic activation.

Lameris TW, de Zeeuw S, Duncker DJ, Tietge W, Alberts G, Boomsma F, Verdouw PD, van den Meiracker AH. Epinephrine in the heart; uptake and release but no facilitation of norepinephrine release. *Submitted.*

Introduction

Several studies have shown that cardiac epinephrine is released into the coronary circulation of the human heart during exercise, at rest with advancing age and in conditions like hypertension, heart failure and panic disorders.[1-3] In accordance with these observations we recently have shown that occlusion of the left anterior descending coronary artery (LAD) for 60 minutes in the intact porcine heart is associated with a progressive increase of epinephrine concentrations (EPI) in the myocardial interstitial fluid (EPI_{MIF}).[4] Whether cardiac epinephrine is released from sympathetic nerve terminals after it has been taken up from the circulation or whether it is released from extraneuronal stores remains unclear.[5,6]

Another point of debate relates to the role of this locally released epinephrine. Several *in vitro* as well as *in vivo* studies have suggested that epinephrine enhances sympathoneural norepinephrine release through stimulation of presynaptic β_2-adrenergic receptors located at the sympathetic nerve terminals.[7-10] This presynaptic facilitation of norepinephrine release by epinephrine is essential to the "epinephrine hypothesis", which proposes that excessive adrenomedullary activation leads to the development of hypertension through increasing sympathoneural norepinephrine release.[10] Other studies, however, have failed to confirm this mechanism.[11-14]

In the present study we have used the microdialysis technique to measure interstitial epinephrine and norepinephrine concentrations in the intact porcine heart. To unravel the source of cardiac epinephrine, we investigated the effect of intracoronary tyramine infusions on interstitial epinephrine concentrations before and after loading the heart with epinephrine by means of intracoronary epinephrine infusions. As tyramine only displaces catecholamines from their storage vesicles in the sympathetic nerve terminals after it has been taken up by the neuronal reuptake (U1) mechanism,[15,16] catecholamines released by tyramine are exclusively from neuronal origin. In addition, we investigated to which extent epinephrine is taken up by and released from cardiac sympathetic nerves similarly to what we have shown for norepinephrine in a previous study.[15]

Finally, as adrenomedullary activation in conditions like hypertension and heart failure would contribute to the deterioration of cardiac function through increasing sympathoneural norepinephrine release, and evidence pertaining to this mechanism in the heart is scarce and conflicting,[10,12] we explored whether intracoronary epinephrine infusions can increase myocardial interstitial norepinephrine (NE_{MIF}) concentrations under basal conditions and during sympathetic activation induced by electrical stimulation of the left stellate ganglion. As over 80% of neuronally released norepinephrine is taken

up by sympathetic nerves of the porcine heart through the U1-mechanism,[15] the U1-inhibitor desipramine was added to the perfusate of one the microdialysis probes to provide local U1-blockade. In addition, we also accounted for a possible inhibition of norepinephrine release through stimulation of presynaptic α_2-adrenergic receptors by adding the non-selective α-adrenergic receptor antagonist phentolamine to the perfusate of another probe in combination with desipramine.

Methods

Animal care
All experiments were performed in accordance with the "Guiding Principles for Research Involving Animals and Human Beings" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam (The Netherlands).

Surgical procedure
Crossbred Landrace x Yorkshire pigs of either sex (30-35 kg, n=15) were sedated with ketamine (20-25 mg/kg i.m., Apharma BV), anesthetized with sodium pentobarbital (20 mg/kg i.v., Apharma BV), intubated and ventilated with a mixture of oxygen and nitrogen.

Catheters were positioned in the superior vena cava for infusion of sodium pentobarbital (10-15 mg/kg per hour) and physiological saline drip. A fluid-filled catheter was placed in the descending aorta for monitoring blood pressure and for blood sampling. A micromanometer-tipped catheter (B. Braun Medical BV) was inserted via the carotid artery into the left ventricle for measurement of left ventricular (LV) pressure and LV dP/dt. After a midsternal thoracotomy, an electromagnetic flow probe (Skalar) was placed around the ascending aorta and a Doppler flow probe (Triton) was placed on the proximal LAD segment, while a cannula (∅: 1.3 mm) was inserted into the LAD distal to the flow probe for infusion of epinephrine and tyramine (Dept of Pharmacy). In animals subjected to sympathetic stimulation, the left stellate ganglion was dissected and an electrode was inserted into the ganglion as described previously by Gootman et al.[17] and connected to a nerve stimulator (Grass S9; pulses of 12 V, 10 Hz and 5 msec).

Microdialysis probes were implanted in LV myocardium: one in the region perfused by the left circumflex coronary artery (LCx) and three in the area perfused by the LAD. To achieve local U_1-inhibition, one of the LAD probes was co-perfused with 100 μM desipramine (DMI, Sigma),[16] while one

LAD probe was co-perfused with DMI and the non-selective α-adrenergic receptor blocking agent 100 μM phentolamine (PHA, Dept of Pharmacy) to block presynaptic α-adrenergic receptor-mediated inhibition of norepinephrine release. The microdialysis technique, probe characteristics, probe recovery, handling and analysis of the microdialysis and plasma samples, have been described previously.[15,18]

Experimental protocol
After a 120-min stabilization period, baseline measurements were obtained over a 30-min period. Probes were perfused with Ringer's solution (Baxter) at a flow of 2 μL/min; dialysate was collected at 10-min intervals, in which period blood was collected from the central aorta (Ao) and coronary vein (CV). In 9 animals, tyramine was infused (26.7 $\mu g \cdot kg^{-1} \cdot min^{-1}$) into the LAD before and 30 minutes after intracoronary epinephrine at infusion rates of 10, 50 and 100 $ng \cdot kg^{-1} \cdot min^{-1}$, each for 20 min (group I). Finally, in 6 animals the left stellate ganglion was stimulated electrically before and during concomitant infusion of 50- $ng \cdot kg^{-1} \cdot min^{-1}$ epinephrine (group II).

Data analysis and calculations
Dialysate epinephrine and norepinephrine concentrations were corrected for probe recovery to yield EPI_{MIF} and NE_{MIF}.[4,15] Lower limits of detection were 0.2 nmol/L in dialysate and 0.02 nmol/L plasma concentrations.[18] Baseline values were determined by averaging the three measurements over the 30-min period prior to intervention. Epinephrine plasma concentrations in the LAD (EPI_{CA}) were calculated from epinephrine infusion rate, coronary plasma flow and EPI_{Ao}. The cardiac extraction of epinephrine (E) was calculated as

$$E\,(\%) = \frac{\Delta CA - \Delta CV}{\Delta CA} \cdot 100\% \qquad (I)$$

where ΔCA and ΔCV are the change from baseline of EPI_{CA} and EPI_{CV} during the intracoronary epinephrine infusions. The ratio of the absolute changes of interstitial to the absolute changes of arterial epinephrine concentrations is presented as

$$\Delta MIF / \Delta CA \qquad (II)$$

where ΔMIF is the change from baseline of the myocardial interstitial fluid concentration.

The percentage of epinephrine that can be recovered from the MIF and that is taken up by U1 (FxU1%) can be calculated from the difference between the ΔMIF/ΔCA ratio with and without U1-inhibition

$$FxU1\% = \frac{\Delta MIF/\Delta CA_{DMI} - \Delta MIF/\Delta CA}{\Delta MIF/\Delta CA_{DMI}} \cdot 100\% \qquad (III)$$

in which $\Delta MIF/\Delta CA_{DMI}$ is the ΔMIF/ΔCA ratio with U1-inhibition.

In analogy to Kopin and colleagues [19] who used radiolabeled material to estimate neuronal release, we replaced the specific activity of radiolabeled catecholamines with the relative changes from baseline of epinephrine concentrations in the LAD (R_{CA}), in the interventricular coronary vein that drains the LAD region (R_{CV}) and in MIF (R_{MIF}) during intracoronary epinephrine infusions, similar to what we have shown for norepinephrine.[15]. The use of these relative changes is based on the assumptions that baseline epinephrine concentrations reflect endogenous epinephrine, and that the change in epinephrine release is negligible compared to the change in epinephrine concentrations produced by infusion of epinephrine. Accordingly, spillover (SO) can be calculated as

$$SO = Q \cdot EPI_{CA} \cdot (1 - E) \cdot \left[\left(\frac{R_{CA}}{R_{CV}} \right) - 1 \right] \qquad (IV)$$

where Q is the coronary plasma flow that is calculated from LAD flow and the hematocrit which was estimated using the plasma hemoglobin concentration. The uptake of released epinephrine (Ur) from the interstitium can then be estimated using R_{MIF}.

$$Ur = Q \cdot EPI_{CA} \cdot E \cdot \left[\left(\frac{R_{CA}}{R_{MIF}} \right) - 1 \right] \qquad (V)$$

The sum of equations IV and V provides an estimation of the neuronal release rate (Rr)

$$Rr = SO + Ur \qquad (VI)$$

Subsequently, the ratio of uptake of released epinephrine and neuronal release rate can be used as a measure for the efficiency of total uptake (Eff_U%)

$$Eff_U\% = \frac{Ur}{Rr} \cdot 100\% \qquad \text{(VII)}$$

Statistical analysis

All data are expressed as mean±SEM. For statistical analysis two-way analysis of variance, one-way analysis of variance for repeated measures with Dunnet's multiple comparison test as post-hoc test, Student's *t* test and linear regression analysis were used as appropriate.

Results

Intracoronary epinephrine infusion and tyramine-induced epinephrine and norepinephrine release

Intracoronary infusion of tyramine caused increases in mean arterial pressure (MAP, 20%), heart rate (HR, 20%), LV dP/dt_{max} (190%), LAD flow (40%) and $NE_{MIF,LAD}$ (12.8±2.9 nmol/L, $P < 0.05$). These responses were not affected by a preceding intracoronary epinephrine infusion (Figure 1, Table 1). In contrast, the change in $EPI_{MIF,LAD}$ during tyramine infusion was negligible before, but increased markedly after intracoronary infusion of epinephrine (9.5±3.0 nmol/L, $P < 0.05$). This was also reflected by the tyramine-induced increase in EPI_{CV} from 0.09±0.01 nmol/L before to 8.1±2.7 nmol/L after epinephrine infusion ($P < 0.05$).

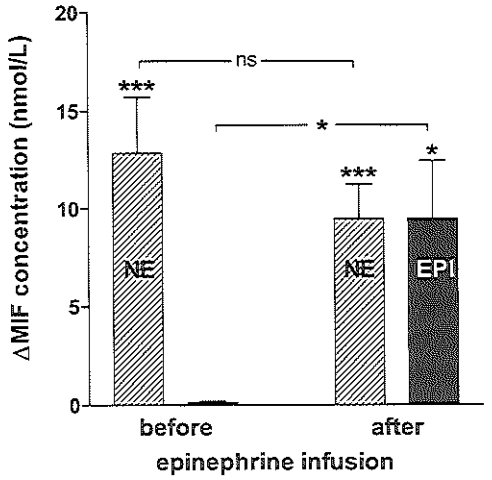

Figure 1. Effect of intracoronary tyramine infusion on the concentrations of norepinephrine (NE) and epinephrine (EPI) in MIF before and after an intracoronary infusion of epinephrine. Data are mean ± SEM, n=9. * $P<0.05$, *** $P<0.001$

Table 1. Cardiovascular function during intracoronary infusion of tyramine before and after intracoronary infusion of epinephrine

	EPI	Baseline	Tyramine
Mean arterial pressure, mmHg	before	90 ± 1	109 ± 4 *
	after	78 ± 3 †	97 ± 4 *†
Cardiac output, L/min	before	2.7 ± 0.3	3.2 ± 0.4 *
	after	2.5 ± 0.2	3.2 ± 0.1 *
Heart rate, bpm	before	128 ± 7	152 ± 9 *
	after	128 ± 8	143 ± 9 *
Systemic vascular resistance, mmHg.min $^{-1}$.L	before	36 ± 4	37 ± 4
	after	32 ± 3	30 ± 2 †
Stroke volume, mL	before	22 ± 2	21 ± 2
	after	20 ± 2	24 ± 2
LV dP/dt_{max}, mmHg/s	before	1683 ± 89	4819 ± 433 *
	after	1558 ± 213	4106 ± 333 *†
LV end diastolic pressure, mmHg	before	6 ± 1	6 ± 3
	after	7 ± 1	7 ± 2
LAD flow, mL/min	before	24 ± 2	34 ± 3 *
	after	24 ± 3	33 ± 4 *

EPI: three consecutive intracorona ry infusions of epinephrine, each for 20 minutes (10, 50 and 100 ng·kg^{-1}·min^{-1}). The intracoronary infusion of tyramine was started 30 minutes after the epinephrine infusions were discontinued. Values are mean±SEM, n=9. * $P < 0.05$ vs. Baseline; † $P < 0.05$ vs. before EPI

Effect of intracoronary epinephrine infusions on norepinephrine and epinephrine concentrations

The intracoronary epinephrine infusions caused dose-dependent increases in LV dP/dt_{max} (130%), HR (15%) and cardiac output (CO, 20%), while MAP (-15%), systemic vascular resistance (SVR, -30%) and left ventricular end diastolic pressure (-20%) decreased (Table 2). In contrast, LAD flow increased 45%, independent of the infused dose.

Intracoronary infusion of epinephrine caused dose-dependent increases in EPI_{CV} from 0.16±0.08 nmol/L at baseline up to 228±39 nmol/L during infusion of 100 ng·kg^{-1}·min^{-1}, and $EPI_{MIF,LAD}$ from 0.31±0.05 nmol/L up to 140±30 nmol/L (Figure 2). U1-inhibition did not affect $EPI_{MIF,LAD}$ at baseline and the lowest infusion rate, but caused an increase in $EPI_{MIF,LAD}$ to similar values as EPI_{CV} at the two higher infusion rates (32-38%, $P<0.001$). Although the cardiac epinephrine extraction was 68 to 78%, there was a marked gradient between interstitial and circulatory concentrations. During the intracoronary epinephrine infusions, the ΔMIF/ΔCA ratio for epinephrine (equation II, Table 2) was 27±4%, 19±3% and 21±3% in the absence of U1-blockade, but

increased to 29±3% in the presence of U1-blockade, irrespective of the epinephrine infusion rate.

Table 2. Cardiovascular Function during Intracoronary Epinephrine Infusion

	Baseline	Epinephrine ($ng \cdot kg^{-1} \cdot min^{-1}$)		
		10	50	100
Mean arterial pressure, mmHg	89 ± 4	85 ± 4	77 ± 4 *	76 ± 3 *
Cardiac output, L/min	2.6 ± 0.2	2.8 ± 0.2	2.8 ± 0.2	3.1 ± 0.3 *
Heart rate, bpm	125 ± 7	130 ± 6	137 ± 6 *	144 ± 7 *
Systemic vascular resistance, $mmHg.min^{-1}.L$	37 ± 4	32 ± 3 *	29 ± 3 *	26 ± 2 *
Stroke volume. mL	21 ± 3	22 ± 2	20 ± 3	27 ± 5
LV dP/dt_{max}, mmHg/s	1604 ± 136	2483 ± 191 *	3068 ± 222 *	3716 ± 186 *
LV End Diastolic Pressure, mmHg	7 ± 2	6 ± 2	6 ± 2*	6 ± 2 *
LAD Flow, mL/min	29 ± 4	41 ± 4 *	41 ± 4 *	43 ± 4 *

Values are mean±SEM, n=9. * $P < 0.05$ vs. Baseline

Despite the large increments in circulatory and interstitial epinephrine concentrations, the pharmacokinetic parameters for epinephrine as spillover, rate of uptake, rate of neuronal release as well as the efficiency of uptake remained unchanged (Table 3). Notwithstanding the aforementioned large increments in EPI_{CV} and $EPI_{MIF,LAD}$, norepinephrine concentrations in MIF, coronary vein and aortic plasma did not change (Figure 2).

Table 3. Spillover, Uptake and Release of Epinephrine Compared to Norepinephrine

	Epinephrine ($ng \cdot kg^{-1} \cdot min^{-1}$)			Norepinephrine ($ng \cdot kg^{-1} \cdot min^{-1}$)	
	10	50	100	110	330
ΔMIF/ΔA, %	27 ± 4	19 ± 3	21 ± 3	10 ± 1	11 ± 1
$\Delta MIF_{DMI}/\Delta A$, %	29 ± 4	29 ± 3 †	31 ± 3 †	21 ± 3 †	36 ± 5 *†
Fx U1, %	17 ± 5	37 ± 6 *	33 ± 7 *	51 ± 7	67 ± 5
Extraction, %	78 ± 3	74 ± 5	68 ± 5 *	79 ± 4	69 ± 3
SO, pmol/min	2.4 ± 1.3	2.8 ± 1.4	3.7 ± 2.2	35 ± 6	39 ± 5
Ur, pmol/min	46 ± 10	51 ± 14	36 ± 9	194 ± 33	204 ± 51
Rr, pmol/min	49 ± 11	54 ± 14	40 ± 10	229 ± 37	243 ± 53
EffU, %	95 ± 3	96 ± 2	94 ± 3	84 ± 2	79 ± 2

Epinephrine values are derived from data during intracoronary infusions of epinephrine in the present study. Norepinephrine values are derived from historic data during systemic intravenous infusions of norepinephrine.[15] Abbreviations are explained in the text. Values are mean±SEM. * $P < 0.05$ vs. lowest dose, † $P < 0.05$ vs. without DMI.

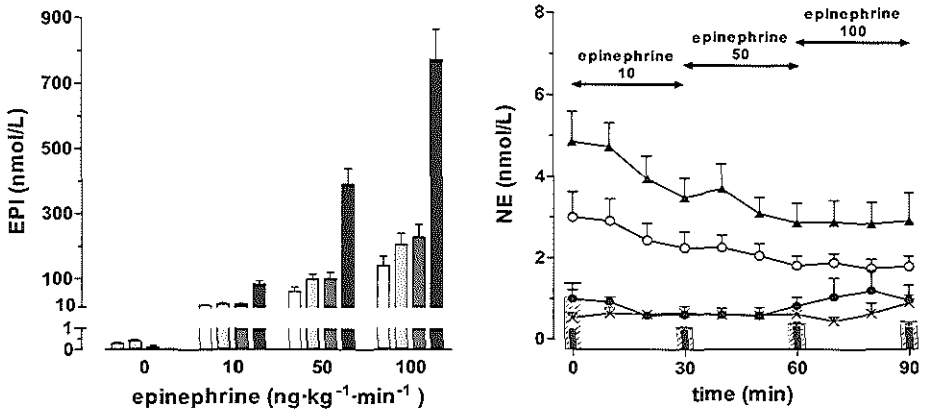

Figure 2. Effect of intracoronary infusion of epinephrine (ng·kg^{-1}·min^{-1}) on epinephrine (left panel) and norepinephrine (right panel) concentrations. Data are shown for $EPI_{MIF,LAD}$ (transparent) $EPI_{MIF,LAD}$ + DMI (light gray), EPI_{CV}(dark gray), EPI_{CA} (black bars), $NE_{MIF,LAD}$ (●), $NE_{MIF,LAD}$ + DMI (○), $NE_{MIF,LAD}$ + DMI + PHA (▲), $NE_{MIF,LCX}$ (x), NE_{Ao} (solid bars) and NE_{CV} (hatched bars). Data are mean ± SEM, n=9.

Intracoronary epinephrine infusion and norepinephrine release during sympathetic activation

Left stellate ganglion stimulation caused increases in MAP (21%), LAD flow (36%) and in particular LV dP/dt_{max} (184%, Table 4 and Figure 3) and caused a rise in NE_{MIF}, particularly in the presence of U_1- and α-adrenergic receptor blockade where $NE_{MIF,LAD}$ increased from 3.4±0.5 to 8.2±1.5 nmol/L (Figure 3). Although intracoronary infusion of epinephrine decreased MAP (-25%) and SVR (-27%) and increased HR (22%), LV dP/dt_{max} (93%) and LAD flow (32%), it did not alter the hemodynamic responses to stimulation.

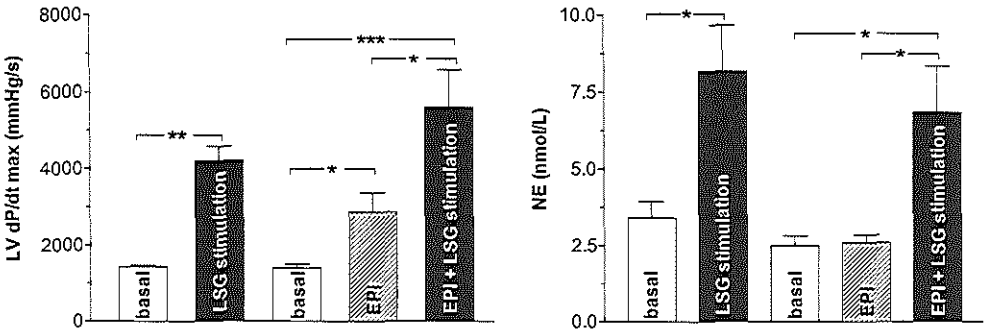

Figure 3. Effect of intracoronary epinephrine infusion on LV dP/dt_{max} (left panel) and $NE_{MIF,LAD}$ + DMI + PHA (right panel) during left stellate ganglion stimulation (LSG). EPI: intracoronary infusion of 50 ng·kg^{-1}·min^{-1}epinephrine. Data are mean ± SEM, n=6.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Similarly, concomitant epinephrine infusion did not enhance the norepinephrine release upon stimulation of the left stellate ganglion (from 2.6±0.3 to 6.9±1.5 nmol/L, Figure 3). In addition, stimulation did not increase $EPI_{MIF,LAD}$ (58±8 vs. 58±5 nmol/L), while EPI_{CV} even decreased (201±16 vs. 158±6 nmol/L, $P < 0.05$).

TABLE 4. Cardiovascular Function during Stellate Ganglion Stimulation before (-) and during (+) Intracoronary Infusion of Epinephrine

	EPI	Baseline	LSG stimulation
Mean arterial pressure, mmHg	-	87 ± 3	105 ± 5 *
	+	67 ± 11 †	84 ± 8 *†
Cardiac output, L/min	-	2.6 ± 0.2	2.7 ± 0.3
	+	2.8 ± 0.5	3.2 ± 0.5
Heart rate, bpm	-	108 ± 6	115 ± 7
	+	132 ± 4 †	137 ± 5 †
Systemic vascular resistance, mmHg.min $^{-1}$.L	-	34 ± 3	41 ± 4
	+	25 ± 2 †	28 ± 3 †
Stroke volume, mL	-	25 ± 1	24 ± 1
	+	22 ± 4	24 ± 4
LV dP/dt_{max}, mmHg/s	-	1485 ± 36	4220 ± 374 *
	+	2864 ± 507 †	5596 ± 980 *†
LV end diastolic pressure, mmHg	-	12 ± 2	10 ± 2
	+	8 ± 3	7 ± 3
LAD flow, mL/min	-	28 ± 5	38 ± 5 *
	+	37 ± 3 †	42 ± 8

EPI: intracoronary infusion of epinephrine (50 ng·kg^{-1}·min^{-1}). LSG: left stellate ganglion. Values are mean±SEM, n=6. * $P < 0.05$ vs. Baseline, † $P < 0.05$ vs. EPI -

Discussion

This study shows that epinephrine is taken up from the circulation by the heart and that it can be released during infusion of tyramine after the heart has been loaded with epinephrine by means of intracoronary epinephrine infusions, indicating that cardiac epinephrine is stored in and released from sympathetic nerve terminals. Our results do not support the concept that myocardial norepinephrine release is facilitated by epinephrine either under basal

conditions or during activation of cardiac sympathetic tone induced by left stellate ganglion stimulation.

Intracoronary epinephrine infusion and tyramine-induced epinephrine release

As tyramine only displaces catecholamines from their storage vesicles in the sympathetic nerve terminals after it has been taken up by the neuronal reuptake (U1) mechanism,[15,16] catecholamines released by tyramine are exclusively from neuronal origin. Under basal conditions, the tyramine-induced epinephrine release was negligible, which is in agreement with earlier results in the intact rabbit heart,[20] and likely reflects the low intra-neuronal epinephrine content (1-2% of cardiac norepinephrine concentrations).[21] After loading the heart with epinephrine by means of an intracoronary epinephrine infusion, tyramine caused substantial increases in EPI_{MIF} and EPI_{CV}, which were comparable to the increases in NE_{MIF} and NE_{CV}. These findings unequivocally demonstrate that in the porcine heart epinephrine can be taken up from the circulation by and released from the sympathetic nerve terminals. This is in agreement with a previous study, in which we showed that myocardial ischemia caused cardiac epinephrine release and that this release is modulated by the U1-mechanism as inhibition of U1 by DMI attenuated epinephrine release by more than 50%.[4] In addition, the similar coronary venous and interstitial epinephrine concentrations during infusion of tyramine indicate an unhindered exchange of neuronally released epinephrine from the interstitial to the vascular compartments.

Effect of intracoronary epinephrine infusions on epinephrine and norepinephrine concentrations

Epinephrine concentrations. Experimental and human studies have reported that the extraction of arterially delivered epinephrine by the myocardium during a single pass (about 50%) is considerably lower than the cardiac extraction of norepinephrine (70-85 %).[1,2,15,22-25] Because U1 is the major determinant of the cardiac clearance of catecholamines and the affinity of epinephrine for the U1 mechanism is lower than that of norepinephrine in the rabbit,[23] it is seems likely that the difference in extraction originates from this difference in affinity for U1. This is also demonstrated by the effect of U1-inhibition on $\Delta MIF/\Delta CA$; depending on the infusion rate, only 17 to 37% of infused epinephrine is cleared by U1, while we have previously shown that in the same experimental model U1 clears 51 to 67% of arterially delivered norepinephrine.[15]

The gradient between coronary venous and interstitial epinephrine concentrations during intracoronary infusion of epinephrine decreased significantly under U1-blockade and can therefore be attributed to U1.

Together with the unhindered exchange of neuronally released epinephrine during intracoronary infusion of tyramine, these results indicate that no endothelial barrier to the diffusion of epinephrine is present in the porcine heart.

Notwithstanding the relatively low clearance of epinephrine by U1, the 70% cardiac epinephrine extraction was still considerably higher than the 50% reported for the human heart,[24] suggesting the presence of a more active extra-neuronal clearance mechanism for epinephrine in the porcine heart, as was also reported for norepinephrine.[15] The modest epinephrine spillover rate of 3.0 pmol/min is in close agreement with that estimated for the human heart and is about 10 times lower than the spillover rate of norepinephrine.[1,3,23,24] Although the spillover rate of epinephrine is low, it should be kept in mind that the rate of neuronal release of epinephrine is more than ten times higher, since about 95% of released epinephrine is already cleared by U1 and extra-neuronal uptake mechanisms before it reaches the circulation.

Norepinephrine concentrations. Although intracoronary epinephrine infusions caused a 450-fold increase in $EPI_{MIF,LAD}$ and a 1400-fold increase in EPI_{CV}, we did not detect any changes in $NE_{MIF,LAD}$ or NE_{CV}. Even under U1-inhibition and α-adrenergic receptor-blockade to prevent respectively rapid clearance and presynaptic α_2-adrenergic receptor-mediated inhibition of norepinephrine release by epinephrine or norepinephrine itself, $NE_{MIF,LAD}$ did not increase during intracoronary infusion of epinephrine. These findings indicate that the potential facilitating effect of epinephrine on norepinephrine release was not masked by rapid clearance by U1 and local sympathoneural inhibition. Our findings agree with studies that also failed to demonstrate enhanced norepinephrine release by epinephrine in the human forearm[11], rabbit aorta,[13] rat mesentery, [14] and in particular with Thompson et al.[12] who demonstrated that epinephrine did not increase norepinephrine spillover in the human heart. Conversely, other studies did show augmentation of pressor responses,[7,9] increased plasma norepinephrine concentrations [26] and forearm norepinephrine spillover in humans,[8,27] as well as increased norepinephrine outflow in rat atria.[10] One may wonder whether the facilitation of norepinephrine release by epinephrine was obscured by an increase in norepinephrine clearance as a result of the epinephrine-induced increase in LAD flow. Indeed, LAD flow increased by 45% during the lowest dose of the intracoronary epinephrine infusion, which might explain the decrease in $NE_{MIF,LAD}$ in the presence of α_2-adrenergic receptor blockade and U1-inhibition (Figure 2). However, there were no further changes in LAD flow and $NE_{MIF,LAD}$ when epinephrine infusion rate was further increased 5- to 10-fold (Table 1 and Figure 2).

Intracoronary epinephrine infusion and norepinephrine release during sympathetic stimulation
It could be argued that in anesthetized animals, facilitation of norepinephrine release by epinephrine is difficult to demonstrate because of the low basal norepinephrine concentrations as compared to awake swine.[28] Hence, we investigated the effects of epinephrine on norepinephrine release during sympathetic activation, which was induced by electrical stimulation of the left stellate ganglion. Left stellate ganglion stimulation resulted in a marked increase in LV dP/dt_{max}, LAD flow and MAP. Although LV dP/dt_{max} was increased by epinephrine prior to stimulation, the hemodynamic response was not enhanced by an intra-coronary infusion of epinephrine (Table 3 and Figure 3). During stimulation of the left stellate ganglion NE_{MIF} increased up to five-fold. The absolute increase in $NE_{MIF,LAD}$ was the most substantial in the presence of U1- and α-adrenergic receptor blockade, underscoring the importance of the α_2-adrenergic receptor-mediated feedback mechanism that inhibits neuronal release of norepinephrine. Similar to the hemodynamic response, concomitant infusion of epinephrine did not augment the response of norepinephrine to left stellate ganglion stimulation, irrespective of the presence of U1- and α-adrenergic receptor blockade (Figure 3).

Conclusions
In summary, the present study shows that epinephrine is taken up by and released from cardiac sympathetic nerves. However, our findings in the porcine heart do not support the concept that myocardial norepinephrine release is facilitated by epinephrine either under basal conditions or during activation of cardiac sympathetic tone induced by left stellate ganglion stimulation. Hence, we hypothesize that the uptake of epinephrine by the heart is principally a mechanism for rapid clearance of circulatory epinephrine, and that the small amount of locally released epinephrine does not modulate left ventricular function.

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Chapter 6

Angiotensin II does not facilitate basal or stimulated norepinephrine release in the porcine heart.

Background - Studies on the effect of angiotensin II on norepinephrine release from sympathetic nerve terminals through stimulation of presynaptic angiotensin II type 1-receptors are equivocal. Furthermore, evidence that angiotensin II activates the cardiac sympathetic nervous system in vivo is scarce or indirect. We investigated whether AngII increases myocardial interstitial norepinephrine concentrations (NE_{MIF}) under basal conditions and during sympathetic activation, and whether it enhances exocytotic and nonexocytotic ischemia-induced norepinephrine release.

Methods and Results – In 27 anesthetized pigs, NE_{MIF} was measured in the perfusion areas of the left anterior descending coronary artery (LAD). Local infusion of angiotensin II into the LAD at consecutive rates of 0.05, 0.5 and 5 $ng \cdot kg^{-1} \cdot min^{-1}$, respectively did not affect NE_{MIF}, LAD flow, LV dP/dt_{max} and arterial pressure despite large increments in coronary arterial and venous angiotensin II concentrations. In the presence of uptake-1 inhibition and α-adrenergic receptor blockade, left stellate ganglion stimulation increased NE_{MIF} from 2.7±0.3 to 7.3±1.2 before and from 2.3±0.4 to 6.9±1.3 nmol/L during infusion of 0.5 $ng \cdot kg^{-1} \cdot min^{-1}$ angiotensin II. 60 min of 70% LAD flow reduction caused a progressive increase in NE_{MIF} from 0.9±0.1 to 16±6 nmol/L, which was not enhanced by concomitant infusion of 0.5 $ng \cdot kg^{-1} \cdot min^{-1}$ angiotensin II.

Conclusions - In conclusion, we did not observe any facilitation of cardiac norepinephrine release by angiotensin II under basal conditions and during either physiological (ganglion stimulation) or pathophysiological (acute ischemia) sympathetic activation. Hence, angiotensin II is not a local mediator of cardiac sympathetic activity in the *in vivo* porcine heart.

Lameris TW, de Zeeuw S, Duncker DJ, Alberts G, Boomsma F, Verdouw PD, van den Meiracker. Angiotensin II does not facilitate basal or stimulated norepinephrine release in the porcine heart. *Submitted.*

Introduction

Activation of the sympathetic nervous system (SNS) simultaneously leads to activation of the renin-angiotensin-system (RAS) via stimulation of β-adrenergic receptors within the kidney resulting in an increased renin release. There is also, albeit conflicting, evidence that the SNS is activated by the RAS.[1-13] This activation supposedly occurs through stimulation of angiotensin II receptors within the central nervous system and/or stimulation of presynaptic angiotensin II receptors located at sympathetic nerve terminals. When investigating the SNS and its interaction with the RAS, the heart is of particular interest. First of all, the mammalian heart has a dense sympathetic innervation. Second, all components of the RAS are present in the heart and most angiotensin II in the heart is formed from locally synthesized angiotensin I.[14] Third, in conditions like hypertension, ischemia and especially heart failure, the RAS and SNS are both activated and this activation likely contributes to the deterioration of cardiac function.[15-17]

Evidence that angiotensin II activates the cardiac SNS *in vivo* is scarce [4] or indirect.[7,18] In a recent study, Teisman et al.[4] have shown with the use of the microdialysis technique that pharmacological (μM) concentrations of locally applied angiotensin II were associated with an increase in norepinephrine concentrations (NE) in the myocardial interstitial fluid (NE_{MIF}) of the *in vivo* rat heart. In the present study, we determined whether physiological (pM) to pathophysiological (nM) concentrations of angiotensin II modulate NE_{MIF} in the porcine heart, in which contrary to the rat heart the parasympathetic system is dominant over the sympathetic system and in this respect is more akin to the human heart. To exclude a masking effect of neuronal norepinephrine reuptake (uptake-1, U1) and negative feedback through presynaptic α-adrenergic receptor stimulation on modulation of NE_{MIF} by angiotensin II, we co-perfused some probes with the U1-inhibitor desipramine (DMI) and the α-adrenergic receptor antagonist phentolamine (PHA), without provoking systemic hemodynamic effects that also may modulate norepinephrine release.[19-21]

In those studies that demonstrated interaction between angiotensin II and the SNS, most evidence points towards direct facilitation mediated by presynaptic angiotensin II type 1 (AT_1) receptors resulting in either a 'classic' calcium-dependent augmentation of exocytotic norepinephrine release,[1-3] or in enhanced nonexocytotic release via activation of the Na^+/H^+ exchanger.[22,23] Therefore, we not only investigated the modulation of NE_{MIF} by angiotensin II under basal conditions but also during enhanced exocytotic norepinephrine release evoked by stimulation of the left stellate ganglion. In addition, we monitored norepinephrine release in MIF during reduction of left anterior

descending coronary artery (LAD) flow, resulting in both exocytotic and nonexocytotic norepinephrine release,[20] while still allowing for intracoronary infusion of angiotensin II.

Methods

Animal care
All experiments were performed in accordance with the "Guiding Principles for Research Involving Animals and Human Beings" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam.

Surgical procedure
Twenty-seven crossbred Landrace x Yorkshire pigs of either sex (30-35 kg) were sedated with ketamine (20-25 mg/kg i.m., Apharma BV), anesthetized with sodium pentobarbital (20 mg/kg i.v., Apharma BV), intubated and ventilated with a mixture of oxygen and nitrogen.[19,20]

Catheters were positioned in the superior vena cava for infusion of sodium pentobarbital (10-15 mg/kg per hour) and physiological saline drip. A fluid-filled catheter was placed in the descending aorta for monitoring blood pressure and for blood sampling. A micromanometer-tipped catheter (B. Braun Medical BV) was inserted into the left ventricle for measurement of left ventricular (LV) pressure and LV dP/dt.[19,20] After midsternal thoracotomy, an electromagnetic flow probe (Skalar) was placed around the ascending aorta and a Doppler flow probe (Triton) was placed on the proximal LAD segment, while a cannula (∅: 1.3 mm) was inserted distal to this site into the LAD for infusion of angiotensin II. In animals subjected to LAD flow reduction, a fluid-filled balloon occluder (In Vivo Metric) was placed around the LAD distal to the Doppler flow probe and attached to a bi-directional roller pump (Ismatec). The voltage output from the Doppler equipment was directed through a custom-built electrical circuit, which steered the roller pump to maintain coronary flow at 30% of baseline values. In animals subjected to sympathetic stimulation, the left stellate ganglion was dissected and an electrode was inserted into the ganglion as described by Gootman et al.,[24] and connected to a nerve stimulator (Grass S9; pulses of 12 V, 10 Hz and 5 msec).

Microdialysis probes were implanted in the LV myocardium: one in the region perfused by the left circumflex coronary artery (LCx) and three in the area perfused by the LAD. To achieve local U_1-inhibition, one of the LAD probes was co-perfused with DMI (100 μM, Sigma),[21] while another LAD probe was co-perfused with DMI and PHA (100 μM, Dept of Pharmacy) to

block presynaptic α-adrenergic receptor-mediated inhibition of norepinephrine release. The microdialysis technique, probe characteristics, handling of the microdialysis and plasma samples for the measurement of norepinephrine concentrations, and probe recovery have been described previously.[19,25] Plasma samples for determination of angiotensin II concentrations (AngII) were rapidly drawn into chilled plastic syringes containing an "inhibitor mix".[14]

Experimental protocol

After a 120-min stabilization period, baseline measurements were obtained over a 30-min period. Probes were perfused with Ringer's solution (Baxter) at a flow of 2 μL/min; dialysate was collected at 10-min intervals, in which period blood was collected from the aorta (Ao) and coronary vein (CV).[19,20] In group I (n=7), the effects of angiotensin II on basal sympathetic norepinephrine release were investigated by infusing angiotensin II (Dept of Pharmacy) into the LAD at consecutive infusion rates of 0.05, 0.5 and 5 $ng \cdot kg^{-1} \cdot min^{-1}$ for 20 min each. In group II (n=7), we assessed the effects of angiotensin II on enhanced exocytotic norepinephrine release by stimulating the left stellate ganglion prior to and during infusion of angiotensin II. To investigate the effect of angiotensin II on nonexocytotic norepinephrine release the LAD flow was reduced by 70% for 60 min without (group III, n=6) and during simultaneous infusion of angiotensin II into the LAD (group IV, n=7). Following 120 min of reperfusion, the LAD perfusion area (area at risk) and infarct size were determined.[20]

Analytical procedures

Norepinephrine concentrations in plasma and microdialysis samples were determined by HPLC with fluorimetric detection.[25] Plasma angiotensin II concentrations were determined with HPLC after Sep-Pak extraction and radioimmunoassay.[14]

Data analysis and statistics

Dialysate norepinephrine concentrations were corrected for probe recovery to yield NE_{MIF}.[19,20] Lower limits of detection in dialysate and plasma were 0.2 and 0.02 nmol/L, respectively.[25] Baseline values were determined by averaging the three measurements over the 30-min period prior to intervention.[19,20] angiotensin II plasma concentrations in the LAD ($AngII_{CA}$) were calculated from angiotensin II infusion rate, coronary plasma flow and arterial angiotensin II concentrations. Results are expressed as mean ± SEM. For statistical analysis two-way analysis of variance, one-way analysis of variance for repeated measures with Dunnet's multiple comparison test as post-hoc test, and Student's *t* test were used as appropriate.

Results

Table 1. Cardiovascular function during intracoronary infusion of angiotensin II.

	Baseline	Angiotensin II, $ng \cdot kg^{-1} \cdot min^{-1}$		
		0.05	0.5	5
Mean arterial pressure, mmHg	83 ± 2	80 ± 5	83 ± 3	85 ± 3
Cardiac output, L/min	2.4 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
Heart rate, bpm	134 ± 5	135 ± 6	135 ± 6	134 ± 6
LV dP/dt_{max}, mmHg/s	1552 ± 76	1472 ± 111	1547 ± 87	1551 ± 87
LV end diastolic pressure, mmHg	12 ± 1	11 ± 1	11 ± 1	12 ± 1
LAD flow, mL/min	32 ± 5	31 ± 4	30 ± 3	28 ± 3

Values are mean±SEM. n=7

Intracoronary angiotensin II infusion and basal norepinephrine concentrations (Group I)
Although angiotensin II caused a dose-dependent increase in angiotensin II concentrations in the coronary vein ($AngII_{CV}$) from 13±2 pmol/L at baseline up to 4150±329 pmol/L during infusion of 5 $ng \cdot kg^{-1} \cdot min^{-1}$ (Figure 1) while angiotensin II in aortic plasma increased from 12±2 to 80±9 pmol/L, there were no significant changes in either hemodynamics (Table 1) or norepinephrine concentrations in MIF, coronary vein and aortic plasma (Table 2 and Figure 1).

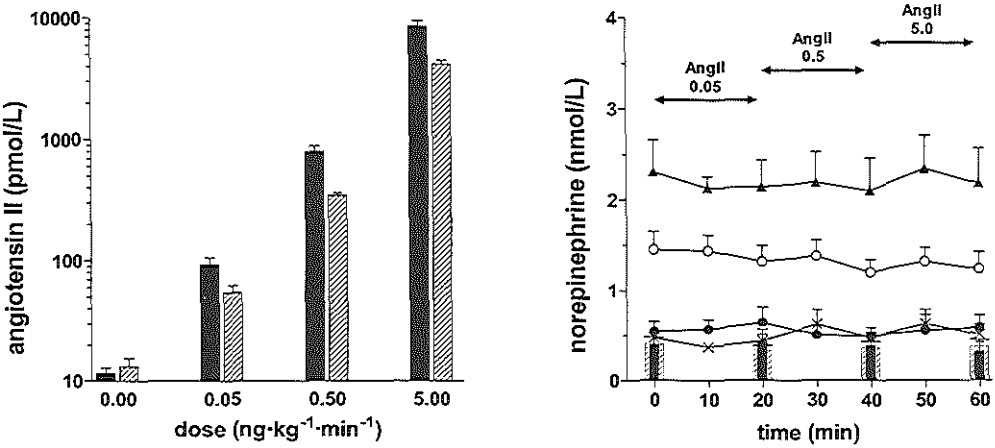

Figure 1. Effect of intracoronary AngII infusion ($ng \cdot kg^{-1} \cdot min^{-1}$) on plasma AngII concentrations (left panel) in the interventricular coronary vein (hatched bars) and LAD (solid bars), and basal cardiac sympathetic tone (right panel); $NE_{MIF,LAD}$ (●), $NE_{MIF,LAD}$ + DMI (○), $NE_{MIF,LAD}$ + DMI + PHA (▲), $NE_{MIF,LCx}$ (x), NE_{Ao} (solid bars) and NE_{CV} (hatched bars). Data are mean ± SEM, n=7.

Table 2. Effect of intracoronary AngII infusion on circulatory and interstitial NE concentrations.

	Arterial Plasma	Coronary Vein	MIF LAD			MIF LCx
			control	DMI	DMI + PHA	
	nmol/L	nmol/L	nmol/L	nmol/L	nmol/L	nmol/L
Group I (n=7)						
Baseline	0.4 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	1.4 ± 0.2 †‡§	2.3 ± 0.4 †‡§‖	0.5 ± 0.2
Ang II 0.05	0.5 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	1.3 ± 0.2 †‡§	2.1 ± 0.2 †‡§‖	0.4 ± 0.1
Ang II 0.5	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	1.2 ± 0.2 †‡§	2.1 ± 0.4 †‡§‖	0.6 ± 0.1
Ang II 5	0.3 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	1.3 ± 0.2 †‡§	2.2 ± 0.5 †‡§‖	0.6 ± 0.1
Group II (n=7)						
Baseline	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.1†‡	1.7 ± 0.4 †‡§	2.7 ± 0.3 †‡§	0.6 ± 0.1 †‡
LSG stimulation	1.0 ± 0.1 *	1.7 ± 0.7 *	1.3 ± 0.2 *	3.1 ± 0.6 *†§	7.3 ± 1.2 *†‡§	1.4 ± 0.5
Ang II 0.5	0.1 ± 0.0	0.3 ± 0.1	0.7 ± 0.1 †‡	1.6 ± 0.2 †‡§	2.3 ± 0.4 †‡§	0.5 ± 0.2 †
LSG stim + Ang II 0.5	1.3 ± 0.1 *	1.7 ± 0.6 *	0.9 ± 0.1 †	2.8 ± 0.6 †§	6.9 ± 1.3 *†‡§‖	1.2 ± 0.6
Group III (n=6)						
Baseline	0.2 ± 0.1	0.3 ± 0.1	0.9 ± 0.1 †‡	4.4 ± 0.8 †‡§	5.0 ± 0.7 †‡§	0.7 ± 0.2 †
Ischemia	0.2 ± 0.0	1.4 ± 0.6	16.2 ± 5.7 †‡*	12.3 ± 4.0 †‡*	14.3 ± 5.4 †‡*	0.5 ± 0.1 †§
Reperfusion	0.4 ± 0.2	0.4 ± 0.1	0.5 ± 0.1 *	1.4 ± 0.2 *†‡§	2.3 ± 0.4 *†‡§‖	0.5 ± 0.1
Group IV (n=7)						
Baseline	0.1 ± 0.1	0.2 ± 0.3	0.9 ± 0.3 †‡	5.2 ± 0.6 †‡§	5.8 ± 0.7 †‡§	0.6 ± 0.1 †
Ischemia + Ang II 0.5	0.2 ± 0.1	0.9 ± 0.2	10.9 ± 3.8 †‡*	11.2 ± 3.5 †‡*	14.1 ± 3.5 †‡*	0.6 ± 0.1 †§
Reperfusion	0.4 ± 0.2	0.3 ± 0.1	1.0 ± 0.5	1.7 ± 0.3 *†‡	2.2 ± 0.4 *†‡§‖	0.7 ± 0.2

AngII: angiotensin II ($ng \cdot kg^{-1} \cdot min^{-1}$); LSG: left stellate ganglion. Intracoronary infusion of AngII had no effect on circulatory or interstitial NE in any of the experimental protocols. Values are mean±SEM. * $P < 0.05$ vs. Baseline; † $P < 0.05$ vs. Arterial plasma; ‡ $P < 0.05$ vs. Coronary vein; § $P < 0.05$ vs. MIF LAD (control); ‖ $P < 0.05$ DMI vs. DMI+PHA.

Intracoronary angiotensin II infusion and norepinephrine release during sympathetic activation (Group II)

Stellate ganglion stimulation caused marked increases in blood pressure (19%), LAD flow (25%) and in particular LV dP/dt_{max} (190%, Table 3 and Figure 2), and caused a rise in NE_{MIF}, particularly in the presence of U_1- and α-adrenergic receptor blockade where $NE_{MIF,LAD}$ increased from 2.7±0.4 to 7.3±1.2 nmol/L (Table 2 and Figure 2). Concomitant intracoronary infusion of angiotensin II did not affect hemodynamic responses to stimulation nor did it modify the stimulation-induced increase in $NE_{MIF,LAD}$ (from 2.3±0.3 to 6.9±1.3 nmol/L).

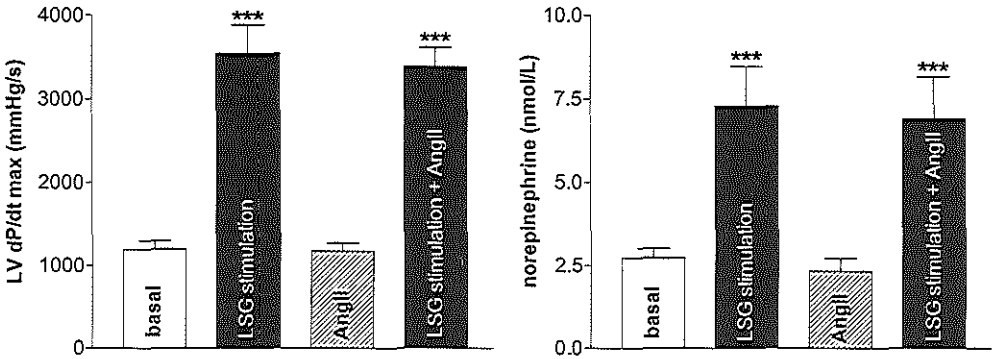

Figure 2. Effect of angiotensin II on LV dP/dt_{max} (left panel) and $NE_{MIF,LAD}$ + desipramine + phentolamine (right panel) during left stellate ganglion (LSG) stimulation. AngII: intracoronary infusion of angiotensin II (0.5 $ng \cdot kg^{-1} \cdot min^{-1}$). Data are mean ± SEM, n=7. *** $P < 0.001$ vs. pre-stimulation values.

Table 3. Intracoronary AngII infusion and cardiovascular function during sympathetic activation.

	Ang II	Baseline	LSG stimulation
Mean arterial pressure, mmHg	-	77 ± 5	95 ± 7 *
	+	75 ± 4	93 ± 6 *
Cardiac output, L/min	-	2.5 ± 0.2	2.8 ± 0.2 *
	+	2.4 ± 0.1	2.7 ± 0.1 *
Heart rate, bpm	-	114 ± 4	115 ± 4
	+	111 ± 5	114 ± 4
LV dP/dt_{max}, mmHg/s	-	1197 ± 103	3445 ± 340 *
	+	1172 ± 102	3383 ± 233 *
LV end diastolic pressure, mmHg	-	11 ± 2	9 ± 2
	+	11 ± 2	9 ± 2
LAD flow, mL/min	-	27 ± 4	36 ± 4 *
	+	27 ± 4	35 ± 4 *

AngII: angiotensin II, 0.5 $ng \cdot kg^{-1} \cdot min^{-1}$; LSG: left stellate ganglion. Values are mean±SEM, n=7; * $P < 0.05$ vs. Baseline.

Intracoronary angiotensin II infusion and norepinephrine release during ischemia (Groups III and IV)

Cardiovascular Function. The 70% LAD flow reduction resulted in 10% reductions of mean arterial pressure and cardiac output, while LV end-diastolic pressure slightly increased (Table 4). Following reperfusion, mean arterial pressure and cardiac output remained depressed while LV end-diastolic pressure returned to baseline. In addition, heart rate increased and LV dP/dt_{max} decreased.

Angiotensin II infusion during ischemia did not alter the hemodynamic response to ischemia and reperfusion. Because LAD flow was kept at 30% of baseline during ischemia any effect of angiotensin II on LAD flow was prevented.

Norepinephrine Concentrations. At baseline $NE_{MIF,LAD}$ and $NE_{MIF,LCx}$ were similar and three times the NE_{Ao} ($P < 0.05$; Table 2). Under U1-blockade with DMI, $NE_{MIF,LAD}$ increased approximately five-fold, irrespective of the presence of α-adrenergic receptor blockade. $NE_{MIF,LAD}$ tripled during the first 20 min of ischemia and continued to rise up to 15-fold at 60 minutes of ischemia (Table 2, Figure 3). Under U1-inhibition, the rate of rise of $NE_{MIF,LAD}$ was attenuated so that from 40 min of ischemia, $NE_{MIF,LAD}$ in the presence of DMI was similar to $NE_{MIF,LAD}$ without DMI. Upon reperfusion $NE_{MIF,LAD}$, $NE_{MIF,LAD}$ in the presence of DMI and NE_{CV} declined rapidly, with the early rate of decline being most pronounced for $NE_{MIF,LAD}$ without DMI (Figure 3).

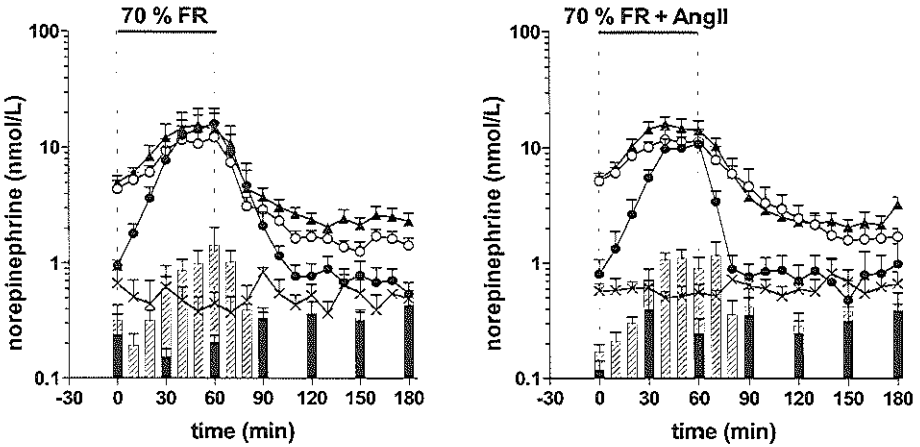

Figure 3. Effect of angiotensin II on the time course of changes in norepinephrine concentrations during 70% LAD flow reduction (70% FR) and reperfusion. Data are shown for $NE_{MIF,LAD}$ (●), $NE_{MIF,LAD}$ + desipramine (○), $NE_{MIF,LAD}$ + desipramine + phentolamine (▲), $NE_{MIF,LCx}$ (x), arterial (solid bars) and coronary venous (hatch ed bars) norepinephrine concentrations. AngII: intracoronary infusion of angiotensin II (0.5 ng·kg^{-1}·min^{-1}). Data are mean ± SEM, n=6 (left panel) and n=7 (right panel).

During ischemia, intracoronary infusion of angiotensin II raised $AngII_{CA}$ from 9±1 to 2323±231 pmol/L while $AngII_{CV}$ increased from 13±3 to 408±46 pmol/L, indicating that 80±5% of angiotensin II was extracted over the coronary bed. However, maximum $NE_{MIF,LAD}$ during the 60-min of ischemia was not modified by concomitant angiotensin II infusion (Table 2 and Figure 3). Within 120 min of reperfusion, $NE_{MIF,LAD}$ and NE_{CV} had

returned to baseline and were similar for groups III and IV. $NE_{MIF,LCx}$ and NE_{Ao} remained unchanged during the course of the experiment in both groups.

Table 4. Intracoronary infusion of angiotensin II and cardiovascular function during ischemia.

	AngII	Baseline	Ischemia 60 min	Reperfusion 120 min
Mean arterial pressure, mmHg	-	95 ± 2	89 ± 1	85 ± 3 *
	+	93 ± 3	86 ± 6	79 ± 4 *
Cardiac output, L/min	-	2.7 ± 0.2	2.4 ± 0.2 *	2.3 ± 0.1 *
	+	2.7 ± 0.1	2.3 ± 0.1 *	2.0 ± 0.2 *
Heart rate, bpm	-	121 ± 4	127 ± 6	136 ± 6 *
	+	117 ± 6	124 ± 6	138 ± 10 *
$LVdP/dt_{max}$, mmHg/s	-	1994 ± 141	1833 ± 85	1615 ± 104 *
	+	1668 ± 111	1554 ± 115	1458 ± 181
LV end diastolic pressure, mmHg	-	12 ± 2	14 ± 2	12 ± 1
	+	6 ± 2	9 ± 2 *	8 ± 2
LAD Flow, mL/min	-	27 ± 4	8 ± 1 *	35 ± 6
	+	35 ± 3	10 ± 1 *	44 ± 5 *

AngII: angiotensin II, 0.5 $ng \cdot kg^{-1} \cdot min^{-1}$. Values are mean±SEM (AngII -, n=6; AngII +, n=7)
* $P < 0.05$ vs. Baseline

Infarct size. The 70% LAD flow reduction resulted in an ischemic area (area at risk) that comprised 32±4% of the LV mass in both groups. Infarct size was 37±7% and 37±4% of the area at risk in group III and IV, respectively.

Discussion

This study provides no evidence for facilitation of cardiac norepinephrine release by angiotensin II under various experimental conditions in the intact porcine heart, as intracoronary infusion of angiotensin II did not modulate (i) basal sympathetic tone, (ii) exocytotic norepinephrine release during sympathetic activation produced by left stellate ganglion stimulation, and (iii) exocytotic and nonexocytotic norepinephrine release during myocardial ischemia.

Intracoronary angiotensin II infusion and basal cardiac sympathetic tone

While the intracoronary angiotensin II infusions in our experiments caused large increments in $AngII_{CV}$, no increments in $NE_{MIF,LAD}$ or NE_{CV} concentrations were observed (Table 2). Inhibition of norepinephrine neuronal reuptake by co-perfusion of microdialysis probes with DMI and inhibition of the presynaptic α_2-adrenergic receptor-mediated negative feedback of norepinephrine release with PHA did not unmask an angiotensin II-mediated increase in $NE_{MIF,LAD}$.

Although our findings agree with studies that also failed to demonstrate an effect of angiotensin II on basal norepinephrine concentration and norepinephrine spillover,[9-11] they are at variance with other studies that have shown angiotensin II to increase basal sympathetic tone.[3,4] It could be argued that in anesthetized animals, facilitation of norepinephrine release by angiotensin II is difficult to demonstrate because of low basal norepinephrine as compared to awake swine. However, Dendorfer and co-workers [3] have stated that facilitation of norepinephrine release by angiotensin II is in fact easier to demonstrate when background sympathetic tone is low.

Angiotensin II may not only facilitate the neuronal release of norepinephrine, but may also inhibit its neuronal reuptake.[18,26] As neuronal reuptake is an important determinant of NE_{MIF} under baseline conditions as well as during increased sympathetic tone,[19,21] an increase in NE_{MIF} concentration through inhibiting neuronal reuptake by angiotensin II would almost certainly have been detected in this study.

We can also exclude that a putative facilitating effect of angiotensin II on norepinephrine release was masked by a hemodynamically mediated increase in norepinephrine clearance. First, we used intracoronary angiotensin II infusions to prevent significant systemic hemodynamic effects. Second, the tendency of LAD flow to decrease would have favored an increase in NE_{MIF} by blunting norepinephrine clearance. In fact, the angiotensin II-associated increase in norepinephrine observed in some studies might be explained by a decrease in clearance caused by angiotensin II-induced vasoconstriction.[4]

Intracoronary angiotensin II infusion and norepinephrine release during sympathetic activation

In the present study, sympathetic stimulation was induced by electrical stimulation of the left stellate ganglion, resulting in marked increases in LV dP/dt_{max}, blood pressure and LAD flow. These hemodynamic effects were not enhanced by an intracoronary infusion of angiotensin II (Table 3 and Figure 2). Similarly, ganglion stimulation increased NE_{MIF} up to five-fold. In the presence of U1- and α-adrenergic receptor blockade, the absolute increase in NE_{MIF} was the most substantial, suggesting an important negative feedback mechanism

through pre-synaptic α-adrenergic receptors, most likely of the α_2-subtype. Again, infusion of angiotensin II did not augment this increase, irrespective of the presence of U1- and α-adrenergic receptor blockade (Table 2 and Figure 2). These results are in agreement with other studies that also failed to demonstrate enhanced norepinephrine release by angiotensin II during sympathetic activation in humans with and without CHF,[10-12] and in particular Rundqvist et al.[8], who demonstrated that intracoronary administration of the angiotensin-converting-enzyme (ACE) inhibitor enalaprilat failed to attenuate the increase in cardiac norepinephrine spillover following sympathetic activation. In contrast, other studies using electrical stimulation in vitro [1,2,5] as well as studies in humans [6,7] did demonstrate an angiotensin II-induced augmentation of sympathetic activation, three of which in the heart.[2,5,7] Of the latter only the study by Saino and colleagues investigated augmentation of sympathoneural activation by angiotensin II in the intact (human) heart. However, since they did not measure norepinephrine spillover or norepinephrine concentrations directly but estimated differences in sympathetic activity by comparing responses of coronary blood flow and coronary vascular resistance to the diving and cold pressure tests with and without simultaneous intracoronary angiotensin II infusion, it can not be excluded that other vasomotor mechanisms than α-adrenergic receptor-mediated vasoconstriction as a result of facilitated norepinephrine release are responsible for the observed hemodynamic responses.

Intracoronary angiotensin II infusion and norepinephrine release during ischemia

As angiotensin II has been reported to enhance either nonexocytotic norepinephrine release via activation of the Na^+/H^+ exchanger [22,23] or exocytotic release via 'classic' calcium-dependent facilitation,[1,3] we monitored norepinephrine release in MIF during myocardial ischemia produced by LAD flow reduction which leads to both exocytotic and nonexocytotic norepinephrine release while still permitting intracoronary infusion of angiotensin II during ischemia. The NE_{MIF} increase during 70% flow reduction (15-fold) was much less than previously described during total occlusion (500-fold),[20] not only because ischemia was less severe but also because washout of released norepinephrine is partially preserved during 70% flow reduction. We kept LAD flow constant at 30% of baseline, thereby preventing any potential effects of angiotensin II on flow-induced changes in norepinephrine clearance (Table 4). During flow reduction, concomitant infusion of angiotensin II did neither augment the ischemia-induced increase in NE_{MIF}, nor did it alter its time course (Table 2, Figure 3).

Our findings are at variance with the attenuation of ischemia-induced norepinephrine release,[23,27,28] as well as the decrease in sympathetic activity in

heart failure,[18,29,30] by ACE-inhibitors or AT_1-receptor blockers (ARB's) that have been reported earlier. Several factors may contribute to these apparent conflicting results. (i) Diffusion limitations for angiotensin II from the bloodstream to the perivascular or myocardial sympathetic nerve terminals could have prevented the infused angiotensin II from reaching the interstitial space and occupying AT_1-receptors.[8] However, this is unlikely, as we have previously shown that the cardiac tissue concentration of radiolabeled ^{125}I-angiotensin II during ^{125}I-angiotensin II infusion was 75% of its arterial concentration and that most of this angiotensin II is bound to AT_1-receptors.[14] (ii) Although RAS-inhibition may exert a direct effect on norepinephrine release in chronic heart failure (CHF),[18,29,30] the decrease in plasma norepinephrine concentrations during CHF treatment with ACE inhibitors or ARB's might also be due to an improvement of cardiac function. (iii) The decrease in sympathetic tone with ACE-inhibitors and ARB's [18,23,27-30] might not be mediated through peripheral presynaptic AT_1 receptors but by other mechanisms. For instance, ACE-inhibitors do not only inhibit AngI to angiotensin II conversion, but also limit bradykinin degradation and stimulate prostaglandin formation. Both bradykinin and prostaglandins have been shown to inhibit norepinephrine release.[27,31] In addition, the interaction between the RAS and SNS might be mediated through central AT_1-receptors in the brain.[32-34] (iv) Facilitation of norepinephrine release by presynaptic AT_1-receptor activation might be counteracted by presynaptic AT_2-receptors, which can inhibit norepinephrine release and are down-regulated in cardiomyocytes of CHF patients.[22,23,35]

In conclusion, we found no evidence to suggest any facilitation of cardiac norepinephrine release by angiotensin II under basal conditions and during sympathetic activation either by ganglion stimulation or acute ischemia. Hence, angiotensin II is not a local mediator of cardiac sympathetic activity in the porcine heart.

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Chapter 7

Cardiac ischemia and local catecholamine release

Background - Elevated concentrations of norepinephrine (NE) have been observed in ischemic myocardium. We investigated magnitude and mechanism of catecholamine release in the myocardial interstitial fluid (MIF) during ischemia and reperfusion *in vivo,* using the microdialysis technique.
Methods and Results – In nine anesthetized pigs, interstitial catecholamine concentrations were measured in the perfusion areas of the left anterior descending coronary artery (LAD) and the left circumflex coronary artery (LCx). After stabilization, the LAD was occluded for 60 min and reperfused for 150 min. During the final 30 min, tyramine (154 $nmol.kg^{-1}.min^{-1}$) was infused into the LAD. During LAD occlusion, NE_{MIF} concentrations in the ischemic region increased progressively from 1.0±0.1 to 524±125 nmol/L. MIF concentrations of dopamine (DA_{MIF}) and epinephrine (EPI_{MIF}) rose from 0.4±0.1 to 43.9±9.5 nmol/L, and from less than 0.2 (detection limit) to 4.7±0.7 nmol/L, respectively. Local U1-blockade attenuated release of all three catecholamines by more than 50%. During reperfusion, MIF catecholamine concentrations returned to baseline within 120 min. At that time, the tyramine-induced NE release was similar to that seen in non-ischemic controls despite massive infarction. Arterial and MIF catecholamine concentrations in the LCx region remained unchanged.
Conclusions - Myocardial ischemia is associated with a pronounced increase of MIF catecholamines, which is at least in part mediated by a reversed neuronal reuptake mechanism. The increase of EPI_{MIF} implies a (probably neuronal) cardiac source, while the preserved catecholamine response to tyramine in post-ischemic necrotic myocardium indicates functional integrity of sympathetic nerve terminals.

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Introduction

Myocardial ischemia is associated with a marked accumulation of norepinephrine (NE) in ischemic tissue.[1-4] *In vitro* studies suggest that this is caused by non-exocytotic release of NE from cardiac sympathetic nerves.[5-7] In contrast to the normally occurring exocytotic NE release, this non-exocytotic NE release is (*i*) calcium independent, (*ii*) not under influence of local or central sympathetic stimulation, and (*iii*) not affected by presynaptic inhibition.[7] Interestingly, these *in vitro* studies in the sympathetically dominant rat heart also suggest that the ischemia-induced non-exocytotic NE release can be attenuated by neuronal uptake-1 (U1) blockade, indicating that under ischemic conditions the U1-mechanism is reversed, and can operate as a carrier for outward instead of inward NE transport.[5,6] However, this has not been investigated in parasympathetically dominant human and porcine hearts *in vivo*. Furthermore, little is known about myocardial release of epinephrine (EPI) and dopamine (DA) in the ischemic heart *in vivo*. This is of particular interest since cardiac E release has been reported in *in vitro* studies, and in healthy elderly men and patients with severe congestive heart failure at rest and during exercise.[8-11]

Microdialysis allows measurement of catecholamine concentrations in the myocardial interstitial fluid (MIF) *in vivo*, and investigation of the mechanisms underlying their local release and clearance.[12] Using an *in vivo* porcine model, we investigated time course and magnitude of changes in MIF concentrations of catecholamines during severe myocardial ischemia and reperfusion. To determine the contribution of reversal of the U1-mechanism to ischemia-induced NE release, one of the microdialysis probes in the ischemic myocardium was co-perfused with the U1-inhibitor desipramine (DMI).[12,13]

Finally, we determined whether sympathetic nerve endings are functionally impaired following reperfusion after severe myocardial ischemia, as has been suggested for the isolated rat hearts.[2] For this purpose, local NE response to an intracoronary infusion of tyramine in the post-ischemic myocardium was compared to the response observed in the non-ischemic porcine myocardium of control animals previously studied under similar experimental conditions.[12] Tyramine is taken up via U1 into the sympathetic nerve endings where it releases NE. Tyramine thus gives information on the NE content as well as U1-function of sympathetic nerve endings.[12]

Methods

Animal care
All experiments were performed in accordance with the "Guiding Principles for Research Involving Animals and Human Beings" as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of the Erasmus University Rotterdam.

Surgical procedure
After an overnight fast, crossbred Landrace x Yorkshire pigs of either sex (30-35 kg, n=9) were sedated with ketamine (20-25 mg/kg i.m.), anesthetized with sodium pentobarbital (20 mg/kg i.v.), intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture of oxygen and nitrogen. Respiratory rate and tidal volume were set to keep arterial blood gases within the normal range.[12,14]

Catheters were positioned in the superior caval vein for continuous administration of sodium pentobarbital (10-15 $mg.kg^{-1}.h^{-1}$) and saline for replacing blood withdrawn during sampling. In the descending aorta, a fluid-filled catheter was placed to monitor aortic blood pressure and blood sampling. Through a carotid artery a micromanometer-tipped catheter (B. Braun Medical BV, Uden, The Netherlands) was inserted into the left ventricle for measurement of left ventricular (LV) pressure and its first derivative LV dP/dt. After administration of pancuronium bromide (4 mg), a midsternal thoracotomy was performed and the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was then placed around the ascending aorta for measurement of cardiac output. After a Doppler flow probe was placed on a proximal segment of the left anterior descending coronary artery (LAD), a cannula (∅: 1.3 mm) was inserted distal to this site into the LAD for administration of tyramine.

Microdialysis probes were implanted in left ventricular myocardium using a steel guiding needle and split plastic tubing: one in the region perfused by the left circumflex coronary artery (LCx) and two in the area perfused by the LAD. In order to achieve local U1-inhibition, one of the LAD probes was co-perfused with DMI (100 μM).[13] Additionally, a microdialysis probe was placed in the interventricular coronary vein that drains the LAD region.[15]

Dialysis methodology
The polycarbonate dialysis membrane of the microdialysis probes (CMA/20, Carnegie Medicine AB, Sweden) has a cut-off value of 20 kD, a length of 10 mm and a diameter of 0.5 mm. Probes were perfused with an isotonic Ringer's solution at a rate of 2 μL/min using a CMA/100 microinjection pump.

Dialysate volumes of 20 μL (sampling time 10 min) were collected in microvials containing 20 μL of a solution of 2 % (w/v) EDTA and 30 nM *l*-erythro-alpha-methyl-NE (AMN) as internal standard in 0.08 N acetic acid. Sampling started immediately after inserting the probes. Plasma samples were drawn into chilled heparinized tubes containing 12 mg glutathione. Microdialysis and plasma samples were stored at –80 °C, until analysis within the next five days.[12,16]

In vivo probe recovery of NE (52±1%) has been determined by retrodialysis using AMN as a calibrator and direct comparison of hemo-microdialysis and plasma samples.[12,17] *In vivo* probe recovery for EPI (68±3%) was determined by comparing EPI concentrations in arterial plasma with EPI concentrations in the dialysate obtained from the carotid artery probe. The *in vivo* probe recovery for DA was not determined directly but was assumed similar to probe recovery of NE because of the similarities of NE and DA in size and charge distribution.

Experimental protocol

After a 120-min stabilization period,[12] baseline measurements were obtained over a 30-min period before the LAD was occluded distal to the first diagonal branch for 60 min, using an atraumatic clip, and thereafter, reperfused for 150 min. During the last 30 min of reperfusion, tyramine (154 $nmol.kg^{-1}.min^{-1}$) was infused directly into the LAD. At the end of the experiment, the perfusion area of the LAD was determined by an intra-atrial infusion of 30 ml of a 5% (w/v) solution of fluorescein sodium during reocclusion of the LAD. During occlusion, ventricular arrhythmias were counted and distinguished as premature ventricular contractions (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF).[18] After inducing ventricular fibrillation with a 9 V battery, the heart was excised and infarct size was determined using para-nitrobluetetrazolium.[14]

Analytical procedures

Plasma catecholamines were determined by HPLC with fluorimetric detection after liquid-liquid extraction and derivatization with the fluorogenic agent 1,2-diphenyl-ethylenediamine (DPE).[19] For microdialysis samples, the catecholamines are not extracted prior to fluorimetric detection with HPLC, but directly derivatized according to the procedure described by Alberts et al.[17] This method suppresses the interference of sulfhydryl compounds on derivatization, thus improving sensitivity.

Reagents and pharmaceuticals
Ketamine and sodium pentobarbital were obtained from Apharmo BV (Arnhem, The Netherlands), pancuronium bromide from Organon Teknica BV (Boxtel, The Netherlands), Ringer's solution from Baxter (Uden, The Netherlands). Tyramine was obtained from the department of pharmacy of the University Hospital Rotterdam. Fluorescein sodium, para-nitrobluetetrazolium, DMI, NE, EPI, DA and AMN were purchased from Sigma (St. Louis, MO), EDTA from Merck (Darmstadt, Germany), L-glutathione from Fluka (Buchs, Switzerland), and acetic acid from Baker (Deventer, The Netherlands). DPE was prepared as reported previously.[19]

Statistical analysis
Five out of the 9 animals encountered ventricular fibrillation during LAD occlusion (between 10 and 30 min of ischemia), but were successfully defibrillated within 1 min using 20-30 W counter shocks and therefore included in the analysis. Since there were no differences in the hemodynamic and catecholamine responses between animals that fibrillated and animals that maintained sinus rhythm, data of all 9 animals were pooled. Catecholamine concentrations obtained with microdialysis were corrected for probe recovery. Lower limits of detection for catecholamines measured with microdialysis and those measured in arterial plasma were 0.2 and 0.02 nmol/L, respectively. Baseline values were determined by averaging the three measurements over the 30-min period prior to occlusion.[12] Results are expressed as mean ± SEM. For statistical analysis two-way analysis of variance, one-way analysis of variance for repeated measures with Dunnet's multiple comparison test as post-hoc test and Student's *t* test were used as appropriate.

Results

Systemic hemodynamics during ischemia and reperfusion
Baseline hemodynamic data and the changes produced by 60-min LAD occlusion and 120 min of reperfusion (Table 1) are in accordance with previously published data from our laboratory.[14]

Infarct size
The LAD occlusion resulted in an ischemic area (area at risk) that comprised 29±2% of the left ventricular mass. Infarct size determined at the end of reperfusion was 84±4% of the area at risk

Table 1. Cardiac and systemic hemodynamics at baseline, at 60 min of ischemia and 120 min of reperfusion.

	Baseline	Ischemia 60 min	Reperfusion 120 min
Mean arterial pressure, mmHg	97 ± 2	82 ± 2*	79 ± 5
Cardiac output, L/min	2.4 ± 0.2	2.1 ± 0.1	1.8 ± 0.1*
Heart rate, bpm	114 ± 4	118 ± 7	137 ± 11*
Systemic vascular resistance, mmHg.min $^{-1}$.L	42 ± 4	41 ± 4	43 ± 5
Stroke volume, mL	22 ± 2	18 ± 1*	15 ± 2*
LV dP/dt_{max}, mmHg/s	1680 ± 100	1460 ± 110	1380 ± 150
LV end diastolic pressure, mmHg	6.6 ± 0.8	9.3 ± 1.4	10 ± 1.9 *
LAD flow, mL/min	34 ± 3	0	46 ± 8

Values are given as mean±SEM, n=9. * = $P < 0.05$, vs. Baseline

Catecholamine concentrations during ischemia and reperfusion

At baseline, NE concentrations in MIF (NE_{MIF}) in the LAD and LCx regions were similar to concentrations in the coronary vein (NE_{CV}), but three times the concentrations in arterial plasma (NE_{art}) ($P<0.05$; Table 2). Dopamine concentrations followed a similar pattern, while EPI was only detectable in arterial plasma. Under U1-blockade, NE_{MIF} increased five-fold, while DA_{MIF} did not change and EPI_{MIF} remained undetectable.

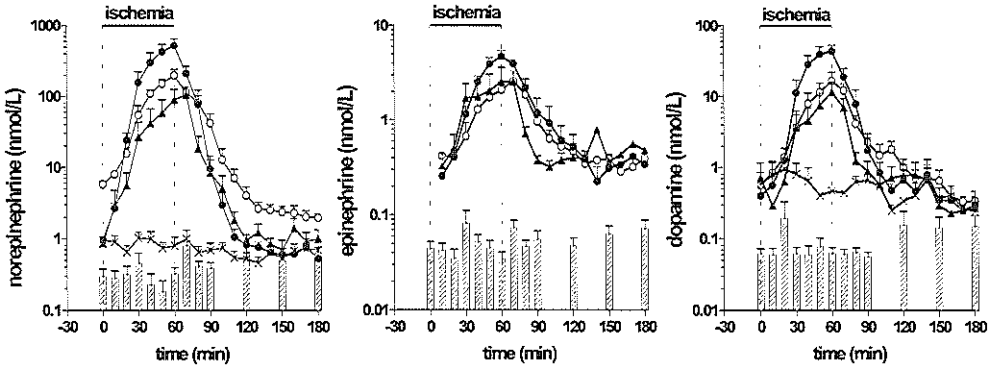

Figure 1. Time course of changes in catecholamine concentrations during ischemia and reperfusion. Data are shown for MIF in the LAD region without (●) and with DMI (○), MIF in the LCx region (×), and concentrations in arterial plasma (hatched bars) and in the coronary vein (π). Data are presented as mean ± SEM, n=9

During the first 10 min of ischemia, NE_{MIF} in the LAD region tripled and continued to rise progressively so that NE_{MIF} had increased 500-fold at the end of ischemia (Table 2, Figure 1). In the presence of U1-blockade, the rate of rise of NE_{MIF} was attenuated so that after 20 min of ischemia, NE_{MIF} under U1-blockade was similar and at 60 min only one-fifth of NE_{MIF} in the absence

of U1-blockade. NE_{CV} increased progressively to 100-fold its baseline value. Upon reperfusion NE_{MIF}, NE_{MIF} under U1-blockade and NE_{CV} declined rapidly, with the early rate of decline being most pronounced for NE_{MIF} in the absence of U1-blockade (Figure 1). Within 120 min of reperfusion, catecholamine concentrations in MIF and coronary vein had returned to baseline values. NE_{MIF} in the LCx perfused area and NE_{art} remained unchanged during the course of the experiment.

Table 2. Circulatory and interstitial catecholamine concentrations at baseline, at 60 min of ischemia and 120 min of reperfusion.

	Arterial plasma nmol/L	Coronary vein nmol/L	MIF LAD nmol/L	MIF LAD + DMI nmol/L	MIF LCX nmol/L
Norepinephrine					
Baseline	0.30 ± 0.08	0.86 ± 0.22 †	0.97 ± 0.11 †	5.76 ± 0.77 †‡§	0.96 ± 0.13 †
Ischemia	0.32 ± 0.07	90 ± 36 *†	524 ± 125 *†‡	198 ± 46 *†‡§	0.79 ± 0.27 †‡§
Reperfusion	0.54 ± 0.23	0.62 ± 0.30	0.54 ± 0.11	1.99 ± 0.28 *†‡§	0.62 ± 0.16
Epinephrine					
Baseline	0.04 ± 0.01	< 0.20	< 0.20	< 0.20	< 0.20
Ischemia	0.03 ± 0.01	2.55 ± 1.07 *†	4.74 ± 0.71 *†	2.09 ± 0.43 *†§	< 0.20 ‡§
Reperfusion	0.07 ± 0.02	< 0.20	< 0.20	< 0.20	< 0.20
Dopamine					
Baseline	0.06 ± 0.01	0.71 ± 0.45 †	0.40 ± 0.12 †	0.57 ± 0.16 †	0.59 ± 0.27 †
Ischemia	0.06 ± 0.01	11.6 ± 4.3 *†	43.9 ± 9.5 *†‡	16.5 ± 5.6 *†§	0.47 ± 0.04 †‡§
Reperfusion	0.15 ± 0.07	0.27 ± 0.11	0.30 ± 0.10 †	0.34 ± 0.13 †	0.21 ± 0.03

Values are given as mean±SEM, n=9. * $P < 0.05$ vs Baseline; † $P < 0.05$ vs Arterial plasma; ‡ $P < 0.05$ vs Coronary vein; § $P < 0.05$ vs MIF LAD

In the LAD region, EPI_{MIF} and DA_{MIF} in the absence and presence of U1-blockade as well as EPI_{CV} and DA_{CV} followed qualitatively similar patterns, but absolute increments during ischemia were substantially less pronounced (Table 2, Figure 1). Again, EPI_{art}, DA_{art} and DA_{MIF} in the LCx region did not change over the course of the experiment, while EPI_{MIF} in the LCx region remained undetectable.

Ventricular arrhythmias during ischemia

Most of the ventricular arrhythmias occurred within the first half-hour of ischemia. The incidence of PVC's was particularly high between 20 to 30 min of ischemia, 661 in total and an average of 73±19 per animal. Five animals suffered from ventricular fibrillation, but were defibrillated successfully within 1 minute. There was no correlation between MIF NE concentrations and the

occurrence of ventricular arrhythmias in general or with ventricular fibrillation in particular.

Post-ischemic catecholamine release by tyramine

Figure 2 shows that infusion of tyramine directly into the LAD after 120 min of reperfusion caused an increase in NE_{MIF} in the post-ischemic myocardium from 0.6±0.1 to 11.5±1.9 nmol/L ($P < 0.05$). This increment was not different from the increase from 0.9±0.2 to 13.4±3.2 nmol/L in the LAD region of the normal (non-ischemic) swine heart.[12] Furthermore, compared to the increment seen in the absence of U1-blockade, the increase in the presence of U1-blockade was minimal.

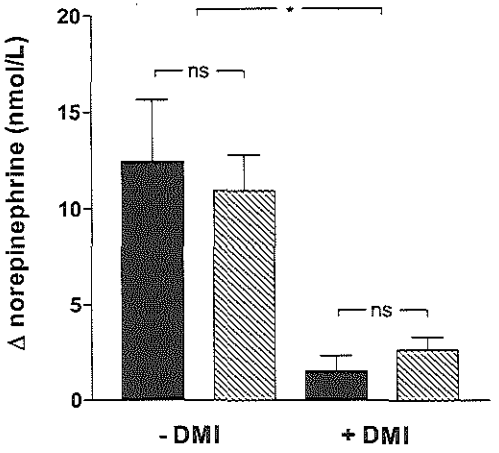

Figure 2. Tyramine induced NE release in the perfusion area of the LAD with and without U1-inhibition. Data are shown for post-ischemic myocardium (n=4, hatched bars) and for historic non-ischemic controls (n=4, solid bars). DMI: desipramine, U1-inhibitor. Data presented as mean ± SEM, n=4, ns: not significant, *: $P < 0.05$

Discussion

This study demonstrates that myocardial ischemia is associated with a rapid and massive increase of the concentration of all three endogenous catecholamines NE, EPI and DA in the myocardial interstitial fluid as measured with the microdialysis technique *in vivo*. As suggested for NE in *in vitro* studies,[5,6] the reversed U1-mechanism plays an important role in the release of all three catecholamines during ischemia *in vivo*. Furthermore, our study shows that after 60 min of ischemia, which results in massive infarction of the jeopardized myocardium, the functional integrity of sympathetic nerve terminals remains intact. Finally, our results suggest that cardiac interstitial EPI has a neuronal origin.

Interstitial catecholamine concentrations during basal conditions

The present study confirms that, at baseline, NE_{MIF} is about 3 times the NE_{art}, and increases about 6-fold in response to U1-blockade,[1,12,20] while EPI_{MIF} was

below the detection limit irrespective of the presence of U1-inhibition. DA_{MIF} did not rise under U1-blockade, suggesting that U1 does not play a predominant role in the clearance of DA from the interstitial compartment under baseline conditions in the heart. Little is known about the affinity of dopamine (DA) for U1 and its relevance to DA clearance in the heart. As the main purpose of the U1 mechanism is to modulate synaptic transmission, it is hard to envisage a substantial role for U1 in the clearance of DA in the absence of any cardiac dopaminergic synaptic transmission. Furthermore, in tissues with known dopaminergic transmission, like brain and kidney DA is taken up by a specific DA neuronal uptake mechanism, which does not take up NE and which is poorly inhibited by DMI.[21,22]

Interstitial catecholamine concentrations during ischemia and reperfusion

In *in vitro* experiments in isolated rat hearts, three phases of ischemia-induced release of NE, each with a different mechanism, have been recognized.[2,5,6,23] During the early phase of ischemia (0-10 min), release of NE, *if present*, is exocytotic and depends on the activation of efferent sympathetic neurons. Accumulation of catecholamines in the extracellular space in this early phase is prevented by the highly efficient U1 mechanism and by presynaptic inhibition by adenosine, which accumulates in cardiac tissue during this phase of ischemia. The latter has shown to be of particular importance in the rat, as adenosine concentrations are considerably higher than in other species.[24] During the second phase of ischemia (10-40 min), the release of NE becomes non-exocytotic and is thought to involve the U1-mechanism in the carrier-mediated efflux of NE in reverse of its normal transport direction.[5,6] During the third phase (> 40 min ischemia) the release of NE is no longer attenuated by U1-inhibitors, which is explained by the occurrence of structural changes in the neuronal membrane of the myocardial neurons.[2]

In the present study, a rapid and pronounced increase of MIF concentrations of all three catecholamines was observed shortly after occlusion of the LAD. As released NE is avidly taken up by the cardiac U1-mechanism, we expected a larger rise of NE_{MIF} in the presence of the U1-inhibitor DMI than without U1-inhibition. However, during this first 10 minutes of ischemia, the increment of NE_{MIF} with U1-blockade (5.8 to 8.5 nmol/L) was similar to the increment without U1-blockade (0.9-2.7 nmol/L). Possibly, in the parasympathetically dominant porcine heart, U1–carrier mediated non-exocytotic NE efflux already occurred in the initial 10 minutes of ischemia, so that in the presence of DMI any decrease in U1-mediated clearance was compensated for by a decrease in the ischemia-induced U1-carrier-mediated NE-efflux. As mentioned, myocardial release of NE in first 10 minutes of ischemia is not an invariable finding. For example, stimulation-evoked NE

release has shown to be suppressed in rat hearts and human atrial tissue, but to be facilitated in guinea pig hearts.[25]

Throughout the ischemic period, MIF catecholamine concentrations rose progressively in the ischemic area. Concentrations of catecholamines changed neither in the non-ischemic LCx area nor in the systemic circulation. Reversal of U1-mechanism continued to contribute to the catecholamine release during the entire period of ischemia. Thus, U1-blockade attenuated the release of all catecholamines by more than 50%, indicating that despite infarction of 83% of the area at risk, U1 was operative after 60 min of ischemia. Our findings vary from those obtained in the ischemic myocardium of the isolated rat heart where the reversed U1-mechanism no longer contributes to release of catecholamines 40 min after induction of ischemia. This difference may be explained by the differences in experimental conditions, e.g. in vivo versus in vitro studies, and the species investigated.[2]

Upon reperfusion, MIF catecholamine concentrations rapidly declined in the post-ischemic myocardium. Washout was probably the predominant factor in the clearance of catecholamines in this early phase of reperfusion. However, the decline in the first 10 min of reperfusion was substantially greater without inhibition of the U1-mechanism, indicating that the U1-mechanism also contributed significantly to the clearance of catecholamine during early reperfusion. Although it should be noted that in contrast to techniques used in *in vitro* studies,[2,25] the time resolution of the MD technique as presently used does not allow making conclusions about minute-to-minute changes of catecholamine concentrations. Compatible with previous findings that EPI is less avidly taken up by U1 than NE,[26] the decline of the EPI_{MIF} during reperfusion was not affected by U1-blockade.

Origin of myocardial interstitial epinephrine

An interesting finding was the ischemia-induced increase of the EPI_{MIF}, albeit small as compared to the increase in NE_{MIF}. Since the concentration of EPI_{art} and EPI_{MIF} in the non-ischemic LCx region did not change during ischemia, this increase must have originated from the heart. It is currently unclear whether this source is neuronal or extra-neuronal. Evidence favoring extra-neuronal synthesis and release of EPI is the presence of the enzyme phenylethanolamine N-methyltransferase in extra-neuronal myocardial tissue.[10,27] Furthermore, an intrinsic cardiac adrenergic cell type outside the sympathetic nervous system, capable of releasing EPI and NE, has been identified in the human heart.[10] Finally, enhanced cardiac EPI spill-over into the coronary circulation of heart failure patients during sympathetic stimulation was disproportionate to the spillover of NE, suggesting that EPI may, in part, be derived from sources other than chromaffin cells or sympathetic nerves.[9,11]

On the other hand, it is known that sympathetic neurons can take up EPI from the circulation and release it upon stimulation.[28] In our study, the pattern of release and clearance of EPI during ischemia and reperfusion was similar to that of NE and DA. Furthermore, inhibition of the neuronal U1-mechanism attenuated the ischemia-induced release of all catecholamines to a similar degree, suggesting a common source, thus favoring a neuronal origin.

Functional integrity of sympathetic nerve endings

The effects of U1 blockade on the NE_{MIF} responses to ischemia and reperfusion suggested that U1-mechanism of the sympathetic nerves was still functioning during and after the 60 min LAD occlusion. This is further substantiated by the NE_{MIF} response to tyramine at the end of reperfusion. Similar to NE, tyramine is taken up by neurons through U1, where it displaces NE from the nerve terminals because of its higher affinity for the neuronal storage proteins. Consequently, the tyramine-induced NE release reflects both neuronal NE content and the efficacy of U1.[12,29] The increase of NE_{MIF} in the post-ischemic LAD region was very similar to that in the non-ischemic control hearts (Figure 2).[12] These findings are in line with those reported by Shindo et al.[4] who studied the tyramine-induced NE release in non- and post-ischemic areas in cat hearts after 40 min of reperfusion following 40 min of ischemia. Additionally, in the present study, the attenuation of the tyramine-induced NE release by U1-inhibition in both post-ischemic and non-ischemic groups was also similar (Figure 2). Although the present experimental setup does not allow for any predictions concerning long-term survival of sympathetic nerves, these present findings indicate that sympathetic nerve terminals remained functionally intact at least during the first few hours after reperfusion. However, functional alterations of the somata of the sympathetic nerves cannot be entirely excluded. Thus, in a canine model of tachycardia-induced heart failure, impairment of the myocardial contractile response to electrical or chemical stimulation of sympathetic somata was observed at a time when the contractile response to tyramine was completely preserved.[30]

Implications

Although the pathophysiological significance of the massive accumulation of catecholamines in the ischemic myocardial tissue was not investigated in the present study, there is evidence from experimental as well as clinical studies that high catecholamine concentrations are deleterious to the heart.[31-34] Several studies have demonstrated NE-dependent anti-arrhythmic effects of U1-inhibition during ischemia either by tricyclic anti-depressant agents like desipramine and imipramine or by structurally unrelated U1-inhibitors like cocaine and nisoxetine.[7,35] This study provides a possible explanation for this

beneficial effect by demonstrating that the reversed U1-mechanism contributes substantially to the release of catecholamines during ischemia.

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Chapter 8

Cardioprotection in pigs by exogenous norepinephrine

Background - Endogenous norepinephrine release induced by cerebral ischemia may lead to small areas of necrosis in normal hearts. Conversely, norepinephrine may be one of the mediators that limit myocardial infarct size (IS) by ischemic preconditioning. Because brief ischemia in kidneys or skeletal muscle limits IS produced by coronary artery occlusion, we investigated whether cardiac norepinephrine release during transient cerebral ischemia also elicits remote myocardial preconditioning.
Methods and Results - In 10 anesthetized control pigs, IS after a 60-min coronary occlusion and 120 min of reperfusion was 84±3% (mean±SEM) of the area at risk. Intracoronary infusion of 0.03 nmol·kg^{-1}·min^{-1} norepinephrine for 10 min before coronary occlusion did not affect IS (80±3%, n=6), whereas infusion of 0.12 nmol·kg^{-1}·min^{-1} limited IS (65±2%, n=7, P<0.05). Neither 10-min (n=5) nor 30-min (n=6) cerebral ischemia produced by elevation of intracranial pressure before coronary occlusion affected IS (83±4% and 82±3%, respectively). Myocardial interstitial norepinephrine levels tripled during cerebral ischemia and during low dose norepinephrine, but increased ten-fold during high dose norepinephrine. Norepinephrine levels increased progressively up to 500-fold in the area at risk during the 60-min coronary occlusion, independent of the pretreatment, while norepinephrine levels remained unchanged in adjacent non-ischemic myocardium and arterial plasma.
Conclusions - Cerebral ischemia preceding a coronary occlusion did not modify IS, which is likely related to the modest increase in myocardial norepinephrine levels during cerebral ischemia. The IS limitation by high dose exogenous norepinephrine is not associated with blunting of the ischemia-induced increase in myocardial interstitial norepinephrine levels.

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Introduction

Ischemic preconditioning, originally described for the myocardium,[1] also occurs in kidney,[2] skeletal muscle,[3] lung[4] and brain.[5] Przyklenk et al.[6] showed that brief regional myocardial ischemia protects not only the jeopardized myocardium during a subsequent coronary artery occlusion, but also the adjacent "virgin" myocardium. Furthermore, it has been shown that brief ischemia in remote organs such as kidney,[7] small intestine[7] and skeletal muscle,[8] is also capable of limiting myocardial infarct size produced by a prolonged coronary artery occlusion.

Norepinephrine (NE) is one of the mediators involved in the signaling pathway leading to ischemic preconditioning,[9,10] and because cerebral ischemia causes a profound release of norepinephrine from sympathetic nerve endings in normal myocardium,[11] this raises the question whether transient cerebral ischemia prior to a coronary artery occlusion may also be cardioprotective. In addition, exogenous administration of norepinephrine before a coronary artery occlusion elicits cardioprotection in rabbits[12] and rats.[13]

The major aim of this study was therefore to investigate the effect of cerebral ischemia on myocardial infarct size produced by a coronary artery occlusion in pigs. Because the cardioprotective effect of norepinephrine has not been established in pigs, we first studied whether intracoronary infusions of norepinephrine are capable of limiting myocardial infarct size. A further aim was, using microdialysis,[11,14] to quantitate the myocardial norepinephrine concentrations during cerebral ischemia and exogenous norepinephrine infusions, and to determine whether limitation of infarct size is mediated by attenuation of myocardial interstitial norepinephrine levels during the infarct-producing coronary artery occlusion.[15]

Methods

Animal care

The present experiments were performed conform with the *"Guide for Care and Use of Laboratory Animals"* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and under the regulations of the Erasmus University Rotterdam.

Experimental protocol

Forty-one crossbred Landrace x Yorkshire pigs of either sex (34±1 kg) were assigned to one of 7 experimental groups of which in 6 groups myocardial infarct size was determined at the end of the protocol (Figure 1). Ten animals

(Control) underwent a 60-min left anterior descending coronary artery (LAD) occlusion followed by 120-min reperfusion, while in 13 animals the 60-min LAD occlusion-reperfusion was preceded by a 10-min norepinephrine infusion into the LAD at a rate of either 0.03 nmol·kg^{-1}·min^{-1} (NE_{low}, n=6) or 0.12 nmol·kg^{-1}·min^{-1} (NE_{high}, n=7). In 3 animals, the effects of the 10-min high dose norepinephrine infusion ($NE_{high,sham}$) on myocardial function and metabolism were evaluated to assess whether this dose produced myocardial ischemia and asynchrony of contraction. Infarct size was not determined in these animals. In 5 animals a 10-min period of global cerebral ischemia (CI_{10}) preceded the 60-min LAD-occlusion by 20 min, while in 6 animals the LAD-occlusion was preceded by a 30-min period of cerebral ischemia (CI_{30}) and 30 min of reperfusion. Finally, in 4 animals, we studied whether 30 min of global cerebral

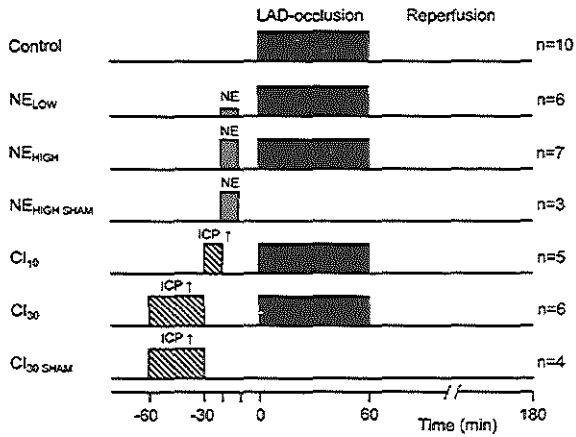

Figure 1. Diagram of the 7 experimental groups. NE was infused at a rate of either 0.03 (NE_{low}) or 0.12 nmol/kg·min^{-1} (NE_{high} and $NE_{high,sham}$) into the left arterial descending coronary artery (LAD). Global cerebral ischemia (CI), produced by elevating intracranial pressure (ICP), was maintained for either 10 min (CI_{10}) or 30 min (CI_{30} and $CI_{30,sham}$). Infarct size was determined at the end of 120 min of reperfusion in all groups except in $NE_{high,sham}$.

ischemia *per se* ($CI_{30,sham}$) damaged normal myocardium. Cerebral ischemia was achieved by infusion of artificial cerebrospinal fluid,[17] such that intracranial pressure increased to approximately 250 mmHg (invariably above the systolic arterial pressure).

In all groups, a 120-min stabilization period followed the surgical procedures, after which baseline measurements were made. Microdialysis was performed in Control, NE_{low}, NE_{high}, and CI_{30}. Dialysate samples were collected over 10-min periods for determination of myocardial interstitial norepinephrine concentrations starting 90 min into the stabilization period, when norepinephrine concentrations had reached stable levels.[14,15] During the subsequent 30 min, baseline dialysate samples were collected. Plasma samples were obtained halfway through each 10-min dialysate collection period. Animals encountering ventricular fibrillation during the protocol were allowed to complete the experiment when sinus rhythm could be restored by DC-countershock within 2 min.

At the end of the 120-min reperfusion period, the area at risk was determined by intra-atrial infusion of 20 ml of 5% (w/w) fluorescein sodium.[18] After the heart was excised, the left ventricle was isolated and cut parallel to the atrioventricular groove into five slices of equal thickness. After the area at risk of each slice was demarcated on an acetate sheet under ultraviolet light, the slices were incubated in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St Louis) per liter of phosphatebuffer (pH 7.4) at 37°C for 30 min, and the non-stained pale infarcted area was also traced onto the sheet. Myocardial infarct size was defined as the ratio of the summated infarct areas and summated areas at risk.[18]

Surgical procedure

Overnight fasted pigs were sedated with ketamine (20-25 mg/kg i.m., Apharmo BV, Arnhem, The Netherlands), anesthetized with sodium pentobarbital (20 mg/kg i.v., Apharmo) and intubated for ventilation with 30% oxygenated room air, while arterial blood gases were kept within the normal range. Catheters were inserted into the superior caval vein for infusion of sodium pentobarbital (10-15 $mg \cdot kg^{-1} \cdot h^{-1}$) and saline. A fluid-filled catheter was placed in the descending aorta for measurement of aortic blood pressure and collection of blood samples, while a micromanometer-tipped catheter was inserted in the carotid artery and advanced into the left ventricle for measurement of left ventricular pressure (LVP) and its first derivative (LV dP/dt). After administration of pancuronium bromide (4 mg, Organon Teknika BV, Boxtel, The Netherlands) and a midsternal thoracotomy, the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta for measurement of cardiac output, while the segment of the LAD between the first and the second diagonal branch was dissected free for placement of a Doppler flow probe (Triton Technology Inc., San Diego, CA) and a microvascular clamp. In NE_{low}, NE_{high}, and $NE_{high,sham}$, a small cannula was inserted into the LAD distal to the flow probe.

One microdialysis probe was implanted in the LAD-area and one in the left circumflex coronary artery (LCx)-area. In CI_{30}, a third probe was placed in the cortex of the brain. Perfusion of the probes started immediately after insertion.[14,15]

In $CI_{30,sham}$ and $NE_{high,sham}$ pairs of ultrasound crystals were implanted in the midmyocardial layer of the LAD- and LCx-areas to assess regional myocardial wall function (Triton Technology Inc., San Diego, CA), while the great cardiac vein accompanying the LAD was cannulated for collection of blood samples. Finally, in $NE_{high,sham}$ also the left atrium was cannulated for injection of radioactive microspheres (^{113}Sn or ^{141}Ce, 15±1 (SD) μm) to

determine the effect of norepinephrine on the distribution of myocardial blood flow.[1]

Two catheters were inserted into the left and right cerebral lateral ventricles through bore holes to produce cerebral ischemia.[16] A fluid-filled catheter was used for infusion of the artificial cerebrospinal fluid to elevate intracranial pressure, which was monitored with a micromanometer-tipped catheter

Microdialysis

The polycarbonate dialysis membrane of the microdialysis probes (CMA/20, Carnegie Medicine AB, Sweden) has a cut-off value of 20 kD, a length of 10 mm and a diameter of 0.5 mm. Cardiac probes were perfused with an isotonic Ringer's solution and the cerebral probe with the artificial cerebrospinal fluid, at a rate of 2 µl/min using a CMA/100 microinjection pump. Dialysate volumes of 20 µl (sampling time 10 min) were collected in microvials containing 20 µl of a solution of 2% (w/v) EDTA and 30 nM l-erythro-alpha-methyl-norepinephrine (AMN) as internal standard in 0.08 N acetic acid. Plasma samples were drawn into chilled heparinized tubes containing 12 mg glutathione. All samples were stored at –80°C, until analysis within the next five days.[14,15] In vivo probe recovery of norepinephrine, determined by retrodialysis and by direct comparison of hemo-microdialysis and plasma samples is 52±1%.[14,15,19]

Data analysis and statistics

Percent systolic shortening (SS) was calculated as the difference in segment length at end-diastole and the minimal segment length during systole divided by the segment length at end-diastole. Asynchrony during norepinephrine infusion was assessed by determining the time interval between the occurrence of minimal segment length (L_{min}) in the LAD- and LCx-areas.

Myocardial O_2-extraction (%) was calculated as the ratio of the arterio – coronary venous O_2-content difference and the arterial O_2-content. At the end of the experiment, the heart was excised, the LAD- and LCx-areas were separated and divided into three layers of equal thickness to determine the subendocardial (inner layer) and subepicardial (outer layer) blood flows and their ratios, using standard techniques.[1]

All results have been expressed as mean±SEM. Statistical significance ($P<0.05$) for changes in hemodynamics and norepinephrine concentrations was determined by two-way ANOVA and one-way ANOVA for repeated measures, followed by Dunnett's multiple comparison test. Statistical significance ($P<0.05$) for differences in infarct size was determined using one-way ANOVA followed by Student's *t* test.

Results

Hemodynamics

Norepinephrine infusions. Intracoronary norepinephrine infusion in NE_{LOW} produced an increase in LV dP/dt_{max}, reflecting an increase in regional contractility as the other cardiovascular variables remained unaffected (Table 1). During the 10-min washout period LV dP/dt_{max} returned to baseline. During norepinephrine infusion in NE_{high} and $NE_{high,sham}$ mean arterial pressure decreased rapidly from 91±2 to 74±6 mmHg followed by a gradual recovery (Table 1). The decrease in cardiac output was responsible for the hypotension, as systemic vascular resistance remained unchanged. Cardiac output decreased, because the increase in heart rate was insufficient to compensate for the decrease in stroke volume. The latter occurred despite the increase in LV dP/dt_{max}, and was likely due to asynchrony of contraction (see below). All parameters recovered during the 10-min washout period that preceded the 60-min LAD-occlusion.

During norepinephrine infusion in $NE_{high,sham}$, SS in the LAD-area increased from 27±2% at baseline to 34±4%, while SS in the LCx-area decreased from 18±1% to 13±1% (both $P<0.05$). These changes were accompanied by asynchrony of contraction between the LAD- and LCx-areas. Thus, whereas under baseline conditions L_{min} of both areas occurred at the end of global left ventricular systole, during norepinephrine the occurrence of L_{min} in the LAD-area preceded L_{min} in the LCx-area by 119±2 ms ($P<0.05$). The latter was due to L_{min} in the LAD-area occurring 56±15 ms before and L_{min} in the LCx-area occurring 63±7 ms after closure of the aortic valves (both $P<0.05$ vs their respective baseline values). During washout all wall function parameters returned to baseline values.

In the LAD-area of $NE_{high,sham}$, O_2-extraction decreased from 64±7% at baseline to 54±7% during norepinephrine infusion, indicating that O_2-delivery increased slightly in excess of the increase in myocardial O_2-demand. In addition the arterio – coronary venous pH difference remained unchanged (0.06±0.01 at baseline and at the end of infusion). Moreover, the subendocardial to subepicardial blood flow ratio remained unchanged in both the LAD-area (1.14±0.25 at baseline and 1.30±0.17 at the end of infusion) and the LCx-area (1.21±0.07 and 1.24±0.05, respectively). Finally, SS in the LAD- and LCx-areas returned to baseline values immediately during the recovery period (24±3% and 17±1%, respectively), indicating that the norepinephrine infusion did not produce myocardial ischemia and stunning.

Table 1. Cardiovascular function during norepinephrine infusion or cerebral ischemia.

			Change from Baseline				
	Groups	Baseline	2 min	5 min	10 min	30 min	recovery
Mean arterial pressure, mmHg	A	93 ± 3	0 ± 3	2 ± 2	3 ± 3	-	-1 ± 2
	B+C	91 ± 2	-15 ± 6 *	-17 ± 5 *	-8 ± 4	-	-2 ± 2
	D	92 ± 5	80 ± 10 *	53 ± 13 *	-11 ± 5	-	-16 ± 12
	E+F	90 ± 4	83 ± 6 *	67 ± 8 *	-19 ± 4 *	-17 ± 5 *	-20 ± 4 *
Cardiac output, L/min	A	2.8 ± 0.2	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	-	-0.1 ± 0.1
	B+C	2.5 ± 0.2	-0.4 ± 0.2 *	-0.5 ± 0.2 *	-0.2 ± 0.1	-	-0.1 ± 0.1
	D	3.8 ± 0.5	0.7 ± 0.2 *	2.1 ± 0.4 *	0.7 ± 0.3	-	-0.3 ± 0.6
	E+F	3.1 ± 0.2	0.7 ± 0.2 *	2.2 ± 0.3 *	0.4 ± 0.1 *	0.9 ± 0.2 *	-0.2 ± 0.4
Systemic vascular resistance, $mmHg \cdot L^{-1} \cdot min^{-1}$	A	33 ± 3	0.2 ± 0.8	0.4 ± 0.7	1.1 ± 0.7	-	1.1 ± 1.1
	B+C	37 ± 3	-0.6 ± 1.4	0.7 ± 0.9	0.6 ± 1.0	-	1.2 ± 0.9
	D	26 ± 3	15.9 ± 4.6 *	1.4 ± 4.4	-7.4 ± 1.9 *	-	-3.6 ± 1.0 *
	E+F	30 ± 2	18.8 ± 4.2 *	1.8 ± 3.2	-8.8 ± 1.3 *	-11.0 ± 1.5 *	-9.0 ± 2.1 *
Heart rate, bpm	A	107 ± 7	1 ± 1	1 ± 2	1 ± 2	-	1 ± 3
	B+C	117 ± 7	13 ± 4 *	15 ± 5 *	12 ± 5	-	2 ± 2
	D	112 ± 5	37 ± 8	68 ± 12 *	19 ± 13	-	17 ± 10
	E+F	101 ± 3	46 ± 5 *	79 ± 6 *	22 ± 4 *	2 ± 3	9 ± 7
Stroke volume, mL	A	27 ± 4	1.1 ± 1.5	1.5 ± 1.4	1.2 ± 1.2	-	0.5 ± 1.1
	B+C	22 ± 2	-5.4 ± 1.9 *	-6.4 ± 1.6 *	-3.8 ± 1.7	-	-1.1 ± 0.7
	D	33 ± 3	-2.6 ± 2.2	0.2 ± 3.8	1.4 ± 3.0	-	-5.8 ± 3.0
	E+F	30 ± 2	-4.4 ± 1.8 *	-0.8 ± 1.5	-1.8 ± 1.2	8.0 ± 1.6 *	-4.2 ±
LV dP/dt_{max}, mmHg/s	A	1560 ± 110	760 ± 100 *	790 ± 90 *	850 ± 60 *	-	-60 ± 70
	B+C	2030 ± 200	860 ± 210 *	890 ± 160 *	1270 ± 120 *	-	-150 ± 70
	D	1790 ± 170	2810 ± 660 *	4650 ± 910 *	-310 ± 410	-	410 ± 560
	E+F	1640 ± 120	2570 ± 440 *	5010 ± 340 *	160 ± 230	-90 ± 170	50 ± 220
Left ventricular end-diastolic pressure, mmHg	A	8 ± 2	1.2 ± 0.5	1.3 ± 0.6	1.9 ± 0.8	-	1.1 ± 0.6
	B+C	8 ± 1	-1.7 ± 0.8	-2.0 ± 0.8 *	-1.4 ± 0.6	-	0.7 ± 0.6
	D	9 ± 1	8.5 ± 1.7 *	1.5 ± 1.2	0.2 ± 2.4	-	-2.5 ± 1.8
	E+F	7 ± 1	10.6 ± 2.5 *	0.9 ± 1.7	-1.2 ± 1.2	1.8 ± 1.5	0.0 ± 0.9
LAD flow, $mL \cdot min^{-1} \cdot g^{-1}$	A	1.7 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.1 *	-	0.0 ± 0.2
	B+C	1.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	-	0.0 ± 0.1
	D	1.0 ± 0.2	1.0 ± 0.2 *	0.7 ± 0.1 *	0.0 ± 0.1	-	-0.1 ± 0.1
	E+F	1.3 ± 0.1	1.3 ± 0.2 *	1.4 ± 0.3 *	-0.1 ± 0.1	0.2 ± 0.2	-0.1 ± 0.1

Values are mean±SEM; n=6 (NE_{low}, group A), n=10 (NE_{high}+$NE_{high,sham}$, groups B+C), n=5 (CI_{10}, group D), n=10 (CI_{30}+$CI_{30,sham}$, groups E+F). * $P < 0.05$ vs. Baseline

Cerebral ischemia. Increasing intracranial pressure (12±2 mmHg at baseline) to 250 mmHg produced an immediate increase in mean aortic pressure in CI_{10}, CI_{30} and $CI_{30,sham}$, which was initially the consequence of increases in both cardiac output and systemic vascular resistance (Table 1). However, after 5 min the tachycardia-mediated increase in cardiac output was exclusively responsible for the hypertension. Despite the increase in afterload, stroke volume was maintained most likely due to enhanced myocardial contractility as LV dP/dt_{max} increased up to four times its baseline value. The increase in coronary blood flow paralleled the increase in myocardial O_2-demand, reflected by the 150% increase in double product (heart rate · systolic arterial pressure).

Table 2. Cardiovascular function during coronary occlusion and reperfusion.

	Treatment	Preocclusion Values	End-Occlusion Values	End-Reperfusion Values
Mean arterial pressure, mmHg	Control	90 ± 2	75 ± 2 *	76 ± 6 *
	NE_{low}	93 ± 4	72 ± 6 *	70 ± 5 *
	NE_{high}	87 ± 4	77 ± 5	77 ± 4
	CI_{10}	75 ± 9	74 ± 5	65 ± 5
	CI_{30}	65 ± 4	61 ± 5	66 ± 4
Cardiac output, L/min	Control	2.6 ± 0.2	2.1 ± 0.1 *	2.1 ± 0.2 *
	NE_{low}	2.8 ± 0.2	2.3 ± 0.2 *	1.9 ± 0.1 *
	NE_{high}	2.2 ± 0.2	1.9 ± 0.2 *	1.8 ± 0.2 *
	CI_{10}	3.5 ± 0.4	2.9 ± 0.3	2.5 ± 0.4
	CI_{30}	2.9 ± 0.3	2.2 ± 0.2 *	2.0 ± 0.1 *
Systemic vascular resistance, $mmHg \cdot L^{-1} \cdot min^{-1}$	Control	38 ± 3	37 ± 3	38 ± 4
	NE_{low}	34 ± 4	32 ± 3	38 ± 4
	NE_{high}	41 ± 4	43 ± 4	45 ± 5
	CI_{10}	22 ± 3	27 ± 4	28 ± 4 *
	CI_{30}	24 ± 2	28 ± 2 *	33 ± 2 *
Heart rate, bpm	Control	115 ± 4	123 ± 7	139 ± 8 *
	NE_{low}	108 ± 9	112 ± 9	119 ± 13
	NE_{high}	124 ± 6	133 ± 11	136 ± 11
	CI_{10}	129 ± 7	119 ± 9	120 ± 7
	CI_{30}	112 ± 6	112 ± 5	115 ± 6
Stroke volume, mL	Control	22 ± 2	18 ± 1 *	16 ± 1 *
	NE_{low}	27 ± 4	22 ± 3	17 ± 2
	NE_{high}	18 ± 2	14 ± 2 *	13 ± 1 *
	CI_{10}	28 ± 4	25 ± 4	21 ± 3
	CI_{30}	25 ± 2	20 ± 2 *	18 ± 1 *
LV dP/dt_{max}, mmHg/s	Control	1610 ± 100	1480 ± 100	1640 ± 260
	NE_{low}	1500 ± 110	1350 ± 110	1250 ± 100
	NE_{high}	1980 ± 280	2060 ± 350	2050 ± 340
	CI_{10}	2200 ± 410	1630 ± 140	1350 ± 140
	CI_{30}	1710 ± 320	1250 ± 160	1170 ± 100
Left ventricular end-diastolic pressure, mmHg	Control	7 ± 1	9 ± 1	10 ± 1 *
	NE_{low}	9 ± 2	10 ± 1	10 ± 1
	NE_{high}	8 ± 2	10 ± 2	12 ± 1
	CI_{10}	7 ± 3	13 ± 3	13 ± 1
	CI_{30}	6 ± 1	9 ± 2 *	12 ± 1 *

Values are mean±SEM; n=10 (Control), n=6 (NE_{low}), n=7 (NE_{high}), n=5 (CI_{10}), n=6 (CI_{30}). Pre-occlusion values of the NE and CI groups correspond to the recovery values of Table 1. * $P < 0.05$ vs. pre-occlusion values.

Similar to earlier observations in dogs[20] and pigs,[11] the transient hyperdynamic phase was followed by a fall in mean arterial pressure below baseline levels at 10 min of cerebral ischemia, which was the result of systemic vasodilatation. Except heart rate, which remained slightly elevated, all other variables had recovered at 10 min. In CI_{30} and $CI_{30,sham}$, mean arterial pressure, cardiac output and systemic vascular resistance did not change further during

the remainder of the 30-min period of cerebral ischemia, while heart rate returned to baseline levels and stroke volume increased. Except for mean arterial pressure and systemic vascular resistance, all other hemodynamic variables and intracranial pressure returned to baseline values during recovery (Table 1).

The increase in intracranial pressure decreased myocardial O_2-extraction from 64±5% at baseline to 58±6% at 5 min, but did not change the arterio – coronary venous pH difference (0.04±0.01 at baseline and at 5 min), indicating the absence of myocardial ischemia. The elevation of the intracranial pressure decreased SS from 24±1% to 16±2% at 2 min, but at 5 min SS had already recovered to 23±1% and to 26±1% at 10 min, with no evidence of depressed regional wall function during the remainder of the 30-min period (27±1%) or the subsequent recovery phase (23±3%).

LAD-occlusion and reperfusion. In Control, mean arterial pressure decreased secondary to the decrease in cardiac output during the 60-min LAD-occlusion and did not change further during reperfusion (Table 2). Heart rate increased slightly, but insufficiently to compensate for the decrease in stroke volume. Pretreatment with norepinephrine had no effect on the hemodynamic responses during the subsequent LAD-occlusion and reperfusion in either NE_{low} or NE_{high}. In CI_{10} and CI_{30}, mean arterial pressure did not further decrease during the LAD-occlusion most likely because systemic vascular resistance, which was still below baseline levels at the onset of the LAD-occlusion, recovered.

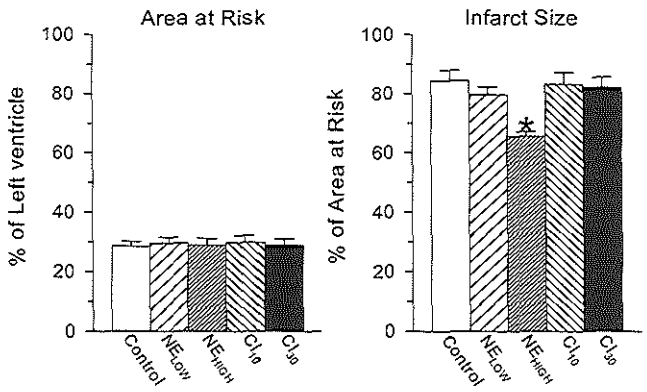

Figure 2. Area at risk and infarct size for the 5 groups in which the LAD was occluded. Global cerebral ischemia alone ($CI_{30,sham}$) did not cause irreversible myocardial damage (not shown). * $P<0.05$ vs. Control. For further details see Figure 1.

Myocardial infarct size

The area at risk was identical in all experimental groups (Figure 2). Infarct size was 84±3%, in Control and 80±3% in NE_{low}, but only 65±2% in NE_{high} ($P<0.05$). Cerebral ischemia had no effect on infarct size development during

the 60-min LAD occlusion as in CI_{10} and CI_{30} infarct size was 83±4% and 82±3%, respectively. Cerebral ischemia *per se* did not cause irreversible damage as in none of the $CI_{30,sham}$ animals infarct tissue was detected.

Myocardial interstitial norepinephrine concentrations

The myocardial interstitial norepinephrine levels in the LAD-area increased from 0.8±0.2 nmol/L to 2.2±0.5 nmol/L in NE_{low}, and to 12.2±5.9 nmol/L in NE_{high} during norepinephrine infusion (both $P<0.05$, Figure 3). Despite the intracoronary route, there was some spillover in NE_{high} as evidenced by small transient increments of norepinephrine in plasma from 0.2±0.1 to 1.0±0.2 nmol/L and in the interstitium of the LCx-area from 1.1±0.3 to 2.4±1.2 nmol/L (both $P<0.05$, Figure 3).

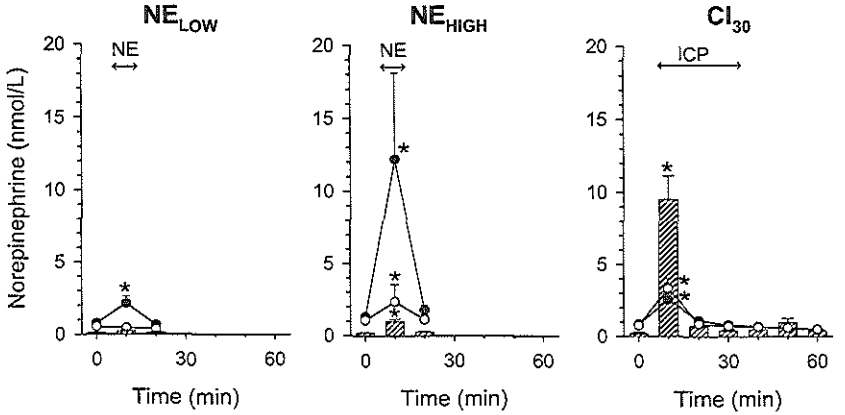

Figure 3. Norepinephrine levels in plasma (hatched bars) and in the interstitium of the LAD - area (●) and the LCx-area (○) during the 10-min norepinephrine infusions and during 30 min of cerebral ischemia. * $P < 0.05$ vs Baseline. For further details see Figure 1.

In CI_{30}, cerebral interstitial norepinephrine levels increased from 0.9±0.4 nmol/L at baseline to 6.1±1.9 nmol/L at 10 min of intracranial pressure elevation and up to 8.3±1.8 nmol/L at 30 min (not shown in Figure 3). Upon cerebral reperfusion, interstitial levels initially increased further to 12.3±2.3 nmol/L, but returned to baseline during the remainder of the 30-min recovery period. Cerebral ischemia resulted in a transient tripling of interstitial norepinephrine levels in both the LAD- and LCx-areas, and a 20-fold increase in plasma norepinephrine levels (Figure 3).

In Control, NE_{low}, NE_{high}, and CI_{30}, norepinephrine levels increased progressively during the LAD-occlusion by up to approximately 500-fold and recovered during reperfusion, independent of the preceding intervention (Figure 4). There was no correlation ($r=0.03$) between the maximum interstitial norepinephrine levels during the LAD-occlusion and myocardial infarct size.

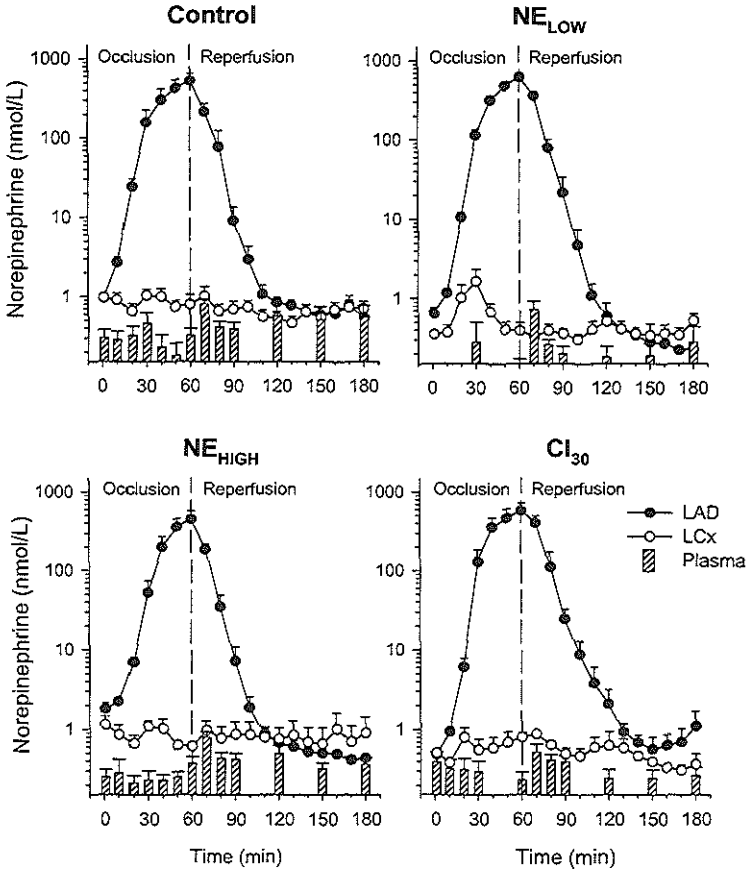

Figure. 4. Norepinephrine levels in plasma (hatched bars), in the interstitium of the LAD -area (●) and the LCx-area (○) during 60-min LAD-occlusion and 120-min reperfusion. Notice that in the LAD-area levels increased 500-fold during LAD-occlusion independent of the preceding intervention. Levels in the LCx -area and plasma remained unchanged.

Discussion

The major findings of the present study are: (*i*) global cerebral ischemia, produced by either a 10-min or a 30-min elevation of intracranial pressure, which by itself produced no irreversible myocardial damage, had no effect on myocardial infarct size produced by a 60-min coronary artery occlusion; (*ii*) intracoronary infusion of 0.03 nmol·kg^{-1}·min^{-1} norepinephrine produced similar increases in myocardial interstitial levels as cerebral ischemia, and did also not limit myocardial infarct size; (*iii*) conversely, intracoronary infusion of 0.12 nmol·kg^{-1}·min^{-1} of norepinephrine that resulted in five times higher myocardial

interstitial norepinephrine levels than cerebral ischemia and low dose norepinephrine was capable of limiting myocardial infarct size; (*iv*) the cardioprotection by exogenous norepinephrine was not caused by *ischemic* preconditioning and (*v*) this protection was not associated with a blunting of the progressive increase in myocardial interstitial norepinephrine levels during the coronary artery occlusion.

Catecholamines and myocardial injury

The relation between catecholamines and myocardial injury was first established by Rona and co-workers,[21,22] who showed some 40 years ago that administration of high systemic doses of isoproterenol produced focal necrotic lesions in normal rat hearts. Elevation of intracranial pressure is well recognized as a cause for myocardial dysfunction and injury. Brain death caused by increased intracranial pressure produces echocardiographic alterations, hemodynamic instability, and contraction band necrosis, all of which have been suggested to be the result of massive neuronal depolarization and release of catecholamines.[23-25] These clinical observations initiated a large number of experimental investigations in which deleterious effects of brain death on function and integrity of normal myocardium were found, but generally no or only minimal focal myocardial necrosis could be demonstrated.[26,27]

In view of the massive myocardial norepinephrine release during coronary artery occlusion,[15,28] it could be hypothesized that catecholamines may contribute to the development of irreversible injury during a coronary artery occlusion. Several,[29,30] though certainly not all,[31,32] studies have reported that β-adrenergic receptor blockade slows the development of myocardial infarction. In contrast, depletion of cardiac norepinephrine stores by reserpination, did not limit myocardial infarct size in rabbits[9] and dogs,[10] suggesting that endogenous catecholamines do not contribute to irreversible damage.

In contrast to the potentially deleterious effects of norepinephrine on normal and ischemic myocardium, this catecholamine has also been implicated in mediating cardioprotection by ischemic preconditioning. Thus, Toombs et al.[9] showed that in rabbits the protection by ischemic preconditioning was abolished when catecholamine stores in sympathetic nerve endings were depleted by reserpine. Furthermore, Thornton et al.[12] demonstrated in the same species that tyramine-induced norepinephrine release 10 min before a 30-min coronary artery occlusion also protected the myocardium. This cardioprotective action of catecholamines has been confirmed in other species such as the rat[13] and the dog.[10] We now show that a high dose of norepinephrine can also protect the porcine myocardium.

Our data on wall function, myocardial blood flow, O_2-extraction and proton release indicate that the high dose of norepinephrine did not produce myocardial ischemia and did therefore not protect the myocardium by ischemic preconditioning. The degree of protection afforded by norepinephrine is less than reported for ischemic preconditioning, but similar to that produced by other non-ischemic stimuli, like ventricular pacing[33] and pharmacological agents such as the K^+_{ATP} channel openers.[34] Since all these stimuli have in common that they ultimately activate K^+_{ATP} channels, it is tempting to speculate that norepinephrine also protected via α_1-adrenergic receptor-mediated protein kinase C activation and subsequent opening of (mitochondrial) K^+_{ATP} channels.[35] Another mechanism by which norepinephrine might protect the myocardium is via a blunted release in catecholamines during the sustained ischemic episode.[36] However, pretreatment with norepinephrine did not modify the release of cardiac norepinephrine during sustained myocardial ischemia in the present study, implying that the norepinephrine-mediated cardioprotection is not related to a blunting of the ischemia-induced increase in norepinephrine levels.

Finally, the present study clarifies another issue on the role of norepinephrine in cardioprotection. Przyklenk et al.[6] demonstrated that myocardial ischemia also elicited cardioprotection in adjacent virgin myocardium and speculated that this might have been triggered by a substantial catecholamine release in that adjacent region. However, we now show that norepinephrine levels in the normal (LCx-perfused) myocardium remained unaltered during and after the 60-min LAD-occlusion (Figure 4), even though the interstitial norepinephrine levels in the LAD-area were 100-fold higher than the value observed after 10 min of ischemia, corresponding to the period used by Przyklenk et al.[6] to precondition the adjacent virgin myocardium.

Cerebral ischemia as a stimulus for cardioprotection

Transient ischemia in small intestines, kidneys and skeletal muscle prior to a coronary artery occlusion can also be cardioprotective.[7,8] We therefore hypothesized that cerebral ischemia might similarly protect the myocardium, especially because cerebral ischemia is associated with substantial norepinephrine release, one of the mediators involved in cardioprotection by ischemic preconditioning. However, transient cerebral ischemia did not reduce myocardial infarct size in the present study. The explanation for the lack of protection might be two-fold. Firstly, 30 min of cerebral ischemia did not produce myocardial ischemia and could therefore not protect the myocardium via ischemic preconditioning. Secondly, although myocardial interstitial norepinephrine levels increased during cerebral ischemia, the rise was much less than during infusion of the high dose of norepinephrine, which elicited

cardioprotection. This is further corroborated by the findings with the low dose of norepinephrine, which produced similar interstitial myocardial norepinephrine levels as cerebral ischemia, and was also ineffective in protecting the heart.

It could be argued that even the 30-min global cerebral ischemia (CI_{30}) was too short to elicit cardioprotection. However, there is ample evidence that the intensity of the preconditioning stimulus is more important than its duration.[37] Moreover, because the elevation of myocardial interstitial norepinephrine levels occurred exclusively during the first 10 min of cerebral ischemia, it is unlikely that extending the period of cerebral ischemia would produce cardioprotection. On the contrary, it might be argued that the duration of the intracranial pressure elevation and recovery phase lasted *too long* as the maximum myocardial interstitial norepinephrine levels reached their peak during the first 10 min, so that a potential effect of that stimulus was lost by the time (50 min later) the LAD was occluded. This is supported by observations that the memory for cardioprotection is shorter when stimuli are used that do not cause myocardial ischemia.[33,34] However, when cerebral ischemia was maintained for only 10 min, and the cerebral reperfusion was shortened to 20 min (CI_{10}), infarct size after the 60-min coronary artery occlusion was also not different from control.

Finally, it can be excluded that a protective effect of transient global cerebral ischemia during the LAD-occlusion was masked by irreversible myocardial damage produced by transient global cerebral ischemia prior to the LAD-occlusion, as in the animals subjected to only 30 min of cerebral ischemia ($CI_{30,sham}$), irreversible damage could not be detected. This observation is in agreement with most experimental studies that have generally reported minimal or no focal myocardial necrosis following cerebral ischemia.[26,27]

Conclusions

In conclusion, global cerebral ischemia preceding a coronary artery occlusion did not modify myocardial infarct size, which is likely related to the modest increase in myocardial norepinephrine levels during cerebral ischemia. The infarct size limitation by the high dose of norepinephrine is not associated with a blunting of the increase in myocardial interstitial norepinephrine levels during the coronary occlusion.

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Chapter 9

Discussion and Summary

For years sympathetic activity in man and experimental animals has been assessed by measuring circulating catecholamines. As their concentration is not only a reflection of release but is also determined by clearance, other more precise methods for assessing sympathetic activity have been developed. The microdialysis technique allows for a semi-continuous measurement of interstitial concentrations in vivo, and is based on diffusion of the substance of interest from the intercellular space through a semi-permeable membrane into a suitable perfusion fluid like Ringer's or Ringer's lactate. Microdialysis can be particularly useful for monitoring sympathetic tone since it allows for the measurement of norepinephrine in the intercellular space, i.e. close to the sites of release and reuptake. In fact, interstitial norepinephrine levels are reported to correlate much more closely with regional sympathetic activity and its subsequent physiologic response than plasma norepinephrine concentrations.[1]

Another advantage of this technique is the possibility of modification of local processes such as neuronal reuptake and presynaptic inhibition of norepinephrine without provoking an unwanted systemic response, by adding the appropriate agent to the perfusion fluid.[2-4] In this thesis, we validated the microdialysis technique for measuring catecholamines (chapter 3) and used it to explore the pharmacokinetics (chapter 4), modulation (chapters 5 and 6) and pathophysiological role of norepinephrine (chapters 7 and 8) in the intact porcine heart.

Chapter 3. *Determination of catecholamines in microdialysis samples*

This chapter describes the validation and technical aspects of the measurement of catecholamines in microdialysis samples. We devised a sensitive and selective method in which natural and synthetic catecholamines in microdialysate samples are simultaneously measured after direct derivatization, without an extraction step, with the fluorogenic agent DPE followed by HPLC separation and fluorimetric detection. When the thus established derivatization procedure was executed on real microdialysis samples, we noticed that the fluorescence of dopamine, *l*-erythro-α-methyl-norepinephrine, norepinephrine

and its neuronal metabolite 3,4-dihydroxyphenylglycol was greatly inhibited, while the fluorescence of epinephrine, epinine and isoproterenol was unaffected. This phenomenon occurred in microdialysis samples from pigs and humans as well as in microdialysis samples from freshly obtained blood, albeit to different extents. Apparently, real microdialysis samples contain (an) unknown compound(s), which inhibit(s) the derivatization reaction. We found that the addition of the sulfhydryl reagent N-ethylmaleimide to the microdialysis samples was sufficient to overcome the inhibitory action of these unknown compound(s) on the derivatization reaction.

As a compromise between high relative but low absolute recovery at a perfusion rate of 0.5 μL/min, and low relative but high absolute recovery at 8 μL/min, a perfusion rate of 2 μL/min appeared satisfactory, and allowed for a sufficiently rapid sampling period of 10 min. At a perfusion rate of 2 μL/min, *in vitro* and *in vivo* recoveries of norepinephrine were similar (50-60%) and compared favorably with the scarce results given in the literature with different, laboratory-made microdialysis probes (23–41%).[5-7]

Chapter 4. *Catecholamine handling in the porcine heart*

This chapter concentrates on the pharmacokinetics of norepinephrine in the heart and its modulation by the neuronal reuptake mechanism. Because the mechanism underlying the large norepinephrine concentration gradient between the interstitial and intra-vascular compartments in the heart was uncertain, we investigated to what extent the concentration of norepinephrine in the myocardial intercellular fluid relates to its concentration in the arterial and coronary venous circulation at baseline and after inducing increments in plasma or interstitial norepinephrine concentration either by systemic infusions of norepinephrine or by an intracoronary infusion of tyramine. In addition, the importance of neuronal reuptake to the interstitial norepinephrine concentration was assessed by perfusing one of the probes with the neuronal reuptake-inhibitor desipramine and by performing studies with isoproterenol, a synthetic catecholamine that is not handled by neuronal reuptake. Finally, adaptation of the method introduced by Kopin et al.[8] provided an estimate of spillover, uptake and consequently, release of norepinephrine.

The experiments largely confirmed the data about norepinephrine kinetics obtained by more indirect but established methods. We concluded that neuronal reuptake as well as extra-neuronal uptake and not an endothelial barrier are the principal mechanisms underlying the concentration gradient of norepinephrine between the interstitial and intra-vascular compartments in the porcine heart.

Chapter 5. *Epinephrine in the heart*

This chapter describes to which extent epinephrine is taken up by and released from cardiac sympathetic nerves and whether it can increase myocardial interstitial norepinephrine concentrations under basal conditions and during sympathetic activation.[9] This study showed that epinephrine is readily taken up from the circulation by the heart, and that 70% is extracted over the cardiac vascular bed. In contrast to norepinephrine (chapter 4) most of the extracted epinephrine is cleared by other mechanisms than neuronal reuptake. In addition, we demonstrated that epinephrine is released during infusion of tyramine after loading the cardiac sympathetic nerve terminals with exogenous epinephrine, indicating that cardiac epinephrine is derived from the storage vesicles of sympathetic nerves within the myocardium. This observation is in line with the results of the study described in chapter 7 demonstrating ischemia-induced epinephrine release, which was modulated by the neuronal reuptake mechanism. With respect to the function of cardiac epinephrine, we did not observe any augmentation of myocardial norepinephrine release by epinephrine either under basal conditions or during activation of cardiac sympathetic tone induced by left stellate ganglion stimulation.

Hence, we hypothesize that the uptake of epinephrine by the heart is principally a mechanism for rapid clearance of circulatory epinephrine, and that the small amount of locally released epinephrine does not modulate left ventricular function.

Chapter 6. *Angiotensin II and norepinephrine release*

In this chapter we investigate the interaction between the sympathetic nervous system and the renin-angiotensin-system. Activation of the sympathetic nervous system simultaneously leads to activation of the renin-angiotensin-system via stimulation of β-adrenergic receptors within the kidney resulting in an increased renin release. There is also, albeit conflicting, evidence that the sympathetic nervous system is activated by the renin-angiotensin-system.[10-22] This activation supposedly occurs through stimulation of angiotensin II receptors within the central nervous system and/or stimulation of presynaptic angiotensin II receptors located at sympathetic nerve terminals. As evidence that the cardiac sympathetic nervous system is activated by angiotensin II *in vivo* is scarce [13] or indirect,[16,23] we determined whether physiological (pM) to pathophysiological (nM) concentrations of angiotensin II modulated interstitial

norepinephrine concentrations in the intact porcine heart under various conditions. Our results provided no evidence for facilitation of cardiac norepinephrine release by angiotensin II, as intracoronary infusion of angiotensin II did not modulate (i) basal sympathetic tone, (ii) exocytotic norepinephrine release during sympathetic activation produced by left stellate ganglion stimulation, and (iii) exocytotic and nonexocytotic norepinephrine release during myocardial ischemia.

Chapter 7. *Cardiac ischemia and local catecholamine release*

In this chapter we explore the fate and potential role of catecholamines and in particular of norepinephrine in myocardial ischemia. This study demonstrated that myocardial ischemia is associated with a rapid and massive increase of the concentration of all three endogenous catecholamines norepinephrine, epinephrine and dopamine in the myocardial interstitial fluid as measured with the microdialysis technique *in vivo*. As suggested for norepinephrine in *in vitro* studies,[24,25] the reversed neuronal reuptake mechanism played an important role in the release of all three catecholamines during ischemia *in vivo*.

Furthermore, this study showed that after 60 min of ischemia, which resulted in massive infarction of the jeopardized myocardium, the functional integrity of sympathetic nerve terminals remained intact. Finally, our results indicated that cardiac interstitial epinephrine has a neuronal origin (see also chapter 5).

Although the pathophysiological significance of the massive accumulation of catecholamines in the ischemic myocardial tissue was not investigated in the present study, there is evidence from experimental as well as clinical studies that high catecholamine concentrations are deleterious to the heart.[26-29] On the other hand, several studies have demonstrated norepinephrine-dependent anti-arrhythmic effects of neuronal reuptake-inhibition during ischemia either by tricyclic anti-depressant agents like desipramine and imipramine or by structurally unrelated inhibitors of neuronal reuptake like cocaine and nisoxetine.[30,31] Our study provided a possible explanation for this beneficial effect by demonstrating that the reversed neuronal reuptake mechanism contributes substantially to the release of catecholamines during ischemia.

Chapter 8. *Cerebral ischemia, norepinephrine and cardioprotection*

This chapter describes the role of norepinephrine in local and remote preconditioning by cerebral ischemia. As norepinephrine is one of the mediators involved in the signaling pathway leading to ischemic preconditioning,[32,33] and because cerebral ischemia causes a profound release of norepinephrine from sympathetic nerve endings in normal myocardium,[34] this raises the question whether transient cerebral ischemia prior to a coronary artery occlusion may also be cardioprotective.

Global cerebral ischemia, induced by a substantial elevation of intracranial pressure, increased cardiac interstitial norepinephrine concentrations three-fold. Cerebral ischemia, which by itself caused no irreversible myocardial damage, had no effect on the myocardial infarct size produced by a 60-min coronary artery occlusion. Intracoronary infusion of 0.03 $nmol \cdot kg^{-1} \cdot min^{-1}$ of norepinephrine induced similar increases in myocardial interstitial levels as cerebral ischemia and did not limit myocardial infarct size either. Conversely, 0.12 $nmol \cdot kg^{-1} \cdot min^{-1}$ of norepinephrine, which resulted in myocardial interstitial norepinephrine levels five times higher than cerebral ischemia, limited myocardial infarct size by more than 20%. The cardioprotection by exogenous norepinephrine was not caused by *ischemic* preconditioning nor was it associated with a blunting of the progressive increase in myocardial interstitial norepinephrine levels during coronary artery occlusion.

Suggestions for future research

The studies described in this thesis show that microdialysis is a valuable tool for measuring as well as for modifying local sympathetic activity. The application of this technique allowed a further extension of the knowledge concerning the kinetics, modulation and pathophysiological role of the cardiac sympathetic nervous system. Nevertheless, many questions remain, of which several will be addressed in this paragraph.

Apart from using microdialysis in acute experiments, i.e. experiments within a timeframe of 6-12 hours, we have also tried to apply the technique in chronic experiments that can take up to 6 weeks. This would have allowed us to monitor cardiac sympathetic activity during the phases of remodeling and the development of heart failure after inducing myocardial infarction. This is of particular interest since it has been suggested that sympathetic activity increases during these two processes.[23,35-41] Unfortunately, up till now the application of microdialysis in these chronic experiments has been unsuccessful because of

two major problems; (*i*) the continuous friction of the beating of the heart for 6 weeks on end (~6 million heartbeats) has proven to be too much for the delicate microdialysis probes; (*ii*) the insertion of a foreign body like a microdialysis probe in the myocardium results in an inflammatory reaction in the surrounding tissue. The subsequent fibrosis progressively hampers the diffusion of the catecholamines from the interstitial space into the perfusate, so that the interstitial norepinephrine concentration no longer reflects myocardial sympathetic activity. Fortifying the area where the in- and outlet tubes are glued to the microdialysis probes with silicone has solved the friction problem, but the problem of fibrosis is practically unsolvable. Hence, the solution lies in conducting acute microdialysis experiments both at the beginning and end of the chronic intervention. While this won't allow for continuous monitoring of sympathetic activity during remodeling and heart failure, it can provide insight into the changes in sympathetic activity associated with these processes. For instance, since some reports suggest that neuronal reuptake is affected in heart failure,[42-44] it might be interesting to investigate this phenomenon in our porcine heart failure model using microdialysis with tyramine and desipramine (see also chapter 7). As there is evidence that the facilitation of norepinephrine release by angiotensin II is particularly apparent in heart failure, [23,45-48] it would also be interesting to verify our findings in chapter 6 during heart failure.

Laser-induced-fluorescence, a new analytical technique that has recently been introduced in our laboratory, allows for an even more sensitive determination of catecholamines. In addition, it is probably also suitable for the determination of peptides like angiotensins and bradykinin. If this technique comes of age, it would open up a vast array of opportunities for future research. For instance, the microdialysis of angiotensins from the cardiac interstitium has proven to be troublesome, as the two studies on this subject report large differences in interstitial angiotensin II concentrations.[49,50] A more sensitive direct determination of angiotensin I and II might be able to resolve this matter and could provide important pharmacokinetic information on the distribution of these peptides over the circulatory, intercellular and intracellular compartments. Moreover, this could facilitate the investigation of the role and modulation of these peptides and their receptors.

Last but not least, while the physiology and pathophysiology of the porcine heart are akin to that of the human heart, it would be very interesting to use the microdialysis technique to measure local sympathetic activity in humans. Obviously, it will not be feasible to monitor the cardiac sympathetic activity in humans with microdialysis, but a more sensitive determination of catecholamines would allow for measuring interstitial catecholamine concentrations in skin and muscle. Until now, we were only able to measure interstitial norepinephrine concentrations in subcutaneous fat of patients with

very high endogenous plasma norepinephrine concentrations (e.g. patients with a norepinephrine-producing pheochromocytoma, Figure 1).

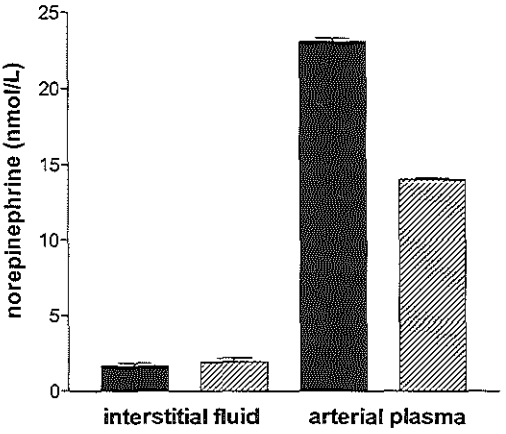

Figure 1. Interstitial (subcutaneous fat) and arterial plasma norepinephrine concentrations of two patients with a norepinephrine-producing pheochromocytoma. Data are shown for patient 1 (solid bars) and patient 2 (hatched bars) as mean±SD (6 samples per patient).

The combination of microdialysis with strain-gauge plethysmography, a technique that is used in our laboratory to measure forearm blood flow, is particularly appealing as it would be possible to monitor and modulate forearm muscle sympathetic activity in a flow-controlled experimental setup, which is essential to pharmacokinetic studies because of flow-induced changes in clearance of norepinephrine.[51] For instance, adding the measurement of muscle sympathetic nerve activity to this combination would allow for the investigation of the relationship between nerve-firing, interstitial norepinephrine concentrations and spillover. This would be of particular interest in patients with heart failure as the mechanism underlying the associated increase in sympathetic tone is still unclear.[43,52] Such an approach could distinguish whether the activation of the sympathetic nervous system is due to augmented sympathetic nerve traffic or to an increase in interstitial norepinephrine concentrations as a result of diminished clearance, e.g. due to a failing neuronal reuptake.

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Nederlandse samenvatting

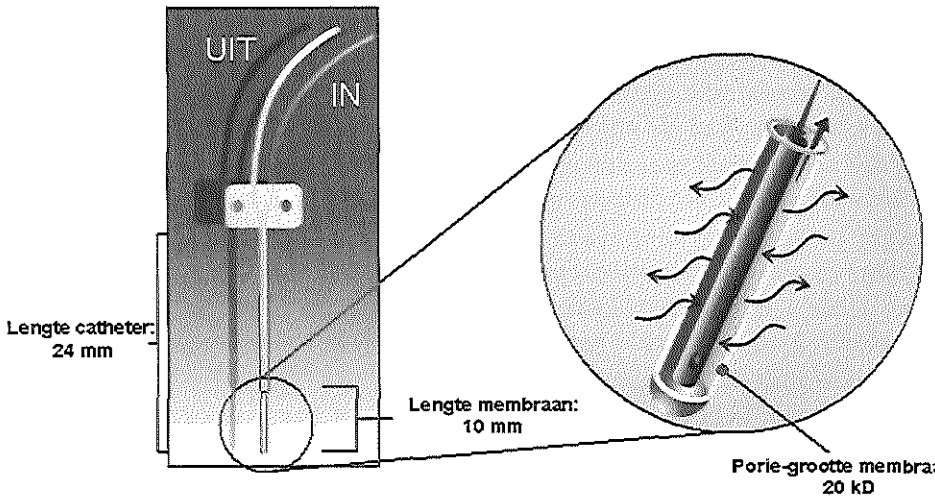
Het hart is de drijvende kracht achter de bloedsomloop en daarmee essentieel voor de zuurstofvoorziening van het lichaam. Het hart klopt spontaan, maar de reactie op verandering in activiteit van het lichaam wordt gestuurd door twee tegengesteld werkende systemen die vanuit de hersenen de hartfunctie en bloeddruk regelen. Aan de ene kant is er het sympathische zenuwstelsel dat de bloedsomloop aanpast aan een verhoogde zuurstofbehoefte van het lichaam. Bijvoorbeeld tijdens hardlopen gaat het hart niet alleen sneller doch ook krachtiger pompen, de bloedvaten in de voor de activiteit benodigde spieren verwijden, terwijl de bloeddoorstroming in andere gebieden van het lichaam die niet bij de activiteit betrokken zijn zoals de ingewanden juist afneemt. Aan de andere kant is er het parasympathische zenuwstelsel dat de bloeddoorstroming aanpast aan inactiviteit en herstel. Wanneer het parasympathische zenuwstelsel overheerst klopt het hart rustiger en verwijden de bloedvaten in organen als de darmen, nieren en lever zodat de bloedtoevoer daar toeneemt.

Toename van de sympathische activiteit leidt tot een verhoogde afgifte van de neurotransmitter noradrenaline uit de sympathische zenuwuiteinden en van vooral adrenaline uit het bijniermerg. Noradrenaline en adrenaline binden zich aan receptoren in bijvoorbeeld hart en bloedvaten en oefenen zo hun werking uit. Vanwege hun chemische structuur behoren beiden tot de catecholamines. Dopamine is een derde catecholamine, en is met name een belangrijke neurotransmitter in de hersenen en tevens de biochemische voorloper van noradrenaline en adrenaline.

Over het algemeen wordt de activiteit van het sympathische zenuwstelsel gemeten door de concentratie van catecholamines in het bloed te meten. De concentratie van deze catecholamines in het bloed hangt echter niet alleen af van de mate waarin zij worden geproduceerd en worden vrijgemaakt, maar ook van de snelheid waarmee zij worden geklaard hetzij door heropname in de sympathische zenuwuiteinden (het neuronale opnamemechanisme) hetzij door afbraak. Ter bepaling van de sympathische activiteit zijn de afgelopen jaren zijn er dan ook methoden ontwikkeld die niet van de catecholamine-concentratie in het bloed afhankelijk zijn, hetzij door specifiek de uitstoot van noradrenaline in het bloed te meten met behulp van radioactief gemerkt noradrenaline, hetzij door de elektrische impulsen van de sympathische zenuwen te registreren.

Microdialyse is een nieuwe techniek waarmee via dunne kathetertjes de concentratie van stoffen in de vloeistof tussen de cellen (de zgn. interstitiële

vloeistof) bijna onafgebroken kan meten. Deze techniek is gebaseerd op diffusie van stoffen vanuit de interstitiële vloeistof door een halfdoorlatende membraan dat alleen kleine moleculen doorlaat, naar een geschikte vloeistof die heel traag (enkele microliters per minuut) door de katheter gepompt wordt (het zgn. perfusaat) zoals de Ringers-oplossing (zie figuur 1).

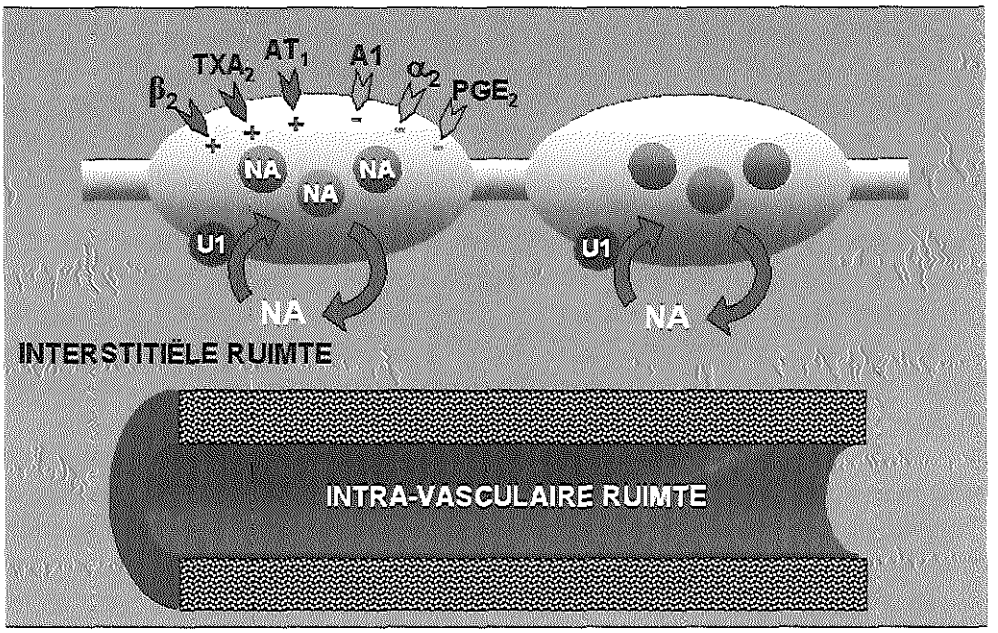


Figuur 1. De microdialyse katheter. **IN**: slangetje waarlangs het perfusaat naar binnen gaat; **UIT**: slangetje waarlangs het dialysaat naar buiten komt. Afgel eid van de internetpagina van Carnegie Medicine AB (http://www.microdialysis.se).

Microdialyse is bij uitstek geschikt om de regionale sympathische activiteit te meten, omdat met deze techniek de concentratie van noradrenaline dichtbij de plaatsen van uitstoot en opname kan worden gemeten (figuur 2). Dit wordt nog eens bevestigd door experimenten die hebben laten zien dat de interstitiële noradrenaline concentratie beter met de regionale sympathische activiteit en de daaraan verbonden fysiologische respons overeenkomt dan de noradrenaline concentratie in het bloed.[1]

Een ander voordeel van deze techniek is dat niet alleen stoffen kunnen worde opgevangen in de dialysevloeistof, maar ook lokale processen als neuronale opname en presynaptische inhibitie (figuur 2) kunnen worden beïnvloed door toevoeging van pharmaca aan het perfusaat. De pharmaca in het perfusaat zullen op dezelfde wijze vanuit het perfusaat naar het interstitium diffunderen als de stoffen in de interstitiële vloeistof naar de dialysevloeistof.[2-4]

Dit proefschrift gaat in op de functie van het sympathische zenuwstelsel in het hart, en dan met name op die van de neurotransmitter noradrenaline. Na de inleiding (hoofdstuk 1), het uiteenzetten van de doelstellingen (hoofdstuk 2), en de bespreking van de techniek (hoofdstuk 3) wordt de rol van catecholamines bij de fysiologie van het hart besproken:



Figuur 2. Sympathische zenuwuiteinden in het hart: presynaptische facilitatie en inhibitie. Getoond zijn de uitstulpingen van de zenuwuiteinden met daarin de blaasjes waarin noradrenaline (NA) opgeslagen is, het neuronale opname mechanisme (U1), de presynaptische receptoren die de afgifte van noradrenaline faciliteren (+), bijv. de β_2-adrenergische receptor (β_2), de thromboxaan type 2 receptor (TXA_2) en de angiotensine-II type 1 receptor (AT_1), evenals de receptoren die de afgifte remmen (-), bijv. de adenosine A1-receptor (A1), de α_2-adrenerge receptor (α_2) en de prostaglandine PGE_2-receptor (PGE_2).

hoeveel noradrenaline (hoofdstuk 4) en adrenaline (hoofdstuk 5) wordt er in het hart opgenomen, hoeveel wordt er door de zenuwuiteinden uitgestoten en welke stoffen kunnen deze opname en afgifte beïnvloeden (hoofdstuk 6). In de laatste twee hoofdstukken wordt ook de rol van noradrenaline bij de pathofysiologie van het hart onderzocht: zowel de associatie met zuurstoftekort en de daarmee gepaard gaande hartritmestoornissen (hoofdstuk 7), als ook de betrokkenheid bij juist de bescherming van het hart tegen zuurstoftekort (hoofdstuk 8). De hoofdstukken 3 tot en met 8 hebben betrekking op de werkelijke studies die in het kader van dit proefschrift gedaan zijn en zullen hieronder per hoofdstuk kort behandeld worden.

Hoofdstuk 3. *De bepaling van catecholamines in microdialyse monsters*

Dit hoofdstuk beschrijft de technische aspecten en validatie van de bepaling van catecholamines in microdialyse-monsters. Wij ontwikkelden een gevoelige en selectieve methode waarmee natuurlijke en synthetische catecholamines

gelijktijdig gemeten konden worden in zeer kleine volumina van kunstmatige oplossingen na derivatisering met een fluorophoor gevolgd door HPLC scheiding en fluorimetrische detectie. Toen de methode werd toegepast op 'echte' (*in vivo*) microdialyse-monsters, bleek dat de fluorescentie van dopamine, α-methylnoradrenaline, noradrenaline en 3,4-dihydroxyphenylglycol, de neuronale metaboliet van noradrenaline, sterk was geremd, terwijl de fluorescentie van adrenaline, epinine and isoprenaline onaangedaan was. Dit fenomeen trad op bij microdialyse monsters van zowel varkens als patiënten. *In vivo* microdialyse monsters bevatten klaarblijkelijk een of meer onbekende stoffen die de derivatiseringsreactie kunnen remmen. De toevoeging van het sulfhydryl reagens N-ethylmaleimide bleek voldoende om de remmende werking op de derivatisering te niet te doen.

Bij wijze van compromis tussen een hoge relatieve maar lage absolute opbrengst bij een perfusiesnelheid van 0.5 µl/min, en een lage relatieve maar hoge absolute opbrengst bij 8 µl/min, bleek een perfusiesnelheid van 2 µl/min optimaal. Bij een perfusiesnelheid van 2 µl/min waren de relatieve *in vitro* en *in vivo* opbrengsten voor noradrenaline nagenoeg gelijk (50-60%)en kwamen goed overeen met die zoals beschreven in de schaarse studies waarin meestentijds gebruik gemaakt werd van zelfgemaakte microdialyse katheters (23-41%).[5-7]

Hoofdstuk 4. *De farmacokinetiek van catecholamines in het varkenshart*

Dit hoofdstuk concentreert zich op de farmacokinetiek van noradrenaline in het hart en de beïnvloeding daarvan door het neuronale opnamemechanisme. Tussen de interstitiële en de intravasculaire ruimten bestaat een grote gradiënt in noradrenalineconcentraties. Het mechanisme dat hieraan ten grondslag ligt is tot op heden nog onopgehelderd. Daarom onderzochten wij hoe de noradrenalineconcentratie in de interstitiële vloeistof van het hartspierweefsel zich verhoudt tot de concentratie in het arteriële en het veneuze bloed onder basale omstandigheden en na het verhogen van zowel de interstitiële noradrenalineconcentratie als de noradrenalineconcentratie in het bloed. De concentratie in het bloed werd verhoogd door noradrenaline intraveneus toe te dienen, terwijl de interstitiële noradrenalineconcentratie werd verhoogd door infusie van tyramine via een van de kransslagaders. Tyramine heeft een hogere affiniteit voor de opslageiwitten van noradrenaline en verdrijft zodoende na opname in het zenuwuiteinde via het neuronale opnamemechanisme noradrenaline uit de opslagblaasjes en vervolgens uit de zenuwuiteinden (figuur 2). Ook werd het belang van de neuronale opname voor de hoogte van de noradrenalineconcentratie onderzocht door een van de microdialyse katheters te perfunderen met de neuronale opnameremmer desipramine, en middels

experimenten met isoprenaline, een synthetisch catecholamine dat niet door het neuronale opnamemechanisme opgenomen wordt. Tenslotte maakte aanpassing van de methode beschreven door Kopin et al.[8] het mogelijk spillover, opname en uitstoot van noradrenaline uit de zenuwuiteinden te berekenen.

De experimenten bevestigden in belangrijke mate de data aangaande de farmacokinetiek van noradrenaline zoals verkregen met indirecte benaderingen. Wij concluderen dat vooral neuronale en in minder mate extraneuronale opname en niet een endotheliale barrière verantwoordelijk zijn voor de genoemde concentratiegradiënt van noradrenaline tussen de interstitiële en intravasculaire ruimten in het varkenshart.

Hoofdstuk 5. *Adrenaline in het hart*

Dit hoofdstuk beschrijft in welke mate adrenaline wordt opgenomen en uitgestoten door de sympathische zenuwuiteinden in het hart. Tevens werd onderzocht of de interstitiële noradrenalineconcentratie onder invloed van adrenaline kan stijgen. Deze studie laat zien dat adrenaline gemakkelijk vanuit de bloedbaan wordt opgenomen door het hart. Van het via de kransslagader aangeboden adrenaline wordt 70% onttrokken. In tegenstelling tot wat we zagen voor noradrenaline (hoofdstuk 4), wordt het grootste deel van dit onttrokken adrenaline geklaard door andere mechanismen dan neuronale opname. Ook kon worden aangetoond dat adrenaline uitgestoten wordt tijdens infusie met tyramine mits het hart tevoren was opgeladen met adrenaline, hetgeen suggereert dat het cardiale adrenaline afkomstig is uit de opslagblaasjes in de sympathische zenuwuiteinden van het hart. Deze waarneming komt overeen met de resultaten van de studie beschreven in hoofdstuk 7, waarin aangetoond wordt dat de door zuurstoftekort geïnduceerde uitstoot van adrenaline gemoduleerd kan worden door het neuronale opnamemechanisme. Alhoewel gesuggereerd is dat het cardiale adrenaline de uitstoot van noradrenaline uit sympathische zenuwuiteinden bevordert, konden wij dat in onze studies niet bevestigen; noch onder basale omstandigheden noch gedurende activatie van de sympathicus tonus door elektrische stimulatie van het linker *ganglion stellatum* (het laatste sympathische neuronale 'tussenstation' tussen de hersenen en het hart). Op grond van deze waarnemingen denken we dat de opname van adrenaline door het hart voornamelijk een mechanisme is voor de snelle klaring van circulerend adrenaline waardoor de werkingsduur van adrenaline zo kort mogelijk is.

Hoofdstuk 6. *Angiotensine-II en de afgifte van noradrenaline*

Dit hoofdstuk beschrijft de interactie tussen het sympathische zenuwstelsel en het renine-angiotensine-systeem in het hart. Activatie van het sympathische zenuwstelsel leidt gelijktijdig tot activatie van het renine-angiotensine-systeem via stimulatie van β-adrenerge receptoren in de nier met als gevolg een toegenomen afgifte van renine aan het bloed. Er zijn echter ook aanwijzingen, hoewel niet eenduidig, dat het sympathische zenuwstelsel kan worden geactiveerd door het renine-angiotensine-systeem.[9-21] Deze wijze van activatie zou bewerkstelligd worden via stimulatie van angiotensine-II receptoren in de hersenen en/of presynaptische angiotensine-II receptoren op de sympathische zenuwuiteinden (figuur 2). Omdat aanwijzingen voor het bestaan van een dergelijke interactie in het hart *in vivo* schaars [12] en indirect [15,22] zijn, onderzochten wij of fysiologische (10^{-12} mol/l) tot pathofysiologische (10^{-9} mol/l) angiotensine-II concentraties onder diverse omstandigheden de interstitiële noradrenaline concentratie in het intacte varkenshart beïnvloeden. In onze studie kon geen facilitatie van de noradrenalineafgifte door angiotensine-II worden aangetoond: toediening van angiotensine-II via een kransslagader veranderde noch de basale sympathicus tonus van het hart, noch de noradrenalineafgifte tijdens sympathische activatie, bewerkstelligd door respectievelijk ganglionstimulatie en acuut zuurstoftekort van het hartspierweefsel.

Hoofdstuk 7. *Zuurstoftekort van het hart en lokale afgifte van catecholamines*

In dit hoofdstuk worden het lot en de mogelijke rol van catecholamines en met name noradrenaline tijdens acuut zuurstoftekort van het hartspierweefsel behandeld. Onze resultaten laten zien dat dit zuurstoftekort is geassocieerd met een snelle massale stijging van de interstitiële concentratie van noradrenaline, adrenaline en dopamine. Zoals eerder gesuggereerd voor noradrenaline in *in vitro* studies,[23,24] wordt deze uitstoot in belangrijke mate veroorzaakt door omkering van het neuronale opnamemechanisme. Bovendien laat deze studie zien dat na een 60 minuten durende afsluiting van de kransslagader waarbij meer dan 80% van het bedreigde hartspierweefsel afsterft, de functie van de sympathische zenuwuiteinden behouden blijft.

Hoewel de pathofysiologische consequentie van de buitengewoon hoge noradrenalineconcentratie in het zuurstofarme hartspierweefsel in deze studie niet is onderzocht, zijn er aanwijzingen vanuit experimentele en klinische studies dat hoge catecholamineconcentraties toxisch zijn voor het hart.[25-28] Ook is uit onderzoek gebleken dat tricyclische antidepressiva zoals desipramine en

imipramine, als ook andere niet-verwante remmers van het neuronale opnamemechanisme zoals cocaïne en nisoxetine, het ontstaan van noradrenaline-afhankelijke ritmestoornissen tijdens zuurstoftekort van het hart remmen.[29,30] Onze studie geeft een mogelijke verklaring voor dit gunstige effect door aan te tonen dat de omkering van het neuronale opnamemechanisme in belangrijke mate bijdraagt aan de uitstoot van catecholamines tijdens zuurstoftekort van het hart.

Hoofdstuk 8. *Zuurstoftekort van de hersenen, noradrenaline en bescherming van het hart*

In dit hoofdstuk wordt de rol van noradrenaline bij lokale preconditionering en preconditionering op afstand door zuurstoftekort van de hersenen beschreven. Korte perioden van zuurstoftekort kunnen het hart beschermen tegen een daaropvolgende langere periode van zuurstoftekort, de zgn. ischemische preconditionering. Deze bescherming is in meerdere organen zoals het hart en de hersenen aangetoond en kan ook op afstand plaatsvinden: kortdurende perioden van zuurstoftekort in een ander gebied binnen het betreffende orgaan of zelfs in een ander orgaan kunnen het orgaan beschermen tegen een volgende periode van zuurstoftekort. Omdat zuurstoftekort van de hersenen gepaard gaat met een forse uitstoot van noradrenaline vanuit de sympathische zenuwuiteinden in normaal hartspierweefsel [33] en omdat noradrenaline betrokken is bij de signaaloverdracht van preconditionering,[31,32] rijst de vraag of een korte periode van zuurstoftekort van de hersenen voorafgaand aan afsluiting van een kransslagader het hart tegen het daaropvolgende zuurstoftekort beschermt.

Zuurstoftekort van de hersenen, bewerkstelligd door een sterke verhoging van de hersendruk, leidde tot een drievoudige stijging van de interstitiële noradrenalineconcentratie in het hart. Het zuurstoftekort van de hersenen had geen effect op de grootte van het hartinfarct veroorzaakt door afsluiting van een kransslagader. Het toedienen van een relatief lage hoeveelheid noradrenaline via de kransslagader met vergelijkbare interstitiële noradrenalinespiegels als tijdens zuurstoftekort van de hersenen verkleinde het hartinfarct evenmin. Echter, toediening van een grotere hoeveelheid noradrenaline, leidend tot vijfmaal hogere interstitiële noradrenalinespiegels dan de eerder genoemde interventies, verkleinde daarentegen het aangedane gebied met meer dan 20%. Deze bescherming door exogeen noradrenaline was niet te wijten aan ischemische preconditionering ten gevolge van noradrenaline-gemedieerd zuurstoftekort noch was deze geassocieerd met verminderde uitstoot van noradrenaline gedurende de afsluiting van de kransslagader.

Suggesties voor verder onderzoek

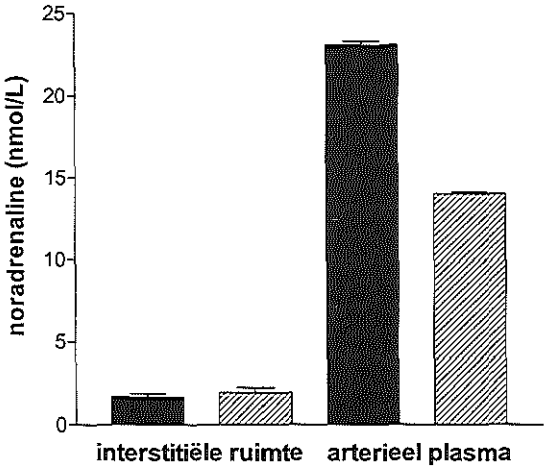
De studies in dit proefschrift tonen aan dat microdialyse een waardevol instrument is om de lokale sympathische activiteit te meten en te moduleren. De toepassing van deze techniek heeft onze kennis over de rol van het cardiale sympathische zenuwstelsel verder verdiept. Desalniettemin blijven nog veel vragen onbeantwoord, waarvan enkele in deze paragraaf behandeld zullen worden.

Naast het gebruik van microdialyse bij kortdurende experimenten (6-12 uur), hebben wij ook getracht de techniek aan te wenden bij chronische experimenten die wel 6 weken kunnen duren. Dergelijke experimenten zouden het mogelijk gemaakt hebben de sympathische activiteit tijdens de verschillende fases van het remodelleringsproces en de ontwikkeling van hartfalen na een hartinfarct te volgen. Dit is met name van belang omdat er duidelijke aanwijzingen zijn dat de sympathische activiteit tijdens deze processen toegenomen is.[22,34-40] Helaas bleek chronische microdialyse in het hart tot op heden niet erg succesvol vanwege twee belangrijke problemen: (*i*) de microdialyse-kathetertjes waren niet bestand tegen de continue frictie van het kloppen van het hart gedurende 6 weken (~6 miljoen hartslagen), met als gevolg lekkage of verstopping; (*ii*) de aanwezigheid van lichaamsvreemd materiaal zoals microdialyse-kathetertjes in het hartspierweefsel leidt tot een ontstekingsreactie in het omliggende weefsel. De daaropvolgende fibrose hindert de diffusie van de catecholamines vanuit de interstitiële ruimte naar het perfusaat zodanig dat de interstitiële noradrenaline concentratie niet langer de lokale sympathische activiteit vertegenwoordigt. Het verstevigen van de plaats waar de slangetjes voor de in- en afvoer in het microdialyse-kathetertje samengelijmd zijn met siliconenkit heeft het frictieprobleem weliswaar opgelost, maar het fibroseprobleem is nagenoeg onoplosbaar. Zodoende ligt de oplossing in het doen van kortdurende experimenten aan het begin en einde van de chronische interventie. Hoewel dit niet een volledig beeld zal kunnen geven van de veranderingen in sympathische activiteit tijdens remodellering en hartfalen, verschaft een dergelijke opzet wel inzicht in de grove veranderingen in activiteit die met deze processen in verband gebracht worden.

Aangezien er enige aanwijzingen zijn dat de neuronale opname van noradrenaline bij hartfalen gestoord is,[41-43] kan het zeker waardevol zijn om dit fenomeen in ons hartfalenmodel bij varkens te onderzoeken met tyramine en desipramine (zie ook hoofdstuk 7). In het zelfde diermodel zou het ook interessant zijn onze resultaten in hoofdstuk 6 nog eens te verifiëren omdat een aantal studies hebben aangetoond dat de facilitatie van noradrenaline door angiotensine-II bij hartfalen in het bijzonder een belangrijk mechanisme is.[22,44-47]

Recentelijk is in ons laboratorium een nieuwe analytische techniek, de laser-geïnduceerde-fluorescentie, geïntroduceerd. Met deze techniek neemt de gevoeligheid van de bepaling van catecholamines belangrijk toe. Daarenboven zou met deze techniek de bepaling van peptiden als bradykinine en angiotensines in microdialyse-monsters wellicht eenvoudiger worden. De twee studies die met conventionele methoden de concentratie van angiotensine-II in microdialyse-monsters hebben gemeten, rapporteren sterk uiteenlopende interstitiële angiotensine-II concentraties in het hart.[48,49] Een gevoeliger en directere techniek om angiotensine-I en II te bepalen zou de werkelijke interstitiële angiotensine-II concentratie wellicht beter kunnen benaderen en bovendien belangrijke informatie kunnen verschaffen over de farmacokinetiek van deze peptiden en hun verdeling over de circulatoire, inter- en intracellulaire compartimenten. Hierdoor zou aan het onderzoek naar de rol en modulatie van deze peptiden en hun receptoren een krachtige nieuwe impuls gegeven kunnen worden.

Hoewel de fysiologie als ook de pathofysiologie van het varkenshart sterk overeenkomt met die van het hart van de mens, zou het zeer aantrekkelijk zijn de microdialyse techniek aan te wenden om de lokale sympathische activiteit bij de mens te meten. Het is weliswaar niet mogelijk zonder meer de sympathische activiteit met microdialyse bij de mens in het hart te meten, doch met een gevoeliger bepaling van catecholamines zou het wel mogelijk zijn de sympathische activiteit in subcutaan vet en spier te meten. Tot op heden is het ons alleen gelukt de interstitiële noradrenaline concentratie in subcutaan vet te meten bij patiënten met sterk verhoogde noradrenalinespiegels in het bloed, zoals bij patiënten met een noradrenaline-producerende bijniertumor (zie figuur 3).



Figuur 3. Interstitiële (subcutaan vet) en arteriële noradrenalineconcentraties van twee patiënten met een noradrenaline-producerende bijniertumor.
Getoond worden de data van patiënt 1 (dichte balken) en patiënt 2 (gearceerde balken) als gemiddelde±SD (6 monsters per patiënt).

De combinatie van microdialyse met "strain-gauge"-plethysmografie, een techniek die in ons laboratorium gebruikt wordt om de bloedstroom (flow) door de onderarm te meten, lijkt zeer aantrekkelijk aangezien het hierdoor mogelijk is de sympathische activiteit in de onderarm te meten en te moduleren onder flow-gecontroleerde omstandigheden. Dit laatste is bij farmacokinetische studies essentieel vanwege de invloed van flow op de klaring van noradrenaline.[50] Het uitbreiden van deze combinatie met het gelijktijdig meten van de (elektrische) zenuwactiviteit in de spieren van de onderarm biedt de mogelijkheid de relatie tussen de werkelijke zenuwactiviteit, de interstitiële noradrenaline concentratie en spillover te onderzoeken. Zulk onderzoek is met name waardevol bij patiënten met hartfalen, aangezien het mechanisme achter de met hartfalen geassocieerde toename van sympathische activiteit nog altijd niet opgehelderd is.[42,51] Met deze benadering zou onderscheid gemaakt kunnen worden of de activatie van het sympathische zenuwstelsel het gevolg is van een toename in elektrische activiteit in de sympathische zenuwen dan wel van verhoogde interstitiële noradrenalineconcentraties, bijvoorbeeld als gevolg van een falend neuronaal opnamemechanisme.

N.B. De voertaal in dit proefschrift is het Amerikaans-Engels, de stofnamen noradrenaline, adrenaline en isoprenaline worden dan ook in de rest van dit proefschrift gespeld als respectievelijk norepinephrine, epinephrine en isoproterenol.

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Zoals op de experimentele cardiologie waren er op mijn eigen afdeling velen die hun bijdrage al dan niet bewust geleverd hebben. Het voormalig hoofd van de afdeling, Prof.dr. M.A.D.H. Schalekamp: Maarten, mijn dank voor het gedogen van mijn aanwezigheid en je waardevolle bijdrage aan mijn angiotensine – komt het er nu wel of niet – probleem. Mijn gewezen promotor en initiator van de microdialyse op onze afdeling, Prof.dr. A.J. Man in 't Veld: Arie, mijn hartelijke dank voor mijn aanstelling; je enthousiasme en creatieve geest worden mijns inziens node gemist. Dr. J. (het geweten) Deinum: Jaap, mijn hartelijke dank voor al onze vurige discussies, weet je zeker dat je geen familie van mijn vader bent? Richard: goede keuze, maar ons onderzoek naar de (explosieve) mogelijkheden van droogijs is nog lang niet afgerond! Marinel: september 2004: ik terug in Dijkzigt, jij Doctor met een nieuwe keuken? Sjors: jammer dat je er niet bent, maar aangezien je het pad volgt van José Martí en Che Guevara: Hasta la Victoria, Siempre! Nicole: mijn ongevraagd (zoals wel vaker) advies: maak het af! Een promotie is straks voor een opleiding wellicht niet meer noodzakelijk maar zal later zeker het onderscheid maken. René, Jeanette, Usha, Angelique (Uhh, ik durf het bijna niet te vragen maar er staan nog wat monsters in de vriezer..), Marjolein, Evelien, Edith, Martin en Jasper.

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Curriculum Vitae

Thomas werd op 5 augustus 1970 in Leiden geboren als Thomas Wiebe Lameris. In 1988 behaalde hij het Gymnasium B diploma op het St. Stanislascollege in Delft, waarna hij geneeskunde ging studeren aan de Erasmus Universiteit Rotterdam. In juni 1993 legde hij tijdens zijn doctoraalstudie de basis voor zijn interesse voor de wetenschap op de afdeling obstetrie en gynaecologie van het St. Catharina ziekenhuis in Eindhoven bij Dr. P.A. de Jong. Kort daarna deed hij zijn keuzeonderzoek "Waarde van de aldosteron-renine-ratio bij diagnostiek primair hyperaldosteronisme" op de afdeling inwendige geneeskunde I van het academisch ziekenhuis Dijkzigt onder begeleiding van Dr. F.H.M. Derkx en Prof. dr. M.A.D.H. Schalekamp. Vervolgens werkte hij op diezelfde afdeling gedurende 14 maanden mee aan het promotieonderzoek van Dr. M.A. van den Dorpel naar de rol van de endotheel-afhankelijke vasodilatatie in de etiologie van hypertensie bij cyclosporinegebruik door niertransplantatiepatiënten onder begeleiding van Prof. dr. A.J. Man in 't Veld en Dr. A.H. van den Meiracker. Na zijn arts-examen in februari 1997 keerde hij in september 1997 terug bij de afdeling inwendige geneeskunde I als arts-onderzoeker. Het grootste deel van het onderzoek dat hij daar de afgelopen vier jaar gedaan heeft, leidde uiteindelijk tot dit proefschrift. Daarnaast is hij betrokken bij het onderzoek van Lt-Kol. R.A. van Hulst van de Koninklijke Marine en de afdeling experimentele anaesthesiologie naar de pathofysiologie van luchtemboliëen in de hersenen, en werkt hij samen met Drs. J de Jong - van der Linden aan een studie naar de prognostische waarde van de flow-reserve van de onderarm voor het welslagen van de Brescia-Cimino shunt onder begeleiding van Dr. M.A. van den Dorpel van de afdeling nefrologie van het Medisch Centrum Rijnmond-Zuid, locatie Clara en Dr. A.H. van den Meiracker. Op 1 oktober is hij begonnen aan de vooropleiding inwendige geneeskunde in het Medisch Centrum Rijnmond-Zuid, locatie Clara in Rotterdam (opleider: Dr. A.F. Grootendorst) in het kader van de opleiding Cardiologie aan het Thoraxcentrum van de Erasmus Universiteit Rotterdam (opleider: Prof. dr. M.L. Simoons).

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