Research Article

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Dysbalance in sympathetic neurotransmitter release and action in cirrhotic rats: Impact of exogenous neuropeptide Y

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Background & Aims: Splanchnic vasodilation is an essential disturbance in portal hypertension. Increased systemic sympathetic nerve activity is well known, but potential corresponding vascular desensitization is incompletely characterized. Release of splanchnic sympathetic neurotransmitters noradrenaline (NA) and co-transmitter neuropeptide Y (NPY) remains to be elucidated. Finally, the effects of exogenous NPY on these mechanisms are unexplored.

Methods: Portal vein ligated cirrhotic, and control rats were used for *in vitro* perfusion of mesenteric arteries. Depletion of vascular pressure response was induced by repetitive electric sympathetic perivascular nerve stimulation (PNS) and performed in the absence and presence of exogenous NPY. Additionally, PNS-induced release of NA and NPY was measured.

Results: Mesenteric PNS-induced pressure response was lower in portal hypertension. Depletion of the pressure response to PNS, representing the degree of desensitization, was enhanced in portal hypertension. NA release was elevated, whereas NPY release was attenuated in cirrhosis. Administration of exogenous NPY led to marked recovery from desensitization and vasoconstrictive improvement in cirrhotic rats, being associated with more pronounced decrease of NA release.

Conclusions: Pronounced depletion of splanchnic arterial pressure-response to repetitive sympathetic nerve stimulation in cirrhosis is partly attributable to altered NA release as well as to deficient NPY release. External NPY restores vascular contractility and attenuates pathologically elevated NA release in the portal hypertensive mesenteric vasculature, revealing post-, and prejunctional effects at the vascular smooth muscle motor endplate; therefore outlining encouraging therapeutic strategies.

Keywords: Neuropeptide Y; Noradrenaline; Perivascular nerve-stimulation; Portal hypertension; Splanchnic circulation.

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Abbreviations: ACH, acethylcholine; b.w., bodyweight; DPP-IV, dipeptidyl-peptidase-IV; ET-1/2, endothelin-1/2; HSP-90, heat-shock-protein-90; LC, liver cirrhosis; NA, noradrenaline (norepinephrine); NPY, neuropeptide Y; eNOS, endothelial-nitric-oxide-synthase; nNOS, neuronal-nitric-oxide-synthase; NO, nitric oxide; PNS, electric perivascular-nerve-stimulation; PR, pressure-response; PVL, portal vein ligation; r, pearson correlation coefficient (pcc); R^2, coefficient of determination; SE, standard error; SMA, superior mesenteric artery; SNS, sympathetic-nervous-system.

Introduction

Splanchnic arterial vasodilation in portal hypertension is attributed to overproduction of vasodilators and hyporeactivity to vasoconstrictors [1], which is mediated partly by defective intracellular vascular smooth muscle cell signaling [2]. Upregulated sympathetic nervous system (SNS), acting as compensatory mechanism, reveals prognostic significance, namely increased serum levels of noradrenaline (NA), correlating with survival of cirrhotic patients [3,4]. In case of portal hypertension, systemic plasma NA levels could mainly depend on splanchnic NA production [5]. Besides systemic NA levels, data in terms of actual splanchnic synthesis/release of neurotransmitters are scarce and controversial [6–12].

Neuropeptide Y (NPY) is co-stored and co-released with NA from secretory vesicles of sympathetic nerve terminals, inducing potentiation of α_1-adrenergic vasoconstriction via G-protein-associated postsynaptic Y_1 receptors [13]. It mediates this effect via sensitizing vascular smooth muscle to NA, although the exact pathway is unknown [14]. We previously found that NPY augments α_1-adrenergic mesenteric vasoconstriction induced by exogenous NA in cirrhotic rats to a greater extent than in healthy animals [15,16].

Prolonged adrenergic tissue stimulation leads to diminished responsiveness to subsequent activation by catecholamines; this general process is termed "desensitization". Consequently, sustained SNS activity, as seen in pheochromocytoma, may lead to vascular desensitization, thereby aggravating splanchnic vasodilation in cirrhosis as well [17,18].

In the isolated rat mesenteric bed, electric sympathetic nerve stimulation mimics extensive and/or prolonged SNS activity. This procedure has been examined previously in healthy rats and causes pressure response by inducing NA and NPY release [19,20]. However, the physiological stimulus for the sympathoadrenergic modulation of vascular tone, namely nerve stimulation, has not been used so far for direct evaluation of SNS neurotransmitter release and associated hemodynamic action in portal hypertensive splanchnic vasculature. Therefore, we aimed firstly at exploring the hemodynamic effect of mesenteric vascular

desensitization caused by repetitive perivascular nerve stimulation with, secondly, concomitant assessment of PNS-induced SNS neurotransmitter release in portal hypertensive rats. Thirdly, the impact of exogenous NPY on PNS-induced pressure response and SNS neurotransmitter release was addressed.

Materials and methods

Animals

Experiments were conducted according to the German Physiological Society principles for the use of laboratory animals (Granted permission number 621-2531.1-23/00, Government of Bavaria).

CCl_4-induced liver cirrhosis (LC)

Cirrhosis was induced in male specific pathogen-free rats (Charles-River) by CCl_4 inhalation along with phenobarbital (0.35 g/L) containing drinking water. After 12–16 weeks, this approach induces cirrhosis. Age- and sex-matched rats were used as control group (Con).

Induction of portal hypertension: Portal vein ligation (PVL)

A prehepatic portal hypertensive animal model extensively studied in our laboratory was used [15,16]. Briefly, after a midline abdominal incision, the portal vein was freed from the surrounding tissue. A ligature was placed around a needle lying alongside the portal vein. Subsequent removal of the needle yielded a calibrated stenosis.

In vitro perfusion

The *in vitro* perfusion system used was a modification of that originally described by McGregor and used extensively in our laboratory [21]. After isolating the superior mesenteric artery (SMA) with its mesentery, splanchnic vasculature was perfused with oxygenated 37 °C-Krebs solution using a roller pump (Isamtec, IPC-8-channel; Glattburg, Zürich, Switzerland). Perfusion pressure was measured with a P-23-Db strain gauge transducer (Statham) and continuously recorded (Powerlab Quadbridge and Powerlab 4/20; AD Instruments).

Perivascular nerve stimulation (PNS)

Nerves surrounding the SMA were stimulated (33 Hz, 50 V, 30 s) using a nerve stimulator (I-ZQ4V, Hugo Sachs Electronics). In preliminary experiments, we investigated the time and frequency dependent induction of the vascular pressure response (PR). We confirm that PNS at 33 Hz and 30 s causes maximal PR, which cannot be increased further by higher frequencies and/or longer stimulation times [19]. PNS provokes reproducible peaks of PR by stimulating endogenous vasoconstrictor release [19].

Quantification of noradrenaline (NA)

Aliquots of 3-ml perfusate were collected (collection tubes containing 60 µl GSH/EGTA solution as preservative). A Prominence HPLC system (Shimadzu, Duisburg, Germany) with an amperometric detector 3500A and Sputnik® detector cell (Recipe, Munich/Germany) was used. Aliquots of 1–1.5 ml perfusate were analysed with a commercially available kit (ClinRep® Catecholamines in plasma, Recipe, Munich/Germany). As low as 5 pg/ml NA could be determined.

Quantification of neuropeptide Y (NPY)

A commercial radioimmunoassay kit by Eurodiagnostica (EURIA-NPY) was used. Crossreaction with other peptides was below 0.1%. The lowest detectable concentration was 3 pmol/L. The mean recovery range was 75–88%.

Protocol I: desensitization to repetitive PNS and neurotransmitter release

This protocol was performed to characterize the extent of pressure response degradation caused by repetitive SNS neurotransmitter release [Con: n = 19, PVL: n = 12, LC: n = 19]. After baseline perfusion at 4 ml/min was established for 30 min, 15 repetitive PNS were implemented (interval-time: 8 min). This first period of repetitive PNS is called the 1st perfusion cycle. To compare endogenous NA and NPY release, perfusate samples were collected after 3rd, 6th, 9th, 12th, and 15th PNS in another series of experiments [Con: n = 12, LC: n = 12]. In PVL rats, hyperdynamic-circulation develops acutely (within 3–5 days) [22]. Portal hypertension decreases afterwards due to the development of portosystemic collaterals, whereas compensatory SNS upregulation and altered NA release in cirrhosis emerge chronically [22]. In cirrhotic rats, systemic NA levels are higher than in PVL rats, indicating that enhanced sympathetic nerve activity depends on the development of cirrhosis [5]. Therefore, PVL rats were not used for NA measurement.

Protocol II: prolonged effects of pressure response (PR) depletion and impact of exogenous NPY

After the 1st perfusion cycle, a 1-h washout period was performed, followed by a 2nd perfusion cycle with 5 sequential PNS (PNS-1b–5b) to investigate the sustained effects of PR depletion. To characterize the influence of exogenous NPY, the groups were divided [Con: n = 8, Con + NPY: n = 8, PVL: n = 6, PVL + NPY: n = 6, LC: n = 11, LC + NPY: n = 11]. In NPY groups, exogenous NPY [50 nM] was added to the perfusion solution halfway through the 1-h washout period. In preliminary studies, we found 50 nM NPY having no direct vasoconstrictive action. NPY was present at the same molar concentration in the perfusion system throughout the 2nd perfusion cycle. For NA measurement, samples were collected similar to protocol I in another series of experiments [Con: n = 12, LC: n = 12]. In the 2nd perfusion cycle, NPY release was not measured, because interacting exogenous NPY would hinder interpretation.

Statistical analysis

Normal distribution was tested by Kolmogorov–Smirnov-test. Values were compared using ANOVA (one-way, with repeated measurements and correction for multiple comparisons) or the Student's-*t* test if appropriate. The significance level was set at $p < 0.05$.

Results

Animals

Spleen weight was elevated in portal hypertension (Table 1). Pressure response to PNS correlated negatively and significantly with spleen weight ($r = -0.86$, $p < 0.01$) being apparently logarithmic ($R^2 = 0.8$).

Repetitive PNS and pressure response (PR) [1st perfusion cycle]

Baseline perfusion pressure was lower in portal hypertensive rats (Table 1). PR was maximal at PNS-3 and decreased with each further PNS, reflecting a desensitization process. PR was blunted in portal hypertension (Fig. 1A). The decline in pressure response was estimated relative to maximum (PNS-3). This process was pronounced in portal hypertensive study groups, indicating an accelerated decline of pressure response to repetitive PNS (Fig. 1B). In fact, in portal hypertensive mesenteric arteries, significantly less numbers of PNS were necessary to achieve, e.g., a 40%-reduction (Table 1) from maximum pressure response.

PNS-induced NA release and associated PR [1st perfusion cycle]

Basal NA release was low in both study groups (Con: 13.6 ± 9.6 pg/ml *vs.* LC: 15.0 ± 6.2 pg/ml; n.s.). PNS-induced NA release was higher in cirrhosis (Fig. 1C). With increasing numbers of PNS, NA release degraded . The slope of decrease and the overall decrease of NA release were more pronounced in cirrhotic rats (477.5 ± 121.6 pg/ml) as compared to controls (219.9 ± 110.9