Norepinephrine Disrupts Cytoskeletal Framework of Microtubules in Rat Hearts

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Sympathetic activations may deteriorate myocardial failure due to progression of myocardial cell injury. In the present study, to test whether microtubules, calcium ion (Ca^{2+}) sensitive cytoskeletons, are disrupted by norepinephrine (NE) and whether beta-adrenoceptor antagonist could attenuate the disruption of microtubules, structures of microtubules are studied in rat hearts with continuous subcutaneous infusions of norepinephrine. In the sham operated rats the microtubules stained by immunohistochemical technique showed normal network structures. A low dose of NE infusion ($2 \mu\text{g/kg/h}$) for 6 h resulted in a minimal change in microtubule structures. However, infusion for 24 h of NE ($2 \mu\text{g/kg/h}$) and a large dose of NE infusion ($20 \mu\text{g/kg/h}$) for 6 h caused disruptions of microtubules in small patchy lesions ($8 \pm 3\%$, $12 \pm 4\%$ of area, respectively). A large dose of NE infusion for 24 h increased systolic blood pressure from 116 ± 6 to 152 ± 4 mmHg and increased plasma NE concentration from 430 ± 40 to 17100 ± 3700 pg/ml and further disrupted the network of microtubules in $40 \pm 6\%$ of the total area. Propranolol ($500 \mu\text{g/kg/h}$) markedly attenuated NE-induced disruptions of microtubules. Disruptions of microtubules may be one of the underlying mechanism of deterioration of myocardial failure in chronic heart failure in which sympathetic activity is markedly activated.

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A large body of evidence suggests that enhanced sympathetic activity in chronic heart failure deteriorates the function of the failing myocardium and that beta-adrenoceptor blocker therapy improves the cardiac function and symptoms of patients with chronic heart failure^{1–5}. These clinical observations are in accordance with the in vitro and in vivo findings that an overdose of catecholamine can induce cellular injury

of the myocardium, leading to cardiac dysfunction^{6–10}. A number of possible mechanisms of cellular injury have been raised, eg, tissue ischemia induced by an increase in myocardial oxygen consumption, microcirculation disturbances due to coronary arterial vasoconstriction and platelet aggregation, enhanced activation of renin-angiotensin-aldosterone system, down-regulation of beta-adrenoceptors and catecholamine cardiotoxicity^{6–10}. Although the subcellular mechanisms of catecholamine injury in heart failure have not been extensively studied yet, intracellular calcium (Ca^{2+})

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overload may play a key role in the progression of cardiac dysfunction. However, abnormal calcium ion handling in failing hearts could not fully explain the morphological changes in the failing myocardium. It is reported that microtubules which compose the cytoskeletal framework of the cell are sensitive to Ca^{2+} overload,^{11,12} and recently, we observed that microtubules are disrupted prior to irreversible injury in ischemic canine hearts.¹³ These results may imply that catecholamine stimulation of the heart can induce disruption of microtubules prior to irreversible injury through augmentation of Ca^{2+} influx, and that treatment with beta-blocker can attenuate the cytoskeletal damage. If this is so, continuous infusion of catecholamine for treatment of heart failure may insult the microtubule structure of the heart. In the present study, we investigated whether microtubules of rat hearts are disrupted by continuous norepinephrine infusion, and whether a beta-adrenoceptor blocker, propranolol, can attenuate the cytoskeletal injury.

MATERIALS AND METHODS

Preparation of the experimental model:

Male Wistar-Kyoto rats weighing 190–210 g were anesthetized with ether. A small incision was made in the back, and a pocket was prepared by blunt dissection. An Alzet Osmotic Minipump (Model 1003D) containing norepinephrine (Aldrich Chem. Co.) and 0.2% ascorbic acid as an antioxidant, was inserted, and the incision was closed with wound clips.¹⁴ The osmotic pump allows the administration of drug at a constant rate of $1.0 \mu\text{l/h}$ for more than 3 days. The concentrations of norepinephrine in the minipumps were $0.4 \mu\text{g}/\mu\text{l}$, $4 \mu\text{g}/\mu\text{l}$ and $40 \mu\text{g}/\mu\text{l}$, so that animals receive doses of $2 \mu\text{g/kg/h}$ (for 6 h: $n=5$, for 24 h: $n=5$), $20 \mu\text{g/kg/h}$ (for 6 h: $n=5$, for 24 h: $n=5$, for 36 h: $n=2$) and $200 \mu\text{g/kg/h}$ for 12 h ($n=2$). Osmotic pumps containing saline were also implanted in 5 rats (sham operated group).

To examine the effect of beta-adrenoceptor blockade, propranolol (Sigma Chemical Co.) was also administered subcutaneously by osmotic pump for 24 h at a rate of $50 \mu\text{g/kg/h}$ ($n=5$) or $500 \mu\text{g/kg/h}$ ($n=5$) to the rats who had received the infu-

sion of norepinephrine $20 \mu\text{g/kg/h}$.

Measurements of blood pressure: Before sacrificing the animals, systolic blood pressure (SBP) and tail pulse (TP) were measured in the conscious state by tail cuff plethysmography.¹⁴ Animals were warmed at 37°C for 10 min and then gently placed in a lutice restraining cage. The tail cuff consisted of a metal tube 14 mm in diameter fitted with a thin inflatable rubber bladder 18 mm long. Maximal cuff pressure and inflation-deflation rate were controlled with a programmed electro-sphyngomanometer (MK-1000, Muromachi Kikai Co LTD, Tokyo, Japan). The average of the first 5 stable measurements was taken as the SBP and TP. Pilot studies verified that the indirect measurements of SBP and TP were virtually identical to simultaneously recorded carotid arterial systolic pressure measured directly with a catheter.

Measurement of norepinephrine concentration: Without anesthesia, animals were killed by a cervical dislocation and blood was quickly sampled for a measurement of norepinephrine concentration. The method of norepinephrine measurement has been described previously.¹⁵ Five milliliters of blood were taken into a tube containing EDTA which was immediately placed in iced water and centrifuged for 20 min. The plasma was kept at -80°C . Within 2 weeks, plasma norepinephrine was adsorbed on alumina and separated by high-performance liquid chromatography (pump, LC-3A; column, Zpax-SCX; Shimazu Seisakusho, Kyoto, Japan). Plasma norepinephrine was determined spectrofluorometrically by the trihydroxyindole method (Shimazu spectrofluorometer RF-500LCA). In this system, sensitivity of the assay is 10 pg/ml plasma and the intra-assay coefficient of variation is 6.8%.¹⁵

Immunohistochemical study: After blood sampling, the heart was excised and the aorta was cannulated. Myocardium was fixed by retrograde perfusion through the aortic canula with a periodate-lysine-paraformaldehyde (PLP) fixative at a pressure of 40 mmHg.¹³ From the mid portion of left ventricle, a thin tissue sample (2 mm in width) was obtained. Samples were immersed in PLP fixative for 6 h and then placed overnight in 4% paraformaldehyde solution (pH

TABLE I SYSTOLIC BLOOD PRESSURE, TAIL PULSE RATE AND PLASMA NOREPINEPHRINE CONCENTRATION DURING NOREPINEPHRINE INFUSION

	<i>n</i>	SBP (mmHg)	TR (/min)	NE (pg/ml)
<i>sham</i>	5	116±5	417±8	430±40
NE infusion (2 µg/kg/h)				
6 h	5	137±5*	432±9	9800±1100*
24 h	5	142±6*	459±17	12600±1700*
NE infusion (20 µg/kg/h)				
6 h	5	148±5*	437±7	14600±2200*
24 h	5	152±4*	417±5	17100±3700*
NE infusion (20 µg/kg/h) with propranolol for 24 h				
50 µg/kg/h	5	117±5+	417±12	9500±900
500 µg/kg/h	5	112±3+	401±4	9300±900

* $P < 0.05$ vs sham, + $p < 0.05$ vs NE infusion for 24 h.

SBP: systolic blood pressure, TR: tail pulse rate, NE: plasma norepinephrine concentration.

Data are mean ± SEM.

7.4). After fixation the tissue samples were rinsed successively in phosphate buffered saline (PBS) solutions containing 10%, 15%, and 20% sucrose (4 h each). Cryosections (2 µm thick) were cut using a tissue-Tek-II microtome/cryostat (miles Inc., Elkhart, Ind.); then they were mounted on gelatin-coated glass slides and rinsed 3 times in PBS (30 min each). The sections were processed for the double staining of microtubules and actin filaments. The sections were incubated overnight at 4°C with monoclonal antibody against beta-tubulin (Amersham Corp., Buckinghamshire, UK; diluted 1:300 in PBS) and then incubated fluorescein isothiocyanate-labeled sheep anti-mouse immunoglobulin (Amersham; diluted 1:30 in PBS) for 1 h at room temperature. Sections were incubated with rhodamine-phalloidin (Wako, Osaka, Japan; diluted 1:70 in PBS) for 20 min at room temperature and rinsed in PBS. All sections were examined by an FX-S RFL fluorescence microscope (Nikon Inc., Tokyo). To test the nonspecific staining, the primary antibody was deleted from the procedures in 2 control samples.

Microscopic views (×80) of randomly selected areas from the transmural samples were photographed (40–80 pairs of photographs for each tissue sample), and the lesions where the microtubule stains were poor or lost were marked by color pens on the photographs. The percentage area of the

lesions of each grade was obtained by the ratio of the area of lesions to total areas examined.

Statistics Blood pressure, tail pulse rate and plasma norepinephrine concentrations were compared by unpaired t-test. All values were expressed as mean ± SEM, and $p < 0.05$ was considered significant.

RESULTS

Systolic blood pressure and tail pulse rate: Table I depicts the systolic blood pressure (SBP), pulse rate and plasma NE concentration in sham operated group and NE infusion group. In the control (sham) group the mean SBP was 116±5 mmHg and tail pulse rate was 417±8/min. In the low dose NE infusion (2 µg/kg/h) group, SBP was significantly higher than the control group. In the large dose NE infusion (20 µg/kg/min), SBP was further elevated, whereas pulse rate was only slightly increased by NE infusion. In contrast, in the NE with propranolol group SBP and pulse rate were almost identical with those in the control group.

Plasma catecholamine concentration: Continuous infusion of NE (2 µg/kg/h) resulted in increases in plasma NE from 430±40 pg/ml to 9800±1100 pg/ml (6 h) and to 12600±1700 pg/ml (24 h). A large dose (20 µg/kg/h) of NE further increased plasma NE levels (Table I).

Fig. 1. Double labeling of ventricular myocardium with mouse monoclonal antibodies against beta-tubulin (panels a and b) and rhodamine phalloidin (panel c) in sham operated rat (a) and norepinephrine infusion (panels b and c). Microtubules stained with antibodies against beta-tubulin form a filamentous network throughout the cytoplasm. Note that the cytoplasmic microtubules are composed of longitudinal and transverse filaments. A part of them forms the circular architecture around the nucleus (panel a). A large dose of infusion of NE ($20 \mu\text{g/kg/h}$) for 24 h caused fragmentations of microtubules (dotted spots) and loss of microtubule immunoreactivity. Network structures of microtubules are unclear (panel b). Panel c shows the actin filaments in the same region as panel b. The alignment of the cross striations is normal although the microtubules are disrupted.

Structure of microtubules: In the sham operated rat, microtubule structures of the heart were observed as tortuous filaments loosely organized throughout the cytoplasm, which were mainly composed of longitudinal and transverse filaments. Both filaments formed reticular structures; some of them were localized at perinuclear space forming a basketlike structure around the nucleus (Fig 1a). Actin filaments labeled with rhodamine-phalloidin form the cross striations of sarcomeres. After the low dose of NE infusion ($2 \mu\text{g/kg/min}$) for 6 h, minimal changes were observed in the immunoreactivities of microtubules. After 24 h, however, small patchy lesions appeared ($8 \pm 3\%$ of the total area) which were composed of several myocytes in which the filamentous structures of microtubules were decreased or fragmented (dotted spots). A large dose of NE infusion ($20 \mu\text{g/kg/h}$) for 6 h resulted in similar disruptions of microtubule networks ($12 \pm 4\%$). NE infusion ($20 \mu\text{g/kg/h}$ for 24 h) caused patchy loss of microtubule structures in $40 \pm 6\%$ of the area

(Fig 1b), in which the cross striations of actin filaments remained normal (Fig 1c). Prolonged infusion of a large dose of NE ($20 \mu\text{g/kg/h}$ for 36 h) and a larger dose NE infusion ($200 \mu\text{g/kg/h}$ for 13 h) resulted in increases in size and number of patchy lesions of microtubular loss (78% and 94%, respectively).

Propranolol infusion ($500 \mu\text{g/kg/h}$) to the rats treated with NE attenuated disruptions of microtubule structures ($17 \pm 5\%$ of the area), although small doses of propranolol ($50 \mu\text{g/kg/h}$) did not attenuate the microtubule disruption ($34 \pm 5\%$ of the area).

DISCUSSION

In the present study we demonstrated that the network structures of microtubules in rat hearts are disrupted by subcutaneous infusion of norepinephrine in a dose- and duration-dependent manner through beta-adrenoceptor stimulation.

Implantation of mini-osmotic pumps to deliver NE increased systolic blood pressure

and did not increase tail pulse rate in our study. These observations are comparable to the results of previous report.¹⁴ Although systolic blood pressure and tail pulse did not seem to respond to NE in a dose-dependent manner, significant elevations of plasma NE level suggest that the doses of NE could adequately stimulate *in vivo* rat hearts.

Disruption of microtubules and its mechanism: In the present study, microtubule structures were minimally insulted with a low dose of NE infusion ($2 \mu\text{g/kg/h}$) for 6 h. However, with longer administration (12 h), patchy lesions were observed, indicating that disruption of microtubules could be induced with even a small dose of NE, which is often administered clinically. Indeed, the resultant increase in plasma catecholamine levels is also similar to that observed in patients with pheochromocytoma.^{16,17} This may also imply that structures of microtubules are disrupted during a long period of uncompensated heart failure in which plasma NE is markedly increased. This is compatible with our observation that microtubules of the biopsied sample from the left ventricle were disrupted to various extents in patients with dilated cardiomyopathy.¹⁸

It is of interest that the disruptions of microtubules in this study seem morphologically different from those during ischemia. In ischemic condition, immunoreactivity of microtubules often completely disappeared associated with irreversible actin filament degeneration.¹³ In contrast, in the present study fragmentations of microtubules (dotted spot) without irreversible actin filament degeneration were observed. This type of microtubule disruption is similar to our previous observation in cultured neonatal rat cardiomyocytes exposed to the beta-adrenergic agonist, isoproterenol.¹⁹ Thus, the underlying mechanisms of disruptions of microtubule structures in the present study may be different from those during ischemia. Although the mechanisms could not be clarified from this study, several lines of evidence suggest that exposure to NE increases intracellular Ca^{2+} concentrations which in turn, disrupt microtubules: Ca^{2+} induced disruptions of microtubules have been demonstrated by microinjections of calcium into the monkey kidney cells and fibroblasts^{11,12} and by treatment with Ca

ionophore in cultured rat cardiomyocyte.²⁰ Ca^{2+} -dependent neutral protease may also be involved in disruptions of microtubules.²¹⁻²⁴ Second possibility is that phosphorylation of tubulin and/or microtubule associated proteins (MAPs) by adrenoceptor stimulation may inhibit polymerization of tubulin to microtubule, since phosphorylation of tubulin and MAPs depolymerizes the microtubule filament *in vitro*.²⁵ Another possibility that mechanical stress²⁶ due to increased SBP and depletion of GTP²⁷ through augmented contractility may disrupt microtubules, also cannot be excluded. In the present study blood pressure response to NE was not dose dependent, however, mechanical stress may be involved in microtubule disruptions.

Effects of beta-antagonist: In this study, administration of propranolol significantly attenuated the disruptions of microtubules induced by NE infusion. During administration of propranolol, SBP and pulse rate were reduced to levels comparable to the control rats. Thus, although hemodynamic effects of propranolol cannot be excluded from the underlying mechanisms, attenuation of Ca^{2+} influx through beta-adrenoceptor blockade may play a key role in the protective effect of propranolol. Whatever the mechanism, our results could provide an important clinical implication in view of a long-term beta-blocker treatment for chronic heart failure.

Technical consideration: Before conclusion we have to exclude the possibility that the disruption of microtubules shown in the present study is an artifact. We used a monoclonal antibody against beta-tubulin to exclude cross-reactions with other components than the microtubules. The filamentous network structures of microtubules stained by the immunohistochemical technique are almost the same as reported in the previous studies.^{13,28} Furthermore, control staining without the first antibody failed to stain the characteristic microtubule structures. However, there is a possibility that disruption of microtubules is due to inadequate perfusion of the fixative to the myocardium because perfusion of the fixative during norepinephrine infusion may not be uniform due to coronary vasoconstriction or thrombus. In our study, however, rhodamine-phalloidin stains of actin fila-

ments did not show the ischemic injury, e.g. disruption of cross-striation and contraction band. Therefore, it is not plausible that the loss of immunoreactivities is an artifact.

In summary, microtubule structures of the rat heart could be disrupted during a long-term infusion of NE or clinical doses. A marked attenuation of the cytoskeletal injury by propranolol administration may provide a rationale for beta-blocker therapy for chronic heart failure in which sympathetic activity is markedly activated.

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