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Hemodynamic and inotropic effects of endothelin-1 in vivo*

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Summary: Endothelin-1 (ET-1) is known to have strong vasoactive properties. Contradictory results have been reported with regard to its inotropic effects.

This study examined the dose-dependent (500, 1000, 2500, 5000 and 10000 ng ET-1/kg vs. NaCl controls) hemodynamic and inotropic effects of ET-1 in 53 open-chest rats during and after a 7-min infusion. Besides measurements in the intact circulation the myocardial function was examined by isovolumic registrations independent of peripheral vascular effects.

A transient ET-1 induced (500, 1000, 2500, 5000 ng ET-1/kg) decrease of the left ventricular systolic pressure (LVSP) and the mean aortic pressure (AoP_{mean}) was followed by a dose-related rise of these pressures (LVSP: –1 %, –1 %, +8 %, +16 % vs. preinfusion values; AoP_{mean}: –11 %, +9 %, +39 %, +52 %). Heart rate (HR) was not influenced by ET-1. Due to the dose-dependent decrease of the stroke volume (SV) the cardiac output (CO) was reduced (CO: –8 %, –23 %, –40 %, –50 %). After an initial vasodilatation ET-1 elevates the total peripheral resistance (TPR: –1 %, +49 %, +139 %, +215 %) dose-dependently. 10000 ng ET-1/kg was a lethal dose resulting in cardiac failure within minutes (low output). Since the maximum of the isovolumic LVSP (peak LVSP) and the corresponding dP/dt_{max} (peak dP/dt_{max}) were unchanged under ET-1, the isovolumic measurements do not indicate a positive inotropic effect of ET-1 in vivo in contrast to published results of in vitro experiments.

It may be possible that a direct positive inotropic effect of ET-1 observed in in vitro studies is counterbalanced in vivo by an indirect negative inotropic effect due to the coronar-constrictive effect of ET-1.

Key words: Endothelin-1 – hemodynamic effects – inotropic effects – isovolumic measurements – rats

Introduction

Endothelin-1 is a 21-amino acid peptide that was originally purified from porcine aortic endothelial cells (38). Endothelin-1 is the most potent vasoconstrictor known to date. Its strong and sustained vasoconstrictive activity can be explained by the fact that endothelin-1 increases the intracellular Ca^{2+} concentration in smooth muscle cells (34, 35). The receptor-mediated endothelin-induced rise in cytosolic Ca^{2+} is, on the one hand, the result of an enhanced influx of extracellular Ca^{2+} through indirectly-activated calcium channels (34); on the other hand, endothelin-1 causes a phospholipase C-mediated release of intracellular Ca^{2+} from the sarcoplasmatic reticulum (15) and an indirect activation of the Na^{+}-Ca^{2+} exchanger (33).

Such an endothelin-induced increase of cytosolic Ca^{2+} was also described in cardiac myocytes (10, 14). Indeed, in vitro experiments on cardiac tissues showed a positive

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inotropic effect of endothelin-1 (12, 14, 17, 22, 26, 37), which can also be explained in part by an endothelin-induced sensitization of cardiac myofilaments to intracellular Ca^{2+} due to stimulation of the sarcolemmal Na^{+}-H^{+} exchanger (22). However, the results with regard to myocardial effects have been controversial in isolated working hearts (2, 17) and in intact animals (19, 39), probably due to the vasoconstrictive action of endothelin-1 (which was overcome in some in vitro heart studies).

The present study was initiated to investigate the dose-dependent hemodynamic and especially the inotropic effects of endothelin-1 in vivo in an open-chest animal model, which permits besides hemodynamic measurements in the intact circulation also the determination of myocardial effects independent of peripheral vascular effects by isovolumic measurements (11).

Methods

The study was performed on 4-month-old normotensive male Wistar rats (n = 53; weight 399 ± 6 g (mean ± SEM)). After anesthesia with urethane 50 % (2.5 ml/kg body weight) intraperitoneally, a venous line for drug infusion was established through the right jugular vein. An ECG (lead II) was recorded to measure the heart rate (HR). A median sternotomy was performed under artificial ventilation with room air (Starling respirator, Braun, Melsungen, FRG). The heart and the great vessels were exposed and the pericardium was opened. A flexible plastic tube advanced through the left carotid artery to the aortic arch was connected to a fluid-filled (heparinized saline) pressure transducer (Statham P23 ID, Gould, Oxnard, CA, USA) to register the aortic blood pressure. To record the left ventricular pressure (LVP) a short fluid-filled 18-gauge metal cannula, positioned through the apex in the left ventricle, was connected to a fluid-filled pressure transducer (Senso Nor 840, Horten, Norway). This transducer was also connected to an amplifier (type 806, Siemens, Munich, FRG) for reading of the left ventricular end-diastolic pressure (LVEDP) and to a differentiator (type 868, Siemens) to calculate the first derivate of the LVP (dP/dt). An electromagnetic flow probe (ID 2 mm, BL 620 Flowmeter, Biotronix, Kensington, MD, USA) was fitted around the ascending aorta to register stroke volume (SV; except for coronary flow).

ECG, the flow signal, the aortic pressures (AoP_s, AoP_d), the left ventricular systolic pressure (LVSP), LVEDP and dP/dt_{max} were recorded on a multichannel ink jet recorder (Mingograf 803, Siemens-Elema, Sweden). The mean aortic pressure ($AoP_m = (2 \times AoP_d + AoP_s)/3$), SV (planimetry of the phasic flow signal, mean of three consecutive beats), cardiac output ($CO = SV \times HR$) and the total peripheral resistance ($TPR = AoP_m/CO$) were then derived. Besides hemodynamic measurements in the intact circulation the myocardial function was examined by isovolumic measurements independently of circulatory changes by cross-clamping the ascending aorta until the maximum of the isovolumic LVSP (peak LVSP) was obtained. From the beat with the highest isovolumic LVSP, the peak dP/dt_{max} and peak LVEDP were determined. The peak LVSP and the corresponding peak dP/dt_{max} are indices of myocardial contractility independent of peripheral vascular effects. At the end of the experiments the pressure-volume relation of the left ventricle was measured as described previously in detail (11). Because the pressure-volume relation was not influenced by endothelin-1, the left ventricular end-diastolic volume (LVEDV) can be derived for a given LVEDP from the pressure-volume relation.

Table 1. Hemodynamic measurements in the intact circulation and isovolumic registrations at the end of stabilization period.

			Endothelin-1			
		1 ml NaCl	500 ng/kg	1000 ng/kg	2500 ng/kg	5000 ng/kg
LVSP	(mm Hg)	124.1 ± 4.9	128.4 ± 7.0	118.2 ± 2.7	133.9 ± 6.6	126.4 ± 3.6
LVEDP	(mm Hg)	3.1 ± 0.4	3.6 ± 0.2	3.4 ± 0.2	3.6 ± 0.4	3.2 ± 0.2
AoP_d	(mm Hg)	48.3 ± 3.3	47.2 ± 7.3	46.0 ± 1.8	49.6 ± 5.3	50.6 ± 2.7
AoP_m	(mm Hg)	64.1 ± 3.4	64.0 ± 6.2	60.6 ± 2.2	63.0 ± 4.9	62.3 ± 3.5
dP/dt_{max}	(mm Hg/s)	6092 ± 364	6490 ± 552	5830 ± 270	6877 ± 657	5779 ± 231
HR	(min^{-1})	249.7 ± 6.3	281.6 ± 17.9	251.0 ± 10.2	276.2 ± 8.8	264.2 ± 16.8
LVEDV	(µl)	293.7 ± 14.1	312.4 ± 12.2	284.9 ± 7.9	283.1 ± 8.9	296.3 ± 12.1
EF	(%)	67.4 ± 7.5	64.4 ± 6.1	62.2 ± 4.7	71.2 ± 5.0	65.4 ± 6.0
TPR	(mm Hg × min × kg/ml)	1.5 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
peak LVSP	(mm Hg)	269.7 ± 6.2	271.7 ± 7.5	275.7 ± 4.2	285.4 ± 5.7	273.4 ± 4.7
peak dP/dt_{max}	(mm Hg/s)	10219 ± 254	10182 ± 612	10352 ± 693	10906 ± 450	9734 ± 359
n		10	10	9	10	10

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; AoP_d, diastolic aortic pressure; AoP_m, mean aortic pressure; dP/dt_{max}; HR, heart rate; LVEDV, left ventricular end-diastolic volume; EF, ejection fraction; TPR, total peripheral resistance; peak LVSP and peak dP/dt_{max}, data derived from isovolumic maximum beat. Values are means ± SEM.

Endothelin-1 was dissolved in a final volume of 1 ml and infused in 7 min with a precision pump (Braun, Melsungen, FRG). To investigate the dose-dependent effects of endothelin-1, we administered doses of 500 ng/kg (n = 10), 1000 ng/kg (n = 9), 2500 ng/kg (n = 10), 5000 ng/kg (n = 10), and 10000 ng/kg (n = 4) endothelin-1. The control group (n = 10) received 1 ml 0.9 % NaCl solution. Endothelin-1 was obtained from Sigma (Sigma, St. Louis, MO, USA). Preinfusion control data of auxotonic and isovolumic measurements were obtained after a 15-min stabilization period. Three minutes after these measurements were made the drug infusion was started. Auxotonic measurements were recorded every minute until termination of the infusion and 5, 10

Table 2. Hemodynamic measurements in the intact circulation and isovolumic registrations at termination of infusion and at 5 and 15 min after infusion.

			Endothelin-1			
		1 ml NaCl	500 ng/kg	1000 ng/kg	2500 ng/kg	5000 ng/kg
LVSP	$7'_{inf.}$	105.9 ± 1.1	98.8 ± 1.7*	99.3 ± 2.1	107.8 ± 3.0	116.0 ± 2.0#
	$5'_{post.}$	101.0 ± 2.3	101.0 ± 1.5	98.3 ± 4.8	98.4 ± 3.5	105.1 ± 1.8
	$15'_{post.}$	98.6 ± 2.3	98.0 ± 1.8	105.1 ± 3.6	106.0 ± 2.1	110.3 ± 4.0*
LVEDP	$7'_{inf.}$	112.8 ± 4.7	104.6 ± 4.4	89.9 ± 9.8*	83.0 ± 5.5#	87.1 ± 5.6#
	$5'_{post.}$	100.7 ± 8.1	103.7 ± 5.2	79.6 ± 11.6	76.8 ± 5.4	72.9 ± 6.8*
	$15'_{post.}$	92.4 ± 5.4	106.8 ± 6.5	101.0 ± 13.3	84.6 ± 5.3	84.9 ± 6.5
AoP_m	$7'_{inf.}$	109.6 ± 3.3	88.7 ± 3.0*	108.7 ± 5.7	139.2 ± 6.0#	152.0 ± 8.5#
	$5'_{post.}$	104.3 ± 3.8	105.1 ± 4.5	115.2 ± 10.3	118.1 ± 5.8	130.6 ± 7.4*
	$15'_{post.}$	97.3 ± 3.7	101.2 ± 3.9	124.4 ± 6.0*	142.2 ± 3.3#	164.8 ± 13.0#
dP/dt_{max}	$7'_{inf.}$	107.6 ± 1.4	97.6 ± 3.1	101.2 ± 3.3	112.7 ± 5.0	127.5 ± 5.4#
	$5'_{post.}$	100.7 ± 2.9	102.7 ± 2.8	100.2 ± 5.1	95.5 ± 3.8	106.1 ± 6.7
	$15'_{post.}$	97.1 ± 3.1	100.1 ± 2.9	106.1 ± 6.0	107.8 ± 4.4	115.9 ± 7.2*
HR	$7'_{inf.}$	100.8 ± 1.6	97.4 ± 2.2	99.8 ± 1.2	100.6 ± 3.8	104.5 ± 3.1
	$5'_{post.}$	101.5 ± 1.7	97.7 ± 2.5	99.1 ± 2.6	96.4 ± 4.3	104.6 ± 4.2
	$15'_{post.}$	98.8 ± 2.8	95.6 ± 2.4	101.8 ± 3.6	95.9 ± 4.6	95.6 ± 2.9
CO	$7'_{inf.}$	121.5 ± 5.6	92.1 ± 4.0#	77.0 ± 4.8#	60.2 ± 3.6#	50.0 ± 3.5#
	$5'_{post.}$	108.7 ± 2.7	89.0 ± 4.9#	67.1 ± 5.6#	40.1 ± 3.3#	29.5 ± 2.7#
	$15'_{post.}$	91.7 ± 2.9	83.6 ± 3.9	81.3 ± 5.2	53.1 ± 5.4#	37.4 ± 3.7#
LVEDV	$7'_{inf.}$	104.7 ± 1.9	101.1 ± 1.6	94.3 ± 4.5*	92.8 ± 2.6#	95.1 ± 2.1*
	$5'_{post.}$	99.5 ± 3.4	100.3 ± 1.8	88.8 ± 6.2	90.7 ± 2.6	89.3 ± 3.1
	$15'_{post.}$	96.3 ± 2.4	101.6 ± 2.1	98.6 ± 5.1	93.8 ± 2.5	93.5 ± 3.2
peak LVSP	$5'_{post.}$	98.7 ± 1.3	94.6 ± 1.7	96.5 ± 3.5	104.1 ± 2.1	104.0 ± 2.2
	$15'_{post.}$	96.8 ± 1.6	93.0 ± 3.2	94.2 ± 3.9	100.2 ± 1.8	100.0 ± 1.9
peak dP/dt_{max}	$5'_{post.}$	98.4 ± 2.6	93.4 ± 2.4	94.0 ± 5.3	97.5 ± 2.5	111.6 ± 5.4
	$15'_{post.}$	96.1 ± 1.5	93.1 ± 2.7	89.9 ± 4.9	93.2 ± 2.6	98.7 ± 3.3
n		10	10	9	10	10

LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; AoP_m, mean aortic pressure; dP/dt_{max}; HR, heart rate; CO, cardiac output; LVEDV, left ventricular end-diastolic volume; peak LVSP and peak dP/dt_{max}, data derived from isovolumic maximum beat; $7'_{inf.}$, at termination of infusion; $5'_{post.}$ and $15'_{post.}$, 5 and 15 min after infusion.
Values are means ± SEM in percentage of preinfusion values. * = $p < 0.05$, # = $p < 0.01$.

and 15 min postinfusion. At termination of infusion and 5 and 15 min postinfusion isovolumic measurements were carried out. At the end of the hemodynamic measurements, blood samples were drawn from the aorta for determination of plasma endothelin-1 levels with a radioimmunoassay (Endothelin-1,2 (high sensitivity) [^{125}I]assay system, Amersham International plc, Amersham, UK).

All data are means ± SEM. Hemodynamic data were normalized to the individual preinfusion control data (100 %; absolute preinfusion control data see Table 1). Normalized data from the endothelin-1 infusion groups were compared with those of the control group by analysis of variance followed by a Dunnett test (6); $p < 0.05$ was accepted as significant.

Results

The four animals which received 10 000 ng ET-1/kg died a short time after infusion: three animals developed low output failure, the other one died due to ventricular fibrillation.

Auxotonic measurements in the intact circulation

The results of the hemodynamic measurements in the intact circulation are shown in Table 2. During the endothelin-infusion the left ventricular systolic pressure transiently decreases (2nd minute of infusion: NaCl: 101.8 ± 0.5 % vs. 500 ng ET-1/kg: 97.3 ± 1.5 %; 1000 ng ET-1/kg: 95.9 ± 2.1 %; 2500 ng ET-1/kg: 93.9 ± 2.3 %, $p < 0.05$;

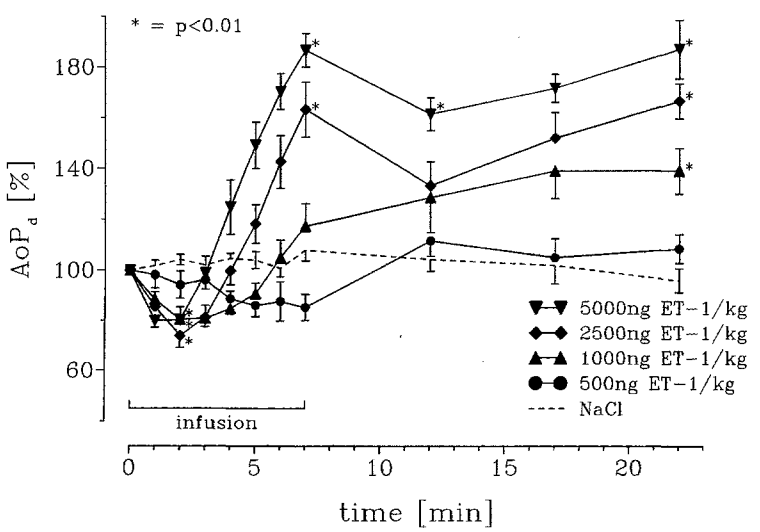

Fig. 1. Effects of different doses of endothelin-1 on the diastolic aortic pressure in the intact circulation. 1 ml NaCl (n = 10); endothelin-1: 500 ng/kg (closed circles, n = 10), 1000 ng/kg (closed triangles, n = 9), 2500 ng/kg (closed diamonds, n = 10), 5000 ng/kg (closed inverted triangles, n = 10); means ± SEM in percentage of preinfusion values; * = $p < 0.01$.

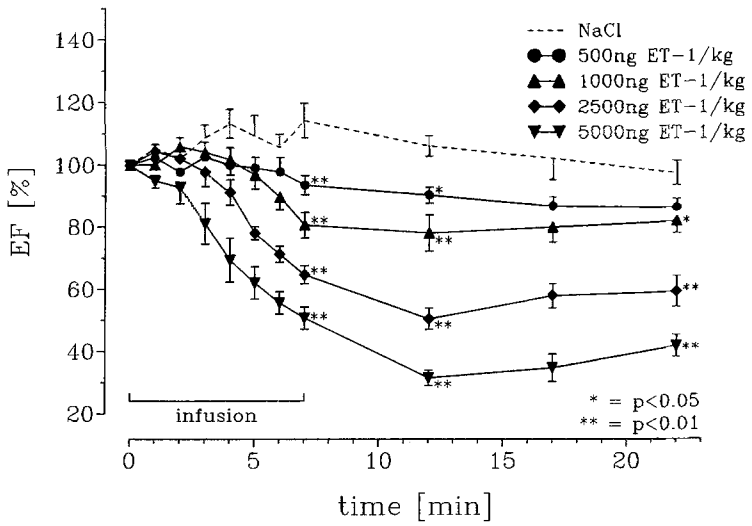

Fig. 2. Effects of different doses of endothelin-1 on the ejection fraction in the intact circulation. 1 ml NaCl (n = 10); endothelin-1: 500 ng/kg (closed circles, n = 10), 1000 ng/kg (closed triangles, n = 9), 2500 ng/kg (closed diamonds, n = 10), 5000 ng/kg (closed inverted triangles, n = 10); means ± SEM in percentage of preinfusion values; * = $p < 0.05$, ** = $p < 0.01$.

5000 ng ET-1/kg: 90.2 ± 3.4 %, $p < 0.01$). Then the left ventricular systolic pressure rises dose-dependently. At the end of infusion the LVSP is still slightly but significantly reduced in the 500 ng/kg-group and significantly elevated in the 5000 ng/kg-group. The

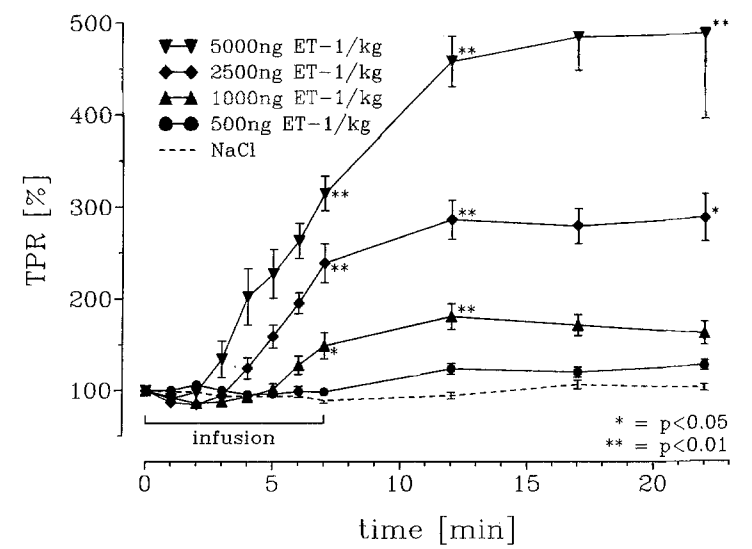

Fig. 3. Effects of different doses of endothelin-1 on the total peripheral resistance in the intact circulation. 1 ml NaCl (n = 10); endothelin-1: 500 ng/kg (closed circles, n = 10), 1000 ng/kg (closed triangles, n = 9), 2500 ng/kg (closed diamonds, n = 10), 5000 ng/kg (closed inverted triangles, n = 10); means ± SEM in percentage of preinfusion values; * = $p < 0.05$, ** = $p < 0.01$.

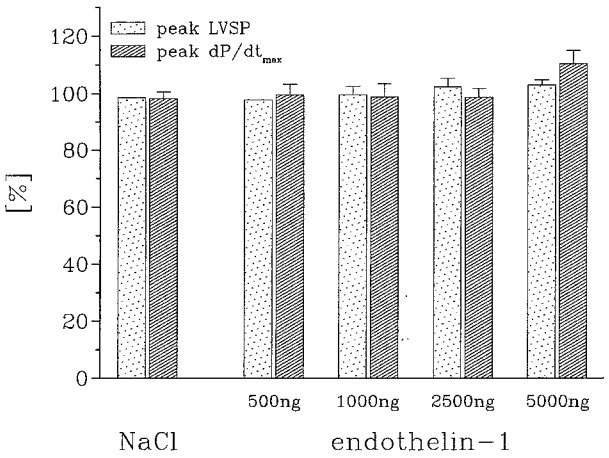

Fig. 4. Peak isovolumic left ventricular systolic pressure (peak LVSP) and the corresponding peak isovolumic dP/dt_{max} (peak dP/dt_{max}) at the end of infusion; means ± SEM in percentage of preinfusion values.

initial fall and the following dose-related increase is more pronounced for the mean and the diastolic aortic pressure, reflecting the peripheral vascular effects of endothelin-1. The fall of the diastolic aortic pressure at the beginning of infusion is even significant in the higher doses (Fig. 1). The decrease is followed by a rise, which is earlier and more pronounced in higher doses of endothelin-1. This increase persists until the end of the experiments. dP/dt_{max} shows the same changes as the pressures.

The heart rate is not influenced by endothelin-1. As a result of the dose-dependent decrease of the stroke volume (end of infusion: NaCl: 119.9 ± 5.8 % vs. 500 ng ET-1/

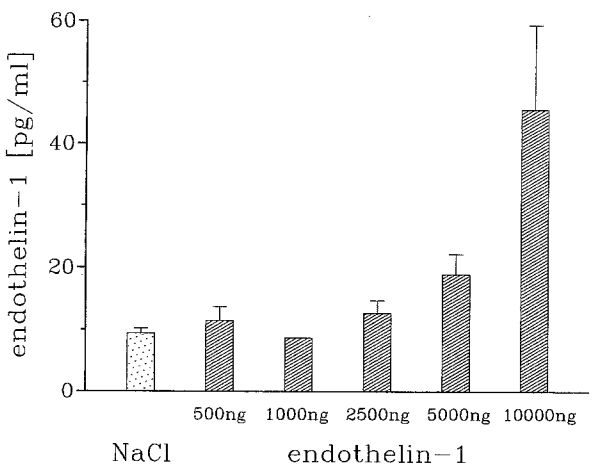

Fig. 5. Venous plasma levels of endothelin-1 at the end of the experiments. 1 ml NaCl (n = 4); endothelin-1: 500 ng/kg (n = 4), 1000 ng/kg (n = 8), 2500 ng/kg (n = 10), 5000 ng/kg (n = 10), 10 000 ng/kg (n = 4); means ± SEM.

kg: 94.6 ± 3.6 %; 1000 ng ET-1/kg: 77.1 ± 4.9 %; 2500 ng ET-1/kg: 59.6 ± 2.0 %; 5000 ng ET-1/kg: 48.0 ± 3.2 %; all $p < 0.01$), the cardiac output is reduced significantly in all endothelin-1 groups until 15 min after infusion. The ejection fraction is significantly lowered in all endothelin-1 groups (Fig. 2), and the left ventricular end-diastolic pressure and the corresponding end-diastolic volume are slightly reduced (Table 2).

After an initial vasodilatation (maximum in the 2nd min of infusion) endothelin-1 markedly elevates the calculated total peripheral resistance (Fig. 3).

Isovolumic measurements

The dose-related effects of endothelin-1 on the isovolumic registrations at the end of infusion are shown in Fig. 4. The peak of the isovolumic left ventricular systolic pressure is not changed under increasing doses of endothelin-1. The little increase of the peak isovolumic dP/dt_{max} in the 1000- and 5000 ng ET-1/kg-group is also not significant. Five and 15 min later these two indices of myocardial contractility are also not altered by endothelin-1 (Table 2).

Plasma endothelin-1 levels

The venous plasma levels of endothelin-1 at the end of the experiments are illustrated in Fig. 5. There is a dose-related increase of the plasma endothelin-1 levels (data after 10000 ng ET-1/kg were obtained directly after the heart has stopped beating in these animals).

Discussion

Endothelin-1 has a potent, dose-dependent contractile effect on vascular smooth muscle (34). Conflicting results are published about the inotropic effects of endothelin-1. In vitro experiments showed a positive inotropic effect of endothelin-1 in animal (12, 14, 17, 22, 26, 37) and human (26) cardiac tissues. Atrial muscle is more sensitive than ventricular muscle (26). But these experiments avoided changes of the myocardial perfusion. Experiments in isolated hearts could not detect a positive inotropic effect of endothelin-1 (16, 27).

In vivo experiments even described a negative inotropic effect of endothelin-1 (5, 39). As endothelin-1 also has a coronary constrictive effect (3, 7, 23, 25) and may cause electrocardiographic signs of myocardial ischemia (5, 25) it is of importance to determine the inotropic effects of endothelin-1 on hearts with an intact regulation of the coronary perfusion. We used an in vivo model which allows (besides making hemodynamic measurements in the intact circulation) the determination of the left ventricular pressure generating capacity as a well-established method to assess myocardial contractility independent of peripheral vascular effects (28) but dependent on changes of the coronary perfusion. To investigate the dose-dependent hemodynamic and inotropic effects of endothelin-1 we administered increasing doses of endothelin-1. Although endothelin-1 has a rather high first-pass effect (1) the plasma-endothelin levels at the end of the experiments indicate that there is also a dose-dependent rise of endothelin-1 in the myocardium.

In our experiments endothelin-1 produces a biphasic blood pressure response. The initial transient hypotension and the following dose-dependent sustained hypertensive response confirm the results of other in vivo studies (9, 19, 20, 25, 30, 39). This biphasic

response is mostly pronounced in the diastolic aortic pressure, reflecting the peripheral vascular effects of endothelin-1. As the cardiac output remained unchanged during the early phase of endothelin-infusion, the reduction of blood pressure is primarily due to a transient vasodilatation. This short-lived vasodilatatory response of endothelin-1 (19, 27, 30, 39) may be explained by the fact that endothelin-1 induces the release of vasodilatatory mediators like endothelium-derived relaxing factor (EDRF) (4, 31, 36), prostacyclin (4, 29) and atrial natriuretic peptide (ANP) (8). The following dose-dependent and sustained endothelin-induced vasoconstriction is in accordance to other in vivo studies. While Yang et al. (39) observed an increase of the LVEDP and Garcia et al. (9) could not detect a change of the LVEDP after endothelin-1, our experiments show an endothelin-induced reduction of the LVEDP. Endothelin-1 may decrease venous return due to reduction of plasma volume by a loss of plasma water to the interstitium (18) or due to a vasoconstriction in the splanchnic vascular bed (24).

In our experiments endothelin-doses up to 5000 ng ET-1/kg have no effect on the heart rate. This data confirm the results of other in vivo studies (5, 20, 39). Ishikawa et al. (13) detected a positive chronotropic response of endothelin-1 in guinea pig spontaneously beating right atrial preparation. In vivo studies of Mir et al. (25) described a positive chronotropic effect by lower doses of endothelin-1 and a hypoxia-induced bradycardia by higher doses of endothelin-1. Indeed, in the animals which received the lethal dose of 10000 ng ET-1/kg the heart rate was also reduced (end of infusion: NaCl: $100.8 \pm 1.6\,\%$ vs. 10000 ng ET-1/kg: $71.9 \pm 6.9\,\%$, $p < 0.01$).

The reduction of the cardiac output in our experiments may be explained in part by the decrease of the preload and the increase of the afterload. Yang et al. (39) supposed that the endothelin-induced fall of CO can be explained in part by a direct depressant effect on cardiac contractility. They supported this hypothesis by the fact that dP/dt_{max} was also reduced in their in-vivo experiments. In contrast to that, Kitayoshi et al. (20) and Garcia et al. (9) measured an endothelin-induced increase of dP/dt_{max} and they concluded that endothelin-1 has a positive inotropic effect. We also measured a significant increase of dP/dt_{max} after 5000 ng ET-1/kg. But dP/dt_{max} from the auxotonic beating heart may be influenced by the pre- and afterload (32). As endothelin-1 has such a tremendous effect on pre- and afterload, it was an important part of our study to determine the peak of the isovolumic LVSP and the corresponding dP/dt_{max} as indexes of myocardial contractility independent of these effects. The results of the isovolumic measurements do not indicate a positive inotropic effect of endothelin-1 detectable in our in vivo model. Endothelin-1 has a strong and dose-dependent coronary constrictive effect (16). So it may be possible that the direct positive inotropic effect of endothelin-1, which is detectable in in vitro studies, is counterbalanced in vivo by an indirect negative inotropic effect due to the coronary constrictive effect of endothelin-1. This has also been suggested previously by us in a review of myocardial actions of endothelin (21) and can also be derived from experiments in isolated hearts, in which the coronary flow decreased due to endothelin-1 (16, 27).

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