List of full papers related to the subject of the thesis

1. Szabó A, Suki B, Csonka E, **Eszlári E,** Kucsa K, Vajda K, Kaszaki J, Boros M: Flowmotion in the intestinal villi during hemorrhagic shock. A new method to characterize the microcirculatory changes. *Shock* 21:320-329, 2004. IF 3,122
2. **Eszlári E**, Czóbel M, Molnár G, Bogáts G, Kaszaki J, Nagy S, Boros M: Modulation of cardiac contractility through endothelin-1 release and myocardial mast cell degranulation. *Acta Physiol Hun* 95:301-319, 2008. IF 0,491
3. Molnár G, **Eszlári E**, Czóbel M, Kaszaki J, Bogáts G, Nagy S, Boros M: A nitrogén-monoxid-szintézis gátlása endothelinfüggő szívkontraktilitás növekedéshez vezet. *Cardiologica Hungarica* 40(1):1-6, 2010. IF 0,0

List of abstracts related to the subject of the thesis

1. Kaszaki J, Molnár G, **Eszlári E**, Csonka E, Nagy S, Petri I, Boros M: The role of mast cells and endothelin-1 in the circulatory effects of colloid-crystalloid resuscitation fluids. *European Surgical Research* 33:155, 2001.
2. **Eszlári E**, Csonka E, Kaszaki J, Molnár G, Szabó A, Boros M: Comparison of the circulatory effects of different hypertonic colloid volume expanders. *European Surgical Research* 34:91, 2002.
3. **Eszlári E**, Csonka E, Kaszaki J, Szabó A, Nagy S, Boros M: Hypertonic colloid volume expanders differentially affect cardiac contractility. *Shock* 18:6-7, 2002.
4. **Eszlári E**, Csonka E, Kaszaki J, Nagy S, Bogáts G, Boros M: Hypertonic dextran volume expanders with different molecular weights differentially affect cardiac contractility. *Experimantal and Clinical Cardiology* 8:38, 2003.
5. Kaszaki J, Czóbel M, **Eszlári E,** Csonka E, Nagy S, Boros M: Interaction between endothelin and nitric oxide in regulation of cardiac contractility. A possible role of cardiac mast cells. *Experimental and Clinical Cardiology* 8:42-43, 2003.
6. Kaszaki J, **Eszlári E,** Csonka E, Czóbel M, Nagy S, Boros M: Endothelin-dependent changes in cardiac contractility after hypertonic colloid infusions. The role of mast cells. *Shock* 21:96, 2004.

ischemia-reperfusion. The microcirculatory effects of endogenous ET can be linked to a Ca^{2+}-dependent signal transduction mechanism. ET-1 causes sustained oscillatory elevations in intracellular Ca^{2+} level *in vitro*, which could be prevented by ET-A receptor antagonism *in vivo*. Thus, the ET-induced Ca^{2+}-mediated catecholamine release could modify microcirculatory responses through an ET-A receptor-dependent mechanism.

In summary, in the presence of time-dependent varying factors, we determined the microcirculatory improvement after hyperoncotic resuscitation and quantitatively compared the microvascular flow-related alterations due to ET-A receptor inhibition.

6. Summary of new findings

I. HSD-induced positive inotropy and CO elevation is accompanied by ET-1 overproduction and a potentially unfavorable cardiac NO-ET-1 balance. Peripheral NO and ET-1 release could significantly modulate the cardiac contractility through myocardial ET-A receptor activation; the ET-A receptor antagonist pretreatment could reduce the late side-effects of HSD infusion (*i.e.* inhibition of NOS activity) without hemodynamic disadvantages.

II. As far as we are aware, this is the first *in vivo* evidence of positive inotropy caused by ET-1 following NOS inhibition. A diminished NO production leads to concomitant ET-1 overproduction in the normal myocardium. A reduced NO synthesis leads to a preponderant ET-1 effect, which decreases CO and increases myocardial contractility through an ET-A receptor-dependent mechanism.

III. Small volume resuscitation with HSD efficiently improves the peripheral tissue microperfusion after experimental HS. ET-A receptor antagonist treatment has favorable microcirculatory consequences in the intestine by increasing the relative duration of high-flow periods in the ileal mucosal villi in the early phase of resuscitation.

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resuscitation, when the gradually decreasing CI was accompanied by a diminished ileal blood flow and a significant increase in plasma ET-1 level. Since this microvascular oscillatory phenomenon was not observed under control conditions, its appearance can be regarded as a manifestation of tissue malperfusion at any later stage of the experiments. Thus, it appears that the beneficial effect of fluid resuscitation is related not only to the temporary restoration of near-normal microcirculatory velocity values, but also to the re-established continuous flow conditions. Accordingly, the increased length of high-flow phases during flowmotion also reflects improved tissue perfusion. The duration of distinct flow periods is therefore a critical factor and should be taken into account in the data analysis and interpretation. These considerations led us to seek a new method with which to quantify the average microcirculatory flow and its fluctuations, which simultaneously takes into account the changes in both amplitude and duration. Using a probabilistic approach, the instantaneous velocity was regarded as a random variable and the duration for which a particular value of the velocity was observed was used to calculate the probabilities as the relative duration of the high and low oscillatory flow periods. Detailed data analysis demonstrated that the average RBCV decreased and its coefficient of variation increased during HS. More importantly, the analysis revealed that hyperoncotic solution supplemented with an ET-A receptor antagonist exerted its beneficial effect by maintaining continuous flow in the villi and prolonging the length of high-flow periods at the onset of resuscitation.

The cause of flowmotion is unclear, but a new balance between vasoconstrictor and vasodilator forces can be presumed behind this phenomenon. Flow oscillations could improve the efficacy of tissue oxygenation during low-flow conditions. HS is characterized by an intense neuroendocrine response and stress hormone release. Norepinephrine induces periodic, intracellular Ca^{2+}-mediated changes in the membrane potential of vascular smooth muscle cells *in vitro*, and increases flow oscillations in the brain. Therefore, it is conceivable that endogenous catecholamines contribute to local flow pattern regulation. Further, it has been shown that endothelial ET-1 production is enhanced by epinephrine. This suggests that the evolving vasoconstriction may be amplified by ET-1 release during HS.

Vasodilator factors are equally important in regulating flowmotion, since vasomotion is dependent on an intact endothelium. It has been proposed that the interstitial accumulation of vasodilator metabolites leads to precapillary sphincter opening during HS. A role of CO_2 can be presumed since inhaled CO_2 reversibly abolishes flow oscillations. It is likely, therefore, that arteriolar vasoconstriction is periodically overcome by metabolic or endothelium-derived dilator forces.

The circulating levels of ET-1 were significantly increased during HS and peaked at the end of the observation period. At the onset of resuscitation, significantly higher ET-1 levels could be demonstrated in both HSD-treated groups, with or without ET-A antagonism, than after saline treatment. This is in agreement with the original report, whereby the ET-A receptor inhibitor compound exerted its inhibitory effects directly through specific receptor-binding rather than influencing the circulating levels of endogenous ET-1. The beneficial effect of ET-A antagonism on the villus microcirculation was also evidenced by the reduced periods of flow cessation. In another study, ET-A receptor antagonism reduced intestinal microvascular injury and PMN leukocyte accumulation during the end of the experiments, which may suggest that, after induction by NNA treatment, it was continuously replaced from the cellular sources. The results also revealed that the ET-1 peptide induced positive inotropy through the activation of ET-A receptors. Recent evidence strongly suggests that the target area of NO and/or ET-1 is the same cellular microdomain for the modulation of cardiomyocyte contractility, via regulation of the L-type Ca^{2+} channels, but in the opposite sense.

ET-A receptor antagonism

ET-A receptor antagonism by the ETR-P1/fl peptide proved effective in reducing the signs of vasoconstriction. As a result, CI displayed an immediate significant increase and did not decline below the control level following NNA treatment, while the NOS inhibition-induced elevation in total peripheral vascular resistance was lowered. This observation is in agreement with the data of Maeda *et al.*, who demonstrated that an exercise-induced increase in ET-1 significantly lowered the plasma NO concentration in the kidney, whereas pretreatment with an ET-A receptor antagonist resulted in significantly higher NO production. This elevation in NO production, presumably through activated ET-B receptor-induced NO release, plays a role in normalizing the LV diastolic-systolic diameter difference. Additionally, the plasma level of ET-1 was significantly lower and in this case the positive inotropic effect was missing. Hence, the volume per cardiac cycle increased, which maintained sufficient perfusion.

It has been shown that ETR-p1/fl peptide infusion in the same dose induces significant increases in CI, LV systolic-diastolic difference and significant decreases in TPR and plasma ET level, while it does not influence MAP, HR and cardiac contractility.

It should be noted that direct and indirect (or peripheral and central) effects of NOS and ET antagonism are difficult to distinguish *in vivo*. Taken together, the increase in LV contractility could be due to several factors, including increases in preload and coronary blood flow or the effects of reflex autonomic changes (*e.g.* baroreceptor responses). However, the results underline the significance of diminished NO production in ET-1 release, and these processes could contribute indirectly to the alterations in cardiac contractility through ET-A receptor activation.

In our further research protocol (Study III), the intestinal macro- and microcirculatory consequences of a 50%-blood volume reduction and the effects of low-volume resuscitation were investigated. The severity of hemorrhage was marked by ~70% decreases in CO and ileal blood flow, together with a significant overproduction of ET-1 in the plasma. Resuscitation caused a partial and transient restoration in macrohemodynamics, which was followed by a gradual deterioration irrespective of the applied therapy. Nevertheless, hypertonic solutions induced a significant microcirculatory improvement in the early phase of resuscitation, marked by a continuous flow pattern and normalization of RBCV in the intestinal villi.

It should be noted that these microcirculatory alterations could be characterized only by means of a novel method of data analysis. Similar to other organs, such as the pancreas, brain and skeletal muscle the deteriorating intestinal macrocirculation was accompanied by periodic fluctuations in capillary blood flow. This occurred not only during the shock state, but also during the later phase of with the ETR-p1/fl peptide has a protective role in maintaining the myocardial NOS activity and preserving the cardiac ET-1 content too, and the PMN accumulation in the cardiac tissue was also reduced. In summary, the data reported here demonstrate that mechanical stimuli involving an HSD-induced volume expansion cause significant peripheral ET-1 release. This response may lead to an ET-1 - NO imbalance, an unfavorable side-effect of HSD fluid therapy, where ET-A receptor-associated effects predominate.

Our next study (Study II) was designed to explore the connection between basal NO synthesis and myocardial function in the unstressed dog, and we have shown that aspecific NOS inhibition leads to an increased myocardial contractility in this setting. The results revealed that the lack of NO is accompanied by significant ET-1 release. These experimental data therefore suggested a suppressive, regulatory role for endogenous NO: it restrains or counteracts these mechanisms, which would otherwise increase the cardiac contractility.

Hemodynamic effects of NOS inhibition

Our study has demonstrated the efficacy of NNA-induced NOS inhibition together with prolonged increases in MAP and total peripheral vascular resistance, and decreased CI. NOS inhibitor treatment caused a decrease in the LV diastolic-systolic diameter difference, which points to an attenuation of the Frank-Starling response and might explain the decrease in CI. Consistent with earlier results, NNA treatment caused a significant increase in heart contractility. Indeed, a detailed analysis of our experimental data suggested that the mechanism of the NNA-induced myocardial contractility elevation is a complex process which involves several variables. Experiments with NO donors have often shown the opposing effects of NO on the myocardial contraction and HR. It has been revealed that the reaction is biphasic and dose-dependent, *i.e.* low concentrations of NO donors (0.1-10 mM) increase the contractility and HR, whereas higher concentrations (> 100 mM) elicit negative inotropic and chronotropic effects. Taken together, an important consequence of NOS inhibition could be an imbalance in the positive-negative inotropy (vasoconstrictor-vasodilator) relationship, which is shifted in the direction of positive inotropy.

ET-1 release and ET-1 effects on cardiac contractility

We have shown that a diminished NO synthesis leads to a preponderant ET-1 effect. Since NO may normally moderate ET-1 production and action, the inhibition of NO synthesis can result in increased plasma levels of ET-1. It has been demonstrated that, after acute NOS blockade, the predominant pressor mechanism is associated with a marked increase in ET-A/ET-B receptor activation, rather than with increases in alpha-1, angiotensin 1 or vasopressin V1/V2 receptor activation.

Several aspects of the model highlight that the NNA-induced circulatory responses are characterized by predominantly ET-related immediate hemodynamic changes, demonstrating that this peptide is an important determinant of the increase in cardiac contractility. This observation supports the *in vitro* finding that NOS inhibition enhances the inotropic response to ET-1. Exogenous ET-1 infusion may increase the *in vivo* cardiac contractility significantly, an effect that can be prevented by ET-A receptor antagonist pretreatment. In our study, the plasma ET-1 level gradually increased up to endothelium or myocardial cells. This possibility is supported by a decreased level of myocardial ET-1 content at the end of the postinfusion period. It should be noted that the positive inotropic ET-1 effect is usually observed only *in vitro*, because this phenomenon is antagonized by the ET-induced myocardial vasoconstriction and subsequent ischemic cardiodepression.

Significance of NO on cardiac contractility

The role of myocardial NO production in regulating the cardiac function is complex and controversial. Previous studies have indicated that excessive NO delivery from inflammatory cells (or cytokine-stimulated cardiomyocytes themselves) may result in profound cellular disturbances leading to attenuated cardiac contractility. However, others have reported that the stimulation of myocardial NO production can offset the increase in contraction in response to a rise in intracellular Ca^{2+}. Cardiac NO production is also activated by stretching and under these conditions NO has been shown to facilitate the Frank-Starling response and to contribute to the increase in intracellular Ca^{2+} transients that mediates the slow increase in contraction in response to a stretch.

However, a considerably decreased NOS activity was observed in the later postinfusion period, to the accompaniment of a TPR elevation and a CI decrease. This marked decrease in NOS activity may be an indirect consequence of the elevation in ET-1 level. It has been proved in a number of diseases involving circulatory failure (myocardial infarction, cardiogenic shock, atherosclerosis and congestive heart failure) and also in surgical interventions that an increased ET-1 level is associated with decreased NO production. One possible explanation of this phenomenon is that the HSD-induced ET-1 release may be attributed to an increase in superoxide radical production, simultaneously with significant NO production, and this leads to the formation of peroxynitrite, a known inhibitor of NOS activity. Furthermore, ET-1 *per se* could inhibit endogenous NO synthesis through enhanced asymmetric dimethylarginine synthesis and ET-A receptor activation. On the other hand, peroxynitrite-mediated myocardial protein nitration has been associated with a depressed cardiac pump function. Borbely *et al.* proposed that alpha-actinin is a target for peroxynitrite in the human myocardium; and its nitration can induce a contractile dysfunction. Additionally, an *in vivo* interaction might occur between the increased nitrite level and myocardial MPO, affording reactive nitrosyl derivatives.

ET-A receptor antagonism

The ETR-p1/fl peptide, used in our study has a special feature: it is an intramolecular complementary peptide of the ET-A receptors; as such, it can specifically recognize and bind the circulating ET-1 molecules, and it is thereby able to decrease the plasma level of released ET-1. ET-A receptor antagonism by the ETR-P1/fl peptide proved effective in reducing the signs of vasoconstriction and it protected the myocardium from excess energy use. As a result of ET-A receptor antagonist pretreatment, the CI did not decline below the control level, and TPR was not increased at the end of the postinfusion period. Moreover, the plasma level of ET-1 was significantly lower and in this case the positive inotropic and chronotropic effects were missing. This group was characterized by a high LVD: the filling volume increased as a result of inhibition of the ET-A receptor-induced venoconstriction. Hence, the volume per cardiac cycle increased, which maintained sufficient perfusion. Pretreatment action on the vascular smooth muscle; a blood fluidity improvement by hemodilution and a stretch-induced ventricular dilatation caused by a pressure or volume overload. In our experiments, the molecular weight of the dextran component of the hyperosmotic–hyperoncotic solution was 40 kD. *In vivo*, solutions of 40 kD dextran are advantageous when an improvement in blood flow is specifically required, while a higher molecular weight dextran (*e.g.* 70 kD) is preferable when a longer circulation time and a plasma volume expansion are the primary goals. The rationale of our approach was to achieve a rapid increase in flow velocity. Indeed, the HSD infusion resulted in prompt increases in MAP and CO and a decrease in peripheral resistance; the HR concomitantly increased, and a significant rise in myocardial contractility was noted in the early phase of the postinfusion period.

The increased LV preload might contribute to the positive inotropy, but the mechanism of the HSD-induced myocardial contractility elevation is rather complex, and this change can not be a simple consequence of an increased intravascular volume. This hemodynamic pattern could increase the coronary blood flow too *(Gregg effect)*, which itself could lead to positive inotropy, and reflex autonomic changes could additionally be involved in this process.

The postinfusion period was characterized by significant rises in the plasma NO and ET-1 concentrations. Given the strong positive inotropic and chronotropic effects of ET-1, it was important to determine how much of the HSD-induced contractility change was due to ET-1. In the later phase of the postinfusion period, the TPR increased significantly. In parallel with this, the HR gradually increased, while the CI decreased and the myocardial contractility returned to a near-baseline level. The increased frequency was probably due to the positive chronotropic effects of the released ET-1. As NO plays a vital role in controlling myocardial contractility, its lack could modify the regulation to a great extent. Since both ET-1 and NO take part in the regulation, their ratio and the bioavailability of these vasoactive mediators are of crucial importance at any given moment.

ET-1 release and ET-1 effects on cardiac contractility

It has been shown that an acute volume expansion caused by a rapid infusion of hypertonic colloid solution results in an increase in plasma ET-1. ET-1 release or synthesis is triggered by various mechanical stimuli, such as the increased osmolarity caused by cell shrinkage, fluid shear stress on the endothelium and physical stretching of smooth muscle vascular cells or cardiomyocytes.

Several aspects of the employed model highlight that the HSD-induced circulatory responses are characterized by predominantly ET-related immediate hemodynamic changes, demonstrating that this peptide is an important determinant of the increase in cardiac contractility, but ET-1-associated late cellular events can lead to a depressed cardiac function. Exogenous ET-1 infusion may increase the *in vivo* cardiac contractility significantly, an effect that can be prevented by ET-A receptor antagonist pretreatment. The results revealed that the plasma level of ET-1 increased significantly in this setup too, and the peptide induced positive inotropy through the activation of ET-A receptors. ET-1 undergoes mainly abluminal release, and its half-life in the circulation is very short as a consequence of effective eliminating mechanisms. The plasma ET-1 level subsequently remained elevated until the end of the experiment, which may suggest that, after induction by HSD, it was continuously replaced from the were observed. During HS, the RBCV during the high-flow phases was significantly lower than at the baseline, and returned to the control level in all groups at the onset of resuscitation. However, the RBCV was moderately higher in the HSD and HSD + ETR-p1/fl-treated groups during the high-flow periods. A-RBCV (which allows simultaneous consideration of RBCV and the flow pattern changes) was restored at the onset of resuscitation in response to HSD and HSD + ETR p1/fl treatments. Capillary stasis in hemoglobin-containing structures was not observed during HS. Resuscitation was associated with a decrease in FCD in each group, and a return to the control level was observed only in the HSD and HSD+ETR-p1/fl-treated groups.

During HS, periods of high RBCV (~500-600 $\mu m\ s^{-1}$) were followed by periods of low RBCV (~100-150 $\mu m\ s^{-1}$). The average duration of high and low-flow periods was 9.9±0.4 and 7.4±0.5 s, respectively. For a 60-s interval, this equals 3.5 cycles min^{-1}, or corresponds to an ~ 43% decrease in relative duration of high-flow periods as compared with the continuous baseline flow (100%). At the onset of resuscitation, continuous flow periods were transiently seen in 33%, 40% and 50% of the experiments after saline, HSD and HSD + ETR p1/fl treatment, respectively. During the later stages of resuscitation, the relative duration of high RBCV periods was decreased, indicating the predominance of oscillatory flow in the villi. The ET-A receptor antagonism significantly increased the relative duration of the high-RBCV periods at the onset of resuscitation by prolonging either the continuous flow or the duration of high-flow periods during oscillation. In the event of prolonged high-flow periods, the use of cycles min^{-1} to express the periodicity would be particularly misleading since it would underestimate or mask these favorable alterations. The plasma ET-1 level was elevated significantly by the end of the shock period in each group (from 3.93 pg ml^{-1}±0.52 to 6.77±0.5 pg ml^{-1}; p=0.0016). At the onset of resuscitation, the high level of ET-1 persisted in both HSD-treated groups, but, a decrease occurred in response to resuscitation with saline (to 2.87±0.71 pg ml^{-1}; p=0.353 *vs*. baseline; p<0.05 *vs* HSD groups). These changes were followed by a gradual increase until the end of the examination period in each group, reaching maximal values of 8.95±2.86 pg ml^{-1} in the saline, 12.84±1.68 pg ml^{-1} in the HSD and 12.12±1.94 pg ml^{-1} in the HSD+ETR p1/fl-treated groups, respectively. HS followed by 180 min of resuscitation was accompanied by a 54.2%, 116.4% and 52.4% increase in MPO activity in the intestine in animals resuscitated with saline, HSD, and HSD combined with the ET-A receptor antagonist, respectively (from 2.25±0.25 to 3.47±0.43, 4.87±0.82; p<0.05 vs baseline and 3.43±0.72 U mg^{-1} protein, respectively).

5. Discussion

In our research protocol we used the ESPDR as a preload-independent index of cardiac contractility, in order to study the circulatory effects of volume therapies. The results of our first experimental series (Study I) revealed the early and late consequences of HSD infusion on cardiac contractility alterations, and confirmed the potential connection between cardiac NO synthesis and ET-1 release. In general, hypertonic–hyperoncotic solutions bring about rapid changes in the macro- and microhemodynamics in various circulatory beds. This could be related to a number of mechanisms, including the redistribution of fluid from the interstitium to the intravascular space; a direct relaxing

4.2. Study II Cardiac effects of decreased NO production

In the control group, there were no significant hemodynamic changes as compared with the baseline values during the 180-min observation period. The infusion of 4 mg kg^{-1} NNA resulted in sustained increases in MAP. The ETR-P1/fl peptide pretreatment mitigated the NNA-induced MAP elevations, but the differences between the values for the NNA and ETR-P1/fl peptide+NNA groups were statistically not significant. Nonspecific NOS inhibition caused an ~ 25% decrease in CI, while the difference between the LV diastolic and systolic diameters (as a percentage of the baseline) was also reduced significantly. The cardiac effects of ET-A receptor antagonist pretreatment included an immediate, significant increase in CI at 30 min of the experiment. Moreover, ETR-P1/fl peptide pretreatment significantly inhibited the NNA-induced decrease in CI and the LV diastolic-systolic diameter difference. Nonspecific NOS inhibition caused a significant increase in myocardial contractility up to the end of the observation period. However, ETR-P1/fl peptide pretreatment significantly inhibited the NNA-induced decrease elevation in myocardial contractility. The plasma ET-1 concentration gradually rose to approximately 1.5-fold following NNA infusion and remained significantly higher than in the control group up to 120 min in the observation period. The ETR-P1/fl peptide pretreatment prevented the NNA-induced increase in plasma ET-1 level throughout the observation period.

4.3. Study III

Effects of HSD and ET-A receptor antagonist treatment in hemorrhagic shock

Macrocirculatory changes

In the sham-operated group, the macro- and microhemodynamic parameters did not change significantly during the 330-min observation period. Macrohemodynamic data on the treated groups are presented in Fig. 10A-C. During HS blood was additionally withdrawn or retransfused to maintain the set MAP value between 40-45 mmHg for 60 min. The ~ 50% reduction in the calculated blood volume was accompanied by an ~ 70% decrease in CI. Resuscitation was followed by a partial recovery in MAP, irrespective of the therapy applied. Resuscitation with saline resulted in a short-term restoration of CI at the onset of resuscitation, but this was followed by a gradual decline. A similar macrohemodynamic deterioration was observed after HSD treatment. ET-A antagonisms, however, resulted in a lesser degree of recovery in CI as compared with saline or HSD treatment at the onset of reperfusion. The ET-A antagonist treatment did not influence the HR. The intestinal perfusion was reduced by ~ 70% during HS. Although the blood flow exceeded the baseline after the start of resuscitation with saline, a gradual deterioration was then observed, parallel to the CI changes and no difference was detected between the experimental groups after 15 min.

Microcirculatory changes

Microcirculatory flow was continuous at the villus tips under the control conditions, while cyclic fluctuation appeared during HS. This time-dependent flowmotion was not confined to the capillaries: it could be also observed in subepithelial venules and central arterioles as well. Additionally, alternating (on-off) flow evolved within adjoining villi, *i.e.* spatially and temporally synchronized flow periods was used for all pairwise comparisons of the mean responses between the different treatment groups. In the Figures, mean values and standard errors of means are given. P values < 0.05 were considered significant.

4. Results

4.1. Study ICardiac and peripheral effects of HSD treatment

Initial effects of surgery

The concentration of the HSD solution and the optimal conditions for the volume expander protocol were determined in pilot studies. In the control group, there were no significant hemodynamic changes as compared with the baseline values, and the ET-1 level did not change significantly during the120-min observation period. The HSD-induced peripheral circulatory reaction was characterized by MAP increases and a biphasic change in TPR: an initial decrease was followed by a return to the baseline during the early phase of the postinfusion period, and the TPR was significantly elevated at 120 min in the postinfusion period. After the ETR-p1/fl peptide pretreatment, the HSD infusion caused a slight, nonsignificant rise in MAP for 45 min as compared with the baseline (15 min: 137±6 mmHg *vs* baseline 123±4 mmHg) or with the HSD-only group. This pretreatment did not influence the early, transient decrease in TPR, but significantly decreased the late TPR elevation observed in the HSD-only group. The cardiac consequences of the HSD-induced volume loading included an increased LVD until 60 min of in the postinfusion period, a significant increase in CI together with a significant myocardial contractility elevation (lasting for 60 min), and a gradually elevated HR. The ETR-p1/fl peptide pretreatment resulted in a lower HR as compared with the HSD infusion, especially in the later postinfusion phase (149±8 *vs* 175±4). In this group, the CI had returned to the baseline level by the end of the observation period. The ET-A receptor antagonist pretreatment caused a noteworthy enhancement of the HSD-induced LVD increase (7.5±1.99%), but this preload index did not differ significantly from the result for the HSD-only group. The ETR-p1/fl peptide pretreatment significantly inhibited the HSD-induced elevation in myocardial contractility. The plasma ET-1 concentration was significantly increased by the end of the infusion (HSD group: 3.095±0.213 *vs* control group: 1.72±0.107 fmol ml^{-1}), and remained significantly higher than in the control group up to the end of the 120-min observation period. The ETR-p1/fl peptide pretreatment prevented the HSD-induced increase in plasma ET-1 level throughout the whole observation period. However, 120 min after the HSD infusion, the myocardial ET-1 content and cNOS activity were significantly lower as compared with those in the control group. In these biopsies, the tissue MPO activity was significantly higher than in the control group. The ET-A antagonist pretreatment prevented the HSD-induced decrease in myocardial ET-1 content and the cNOS activity changes at 120 min in the postinfusion period, as the NOS values were not significantly different from those in the control group. The administration of the ET-A receptor antagonist decreased the tissue MPO activity (30.1±2.6 *vs* 61.7±7.6 mU (mg protein)$^{-1}$ min^{-1}). These differences were statistically significant as compared with the HSD group.

0.1 mM phenylmethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 2000g. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan) and the data were referred to the protein content.

3.4. Experimental protocols

In Study I, surgery was followed by a recovery period for cardiovascular stabilization, and baseline variables were then determined during a 30-min control period. The animals were randomly allocated to one or other of three groups. Group 1 (n = 10) served as control and was treated with 0.9% saline (4 ml kg^{-1}), while Groups 2 (n = 7), and 3 (n = 7) were infused iv with 4 ml kg^{-1} HSD during 15 min. The solution was prepared from isotonic 10% dextran-40 (Baxter, Munich, Germany) and 7.2% NaCl solution. The animals in group 3 were additionally treated with the selective ET-A receptor antagonist ETR-p1/fl peptide (VLNLCALSVDRYRAVASWRVI; Kurabo Ltd, Osaka, Japan; 100 nmol kg^{-1} iv bolus in 1.5 ml kg^{-1} saline 15 min before HSD treatment. The beginning of HSD infusion served as the zero point of the experiments, and the animals were observed for a further 120 min in all groups.

In Study II, the animals were randomly allocated to one or other of three groups. Surgery was followed by a recovery period for cardiovascular stabilization. Baseline variables were determined during a 15-min control period. Group 1 (n=7) was treated with 0.9% saline iv, while in Groups 2 and 3 (n=7 each), the animals received 4 mg kg^{-1} NNA (Sigma Chem. U.S.A.) in 2 ml kg^{-1} saline during a 15-min iv infusion. The animals in Group 3 were additionally pretreated (100 nmol kg^{-1} iv bolus in 1.5 ml kg^{-1} saline) with the selective ET-A receptor antagonist ETR-P1/fl peptide 30 min before NNA treatment. The animals were observed for 135 min after the end of the treatment period; hemodynamic measurements were performed every 30 min.

In Study III, the animals were randomly assigned into 3 groups, which were subjected to HS (Groups 1-3). Surgery was followed by a 60-min stabilization period. Baseline variables were recorded for 30 min, and blood was then withdrawn from the femoral artery into a heparinized (25 IU ml^{-1}) reservoir until the MAP reached 40 mmHg. Blood was additionally withdrawn, or retransfused to maintain the set MAP value for 60 min. The animals were monitored for 180 min after HS. In Group 1 (n=11), the animals were resuscitated with 0.9% saline (150% of the lost blood volume) over 10 min, followed by a low-rate infusion of saline (1 ml kg^{-1} hr^{-1}). Group 2 (n=10) was treated with HSD (7.2% NaCl-10% dextran, 4 ml kg^{-1}) over 10 min, followed by a continuous infusion of saline (1 ml $kg^{-1}hr^{-1}$). The animals in Group 3 (n=8) were treated with the selective ET-A receptor antagonist ETR-p1/fl peptide (100 nmol kg^{-1} iv bolus in 1.5 ml kg^{-1} saline) 5 min before resuscitation and then HSD (4 ml kg^{-1} over 10 min) and saline infusion (1 ml $kg^{-1}hr^{-1}$) was given.

3.5. Statistical analysis

In Studies I and II, data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Changes in variables within and between groups were analyzed by two-way ANOVA. Time-dependent differences from the baseline (time 0) for each group were assessed by the Holm-Sidak test, while in Study III, changes in variables within groups were analyzed by two-way ANOVA tests followed by the Bonferroni test. The Student-Newman-Keuls test flow (or stop) conditions. All data were expressed as means of a minimum of 10 measurements at each time point.

3.3. Biochemical measurements

Plasma and cardiac tissue ET-1 measurements

Two-ml blood samples were drawn from the jugular vein into chilled polypropylene tubes containing EDTA (1 mg ml^{-1}) and aprotinin (Trasylol, Bayer, Leverkusen, Germany) (500 KIU/mL) before and after ETR-P1/fl peptide and NNA infusions, and at the end of the observation period. The blood samples were centrifuged at 1200g for 10 min at 4 ^oC. The plasma samples were then collected and stored at -70 ^oC until assay. For tissue samples (only in Study I), full-thickness heart biopsies were homogenized in phosphate buffer, and the homogenate was centrifuged at 4 ^oC for 30 min at 24 000g. The supernatants and plasma samples were analyzed for ET-1 with an ELISA kit (Biomedica, Vienna, Austria). According to the manufacturer, the cross-reactivity with ET-1 and ET-2 was 100%

NOS activity measurements

NO formation in cardiac tissues was measured via the conversion of [^{3}H]L-citrulline from [^{3}H]L-arginine according to the method of Szabó (1993). Briefly, heart biopsies kept on ice were homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4 ^oC for 20 min at 24 000g and the supernatant was loaded into centrifugal concentrator tubes (Amicon Centricon-100; 100 000 MW cut-off ultrafilter). The tubes were centrifuged at 1000g for 150 min and the concentrated supernatant was washed out from the ultrafilter with 250 μl homogenizing buffer. The samples were incubated with a cation-exchange resin (Dowex AG 50W-X8, Na^+ form) for 5 min to deplete endogenous L-arginine. The resin was separated by centrifugation (1500g for 10 min) and the supernatant containing the enzyme was assayed for NOS activity.

For the Ca^{2+}-dependent NOS (cNOS) activity, 50 μl enzyme extract and 100 μl reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM NADPH, 10 μM tetrahydrobiopterin, 1.5 mM $CaCl_2$, 100 Uml^{-1} calmodulin and 0.5 μCi [^{3}H]L-arginine (Amersham U.K., specific activity 63 Ci $mmol^{-1}$)) were incubated together for 60 min at 37 ^oC. The reaction was stopped by the addition of 1 ml ice-cold HEPES buffer (pH 5.5) containing 2 mM EGTA and 2 mM EDTA. Measurements were performed with the NOS inhibitor NNA (3.2 mM) to determine the extent of [^{3}H]L-citrulline formation independent of the NOS activity. Ca^{2+}-independent NOS activity (iNOS) was measured without Ca-calmodulin and with EGTA (8 mM). 1 ml reaction mixture was applied to Dowex cation-exchange resin (AG 50W-X8, Na^+ form) and eluted with 2 ml distilled water. The eluted [^{3}H]L-citrulline activity was measured with a scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2100TR/2300TR, Packard Instrument Co, Meriden, CT, U.S.A.). Protein contents of samples were determined by the Lowry method.

Myocardial myeloperoxidase (MPO) activity measurement

The MPO activity, as a marker of tissue PMN leukocyte infiltration, was measured via cardiac muscle biopsies. Briefly, the sample was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing

In the first series of experiments, we set out to examine the consequences of rapid alterations in peripheral flow on the ventricular function with or without ET-A receptor antagonist treatment. To this end, we employed a hypertonic–hyperoncotic solution to induce rapid changes in the macro- and microhemodynamics.

Since a constant NO release will normally suppress ET production and action, we hypothesized that the nonspecific inhibition of nitric oxide synthase (NOS) unveils or enhances ET-1 effects. Hence, in the second series, we planned to characterize the consequences of artificially diminished NO production on the myocardial performance, and examined the ET receptor dependence of the process.

In a further series of experiments, the aim was to investigate the peripheral microcirculatory consequences of acute cardiovascular deterioration with ET-1 release in order to assess the efficacy of fluid therapies with or without ET-A receptor antagonist treatment.

A. Study I outlined the consequences of an HSD-40 infusion-induced peripheral flow stimulus on the ventricular function, together with detailed biochemical analyses of the myocardial tissue with or without ET-A receptor antagonist pretreatment.

B. Study II characterized the ET-A receptor dependence of artificially diminished NO production on the myocardial performance in a large animal model by using the nonselective L-arginine analog NOS inhibitor N-ω-nitro-l-arginine (NNA).

C. Study III assessed the microcirculatory efficacy of HSD with or without ET-A receptor antagonist treatment during acute circulatory deterioration and compromised peripheral perfusion.

3. Materials and Methods

3.1. Macrohemodynamic measurements

All hemodynamic signals (pressures, LVP and LVD) were registered with a computerized data-acquisition system (SPEL Advanced Haemosys 2.72, Experimetria Ltd., Budapest, Hungary). The MAP and central venous pressure (CVP) were monitored with Statham P23Db transducers. The HR was calculated from the MAP curve. The CO was determined by thermodilution, using a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary), normalized for body weight and expressed as CI (ml kg^{-1} min^{-1}). The total peripheral vascular resistance (TPR) was calculated via the standard formula.

The ultrasonic dimension crystals were connected to a sonomicrometer (Triton Technology, Inc., San Diego, CA, U.S.A.). Via the LVP and LVD signals, the end-systolic elastance, as a parameter of the LV myocardial contractility, was estimated from the slope of the end-systolic pressure *vs* diameter relationship with a computer program developed by our group (only in Studies I and II).

3.2. Microcirculatory measurements

The intravital OPS technique (Cytoscan A/R, Cytometrics, PA, USA) with a 10x objective was used for continuous visualization of the microcirculation of the intestinal villi. Images were recorded by an S-VHS video recorder (Panasonic AG-TL 700) and evaluated off-line by frame-to-frame analysis. FCD (length of perfused nutritive capillaries per observation area (cm^{-1}), and RBCV ($\mu m\ sec^{-1}$) were determined in 3 separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). During capillary flowmotion, RBCV was determined during high-flow and low-

Once the ETs are formed, their physiological and pathophysiological actions are mediated by at least two distinct receptor subtypes, ET-A and ET-B, both from the family of G-protein-coupled receptors. The ET-A receptor has a higher affinity for ET-1 and ET-2 than for ET-3, whereas the ET-B receptor displays equal affinities for all the isopeptides. The receptors mediate different circulatory effects, depending on their localization, but it is suggested that vasoconstriction is mediated predominantly via the ET-A subtype, while the activation of ET-B receptors elicits both vasodilator and vasoconstrictor responses.

In the vasculature, ET-A receptors are localized on smooth muscle cells, whereas ET-B_1 receptors are found on endothelial cells, and to a lesser extent, ET-B_2 in smooth muscle cells. ET-A receptors exhibit exclusive affinity for the ET-1 peptide, but ET-B receptors have no isoform preference. Stimulation of ET-A and ET-B_2 receptors leads to the activation of phospholipase C, with a subsequent accumulation of inositol triphosphate and intracellular Ca^{2+}, resulting in vasoconstriction. In contrast, ET-B_1 receptors mediate the release of NO and prostacyclin and inhibit ECE expression in the endothelium. ET-B receptors are also involved in the clearance of circulating ET.

The cardiac tissue has been shown to produce a number of substances that modulate myocardial contraction, including ET. The synthesis of ET occurs in both the vascular and cardiac chamber endothelium and by cardiac myocytes. The effects of ET on the heart are therefore thought to be paracrine and autocrine, at least in non-pathological states. Within isolated myocardial tissue, ET is a potent inotrope, with effective concentrations in the subnanomolar range. However, the *in vivo* relevance of these data remains unclear.

1.7. The ETR-p1/fl peptide

The ETR-P1/fl peptide is an antisense homology box-derived peptide with strong inhibitory potency against the ET-A receptor. The ETR-P1/fl peptide was developed by using the antisense homology boxes of the human ET-A receptor. The sense-antisense interaction means that the peptide synthesized from the inactive DNA strand is complementary to the peptide translated from the active DNA strand. Peptides therefore can recognize and bind to each other. Moreover, a sense-antisense interaction exists not only between individual peptides, but also within two parts of the one peptide chain.

1.8. Significance of fluid resuscitation in circulatory disturbances

In the treatment of acute circulatory failure, fast, efficient and self-tailored fluid replacement is a primary goal. The administration of intravenous fluid to avoid dehydration, maintain an effective circulating volume, and prevent inadequate tissue perfusion should be considered a core element of perioperative practice. The main goal of fluid therapy is to ensure an adequate O_2 supply for the organs.

2. Main goals

The general aim of our studies was to analyze the effects of ET-A receptor antagonism on the central and peripheral circulatory patterns in physiological states and in conditions associated with endogenous ET-1 overproduction.

Many clinicians use ejection fraction measurements to evaluate the cardiac contractility. However, the ejection fraction is influenced by preload and afterload alterations without any change in contractility. Depending on the loading conditions, hearts with a lower ejection fraction can produce a greater CO. Although roughly indicative of the cardiac reserve, the ejection fraction is an inconsistent marker for the overall cardiac function perioperatively.

1.4. Analysis of microhemodynamics

The recent development of new medical imaging techniques, together with data from clinical investigations, has helped to identify the microcirculation as playing a key role in pathological conditions. The most convincing data concerning the significance of this system derive from intravital microscopy (IVM) studies. This technology allows real-time imaging of the microcirculation and exact determination of the consequences of circulatory disorders. Disturbances of the microcirculatory perfusion are characterized by changes in the functional capillary density (FCD) and the red blood cell velocity (RBCV, $\mu m\ s^{-1}$). The FCD is defined as the length of red cell-perfused capillaries in relation to the observation area, which accurately describes the decrease in efficacy of tissue perfusion when the corresponding area is unchanged.

1.5. The vascular endothelium

Endothelial cells as the bricks of vascular lining are involved in many aspects of vascular biology, including vascular tone, hemostasis, immune and inflammatory responses, barrier function and angiogenesis. These functions range from relatively long-lived features to minute-by-minute responses to stimuli such as the synthesis and secretion of vasoactive mediators. The blood flow regulates the internal diameter of the arteries, both chronically through reorganization of the cellular and extracellular components of the vascular wall, and acutely through relaxation/contraction of the smooth muscle cells. In both cases, the presence of the endothelium is required. Vasodilatation/vasoconstriction are regulated by many reflexes, neuronal and hormonal substances; they usually have systemic responses.

1.6. Vasoregulatory role of endothelins

ET release is regulated by both rheological and chemical factors, such as pulsatile stretch, shear stress and pH. Hypoxia is considered one of the basic stimuli for ET synthesis. Cytokines, adhesion molecules and vasoactive agents also stimulate ET production. Under physiological conditions, ET is produced predominantly by the endothelium, but in pathophysiological states other cells, such as PMNs, macrophages, smooth muscle cells, cardiomyocytes and mesangial cells can also be the source of ET release.

ET isoforms are coded by at least three distinct genes; following transcription, ETs are formed by multiple cleavage via prepro-ET and then big-ET. The process may be influenced considerably by the activity of ET converting enzyme (ECE) isoforms present in endothelial, smooth muscle cells, cardiomyocytes and macrophages. Due to the functional and structural similarities with neutral endopeptidases, ECE-independent mechanisms may also contribute to ET production. The process results in 21-residue peptide isoforms of ET-1, ET-2, and ET-3. The responsiveness to ET isopeptides is heterogeneous in a variety of vascular and nonvascular tissues.

1. Introduction

The endothelins (ETs) are powerful vasoactive peptides, of which ET-1 is the major isoform. There is a substantial and growing body of evidence that ET-1 and the activation of ET receptors play decisive roles both in physiological vasoregulation and during acute disorders of the cardiovascular system. The peptide ET-1 was originally described as the most potent vasoconstrictor agent produced by endothelial cells. Nevertheless, it has been established that other cells, such as leukocytes, macrophages, smooth muscle cells and cardiomyocytes, can also be sources of ET-1 release in pathophysiological circulatory states. It has further been demonstrated that plasma ET-1 levels correlate linearly with the severity of circulatory disorders; accordingly, the possibility of ET receptor antagonist therapies has received increasing attention.

1.1. Regulation of cardiac performance

The cardiac pump function or performance depends on many parameters, which act in parallel and synergy normally exists to some degree. Examples of performance parameters include cardiac output (CO), stroke volume (SV), rate of fiber shortening, stroke work, myocardial compliance and ejection fraction. Myocardial performance is dependent on *preload, afterload, heart rate (HR)* and *contractility,* and is influenced by neurohormonal and local endothelial-derived factors.

1.2. Analysis of cardiac function

As many of the indices of myocardial performance are independent, qualifying the contribution of each component to the overall cardiac function is not possible at present, and the clinical utility of monitoring each individually is not therefore established. Bedside measurements of left ventricular (LV) dimensions, volumes and ejection fraction and the other indices of systolic and diastolic function can now be carried out, but their routine use in clinical practice remains unproven.

Examinations of cardiac contractility in clinical practice would be extremely beneficial, but at present direct measurement is not possible. In practice, there are few invasive or non - invasive monitoring techniques to evaluate or calculate this parameter. The end-systolic pressure-volume relationship (ESPVR; for details see below) is a fundamental description of systolic cardiac mechanics, with especial regard to the preload recruitable stroke work (PRSW) relationship. PRSW is another index of contractility, which is perhaps less influenced by other parameters. The stroke work is the area of the pressure-volume loop. For each pressure-volume loop derived by vena caval occlusion, the stroke work is plotted relative to its end-diastolic volume. The slope of the derived linear relationship is a measure of contractility independent of preload and afterload. The PRSW relationship reflects the overall performance of the left ventricle, combining the systolic and diastolic components. It follows that the degree of contractility can be assessed, but, unlike blood pressure, an ideal number or range to describe it can not be derived. Since ESPVR and PRSW are unique for each ventricle, these parameters more accurately measure changes in contractility. The greatest impediment to the clinical application of ESPVR and PRSW is the difficulty in measuring ventricular volume and inducing a preload reduction to derive the pressure-volume loops. More easily measurable indices of contractility have been actively sought.

Cardiac and peripheral hemodynamic effects of selective endothelin-A receptor antagonism

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