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Sympathetic Activation in Adipose Tissue and Skeletal Muscle of Hypertensive Rats

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Abstract—The activation of the sympathetic nervous system is a common feature of arterial hypertension and other cardiovascular diseases. This activation might be dependent on an altered baroreflex control of vascular resistance of which the inhibitory response on sympathetic activity appears impaired. The aim of the study was to monitor during the natural course of arterial hypertension in spontaneously hypertensive (SHR) and age-matched Wistar Kyoto (WKY) rats (5, 16, 30, and 54 weeks of age) the peripheral sympathetic activity expressed as interstitial norepinephrine (NE) release and as tyrosine hydroxylase (TH) activity, the rate-limiting enzyme of NE synthesis, in the differently baroreflex-controlled subcutaneous adipose tissues and skeletal muscles. Blood pressure and plasma NE in SHR were similar to WKY at 5 weeks of age but increased at all other ages. Body weight was similar in both 5-week-old rats but reduced in SHR at all other ages. The interstitial NE levels were greater in both SHR tissues at all ages as compared with WKY. In adipose tissue of SHR, TH activity was higher at all ages as compared with WKY, whereas TH activity in skeletal muscle was higher only after the development of hypertension. These data show that in both SHR tissues, an increase of interstitial NE release is always present during its lifespan. This suggests that increased sympathetic activation in the SHR model is not specific to baroreflex-controlled tissues such as skeletal muscle but involves also subcutaneous adipose tissue, the sympathetic efferents of which are independent from baroreflexes. (*Hypertension*. 2002;36[part 2]: 656-661.)

Key Words: norepinephrine ■ muscles ■ adipose tissue ■ rats, spontaneously hypertensive

The increased peripheral sympathetic outflow represents a common feature of many hypertensive patients and of spontaneously hypertensive rats (SHR), especially in the early phases of development of the disease.[1–5] Preferential activation of sympathetic neural outflow to the heart, the kidney, and the skeletal muscle has been reported in both essential and experimental hypertension in association with altered mechanisms of norepinephrine (NE) synthesis, including tyrosine hydroxylase (TH) and dopamine β-hydroxylase activity, and reuptake and degradation of NE when released from nerve endings.[4–10] Cardiac, but especially skeletal muscle, sympathetic outflow are strongly modulated by baroreflexes, and some authors suggest that the increase of sympathetic neural activity could be the result of an early impaired inhibitory influence of arterial baroreceptors.[11–13] However, the increased peripheral sympathetic discharge does not seem to be localized to tissues under baroreflex control, because we have recently demonstrated in prehypertensive and hypertensive SHR that an increased release and metabolism of NE is also detectable in the subcutaneous adipose tissue,[14–15] where sympathetic activity is relatively independent from the baroreflex function.[16,17,18] In contrast to skeletal muscle

sympathetic activity, the impulses to subcutaneous adipose tissue show no signs of pulse-synchronous grouping and no correlation with spontaneous fluctuations of blood pressure, suggesting that baroreflex modulation is weak or absent, depending mainly on metabolic and thermoregulatory influences.[16–19]

In addition, little is known, in in vivo situations, about the time-course of peripheral sympathetic outflow during the lifespan of the SHR model of hypertension.

The purpose of this study was to investigate the sympathetic peripheral outflow in two peripheral tissues, the skeletal muscle and subcutaneous adipose tissues, which are controlled differently by baroreflexes, by combining the microdialysis technique[14,15] for the measurement of interstitial NE with the analysis of TH activity, the rate-limiting enzyme of catecholamine biosynthesis,[20] in the same group of awake and freely moving Wistar-Kyoto (WKY) and SHR during their natural history of arterial hypertension.

Methods

All the experimental procedures were approved by the Institutional Animal Ethics Committee of the University of Parma. Male SHR

Body Weight, Systolic Arterial Pressure, and Heart Rate in the Natural Course of Arterial Hypertension in WKY and SHR of 5, 16, 30, and 54 Weeks of Age

Parameters	WKY	SHR	WKY	SHR	WKY	SHR	WKY	SHR
	5 wk		16 wk		30 wk		54 wk	
Body weight, g	91 (5)†	88 (3)	314 (6)*	246 (7)§	415 (8)*	311 (7)§	504 (9)*	391 (6)
Systolic arterial pressure, mm Hg	113 (3)	117 (2)	122 (4)*	172 (3)§	138 (4)*‡	206 (5)	141 (5)*‡	200 (6)
Heart rate, bpm	411 (7)†	398 (8)§	357 (10)	365 (7)	338 (10)	345 (6)	332 (9)	340 (7)

Data are presented as mean±SEM.

*$P<0.01$ vs age-matched SHR; †$P<0.01$ vs older WKY; ‡$P<0.01$ vs younger WKY; §$P<0.05$, $P<0.01$ vs older SHR.

(n=35) and WKY (n=33) rats were obtained at 4 weeks of age from Charles River Italia, and they were kept 1 per cage in a room with 12-hour light/dark cycle and controlled temperature (23 to 25°C). The animals were fed standard laboratory rat chow (containing 0.54% of sodium chloride) and tap water ad libitum; body weight was measured once a week. Systolic arterial pressure was also measured once a week by the tail-cuff method as previously described.[14] Data values for each rat were taken as an average of at least 4 stable readings.

Microdialysis Experimental Procedure

Under light anesthesia with ether, two flexible concentric microdialysis probes with membranes of 10-mm length and 0.5-mm outside diameter (molecular weight cutoff, 20 Kda, CMA/20; CMA/Microdialysis AB) were inserted into the subcutaneous adipose tissue and skeletal muscle in the parascapular region, as we previously described.[15] A small biopsy, taken after the insertion of the microdialysis probe guide, was weighed, divided in two portions, and quickly frozen. One portion of the biopsy was checked by cryostat sections for histologic confirmation of the position of the probes in the white adipose tissue or in the skeletal muscle, the other was used for TH activity analysis. Microdialysis probes were then connected to a microperfusion pump (CMA100) and perfused at a flow-rate of 2.0 μL/min with a Ringer's solution. The rats took a few minutes to recover from the experimental handling, and after a 30-minute equilibration period, the dialysates were collected at 30-minute intervals over a period of 120 minutes and immediately analyzed for NE. At the end of the each microdialysis procedure, the rats were housed again for the longitudinal study. The same procedure was repeated on the same groups at the age of 16, 30, and 54 weeks. At the end of the microdialysis procedure, an arterial blood sample of 500 μL from the tail artery was taken from rats of each strain to measure plasma NE (5 weeks: SHR [n=10], WKY [n=10]; 16 weeks: SHR [n=9], WKY [n=8]; 30 weeks: SHR [n=8], WKY [n=8]; 54 weeks: SHR [n=6], WKY [n=7]).

NE Analysis and Tyrosine Hydroxylase Activity

NE concentrations in plasma and dialysates from tissue interstitia were immediately analyzed after collection by high performance liquid chromatography using electrochemical detection (HPLC-ECD), as previously described.[6] The probes used for the microdialysis procedure were calibrated in vivo for the relative recovery rate of NE in all rats undergoing experimental procedures by perfusing 3,4-dihydroxy-benzylamine (DHBA), an internal standard that is thought to behave like NE and have similar diffusion characteristics across the dialytic membrane. Hence, NE concentrations measured in the dialysates were corrected for an in vivo recovery of DHBA (mean recovery rate, 45±4%). TH activity in skeletal muscle and adipose tissue was analyzed by the method of Naoi et al.[21] Biopsies of skeletal muscle and subcutaneous adipose tissue were homogenized and incubated at 37°C for 10 minutes with 140 μmol/L L-tyrosine in 880 μL of sodium acetate-acetic acid buffer (100 mmol/L, pH=6.0) containing 1.4 mmol/L (6R)-5,6,7,8-tetrahydrobiopterin, 10 μg of catalase, and 0.7 mmol/L 4-bromo-3-hydroxybenzyloxyamine (NSD1055, an inhibitor of aromatic L-amino acid decarboxylase). The incubation was stopped by the addition of 0.1 mmol/L perchloric acid containing 0.4 mmol/L

sodium metabisulphite and 0.1 mmol/L disodium EDTA. After vortexing, the sample was allowed to stand in an ice bath for 10 minutes and then centrifuged at 1000 g for 10 minutes, and the supernatant was injected in a HPLC-ECD system for L-3,4-dihydroxyphenylalanine (L-DOPA) analysis. TH activity was calculated as the amount of L-DOPA generated from L-tyrosine per minute per milligram of tissue.

Statistical Analysis

Statistical analysis was based on a two-way ANOVA model for repeated measures, in which the dependent variable (NE concentration or TH activity) represents the same measurement taken at various points in the rat's life. Student's t test either for independent or paired samples was used to compare group means when ANOVA showed a significant effect of the factor. Relations between interstitial NE in both tissues and other indices of sympathetic activity were analyzed with linear regression analysis using Pearson correlation coefficients. All data are expressed as mean±SEM, and a value of $P<0.05$ was considered statistically significant.

Results

Body Weight, Arterial Pressure, and Heart Rate

As shown in the Table, 5-week-old SHR showed no differences in systolic arterial pressure and heart rate as compared with age-matched WKY. At that time, body weight was also comparable in both strains but became higher in older WKY as compared with age-matched SHR (t test, $P<0.01$). Systolic arterial pressure was also greater in SHR at 16, 30, and 54 weeks of age as compared with age-matched WKY and to 5-week-old SHR ($P<0.01$, t test). A slight but significant increase was also found in the systolic arterial pressures in WKY at 30 and 54 weeks of age as compared with younger rats. Heart rate was reduced in older rats of both strains as compared with 5-week-old rats (ANOVA, $P<0.01$), without differences between the strains at 16, 30, 54 weeks of age.

Plasma Norepinephrine Level

Five-week-old SHR and WKY showed similar levels of arterial plasma NE (t test, $P=0.46$). However, after the development of hypertension, an increase in plasma NE levels were found in SHR as compared with younger SHR and age-matched WKY (ANOVA, $P<0.01$) (Figure 1). No significant correlations were found between arterial plasma and interstitial NE both in the skeletal muscles and in the subcutaneous adipose tissues of WKY rats of all ages. No correlation between arterial plasma and interstitial NE in both tissues was found in the youngest (5-week-old) and oldest (54-week-old) SHR, whereas at 16 and 30 weeks of age, a slight significant relation was measured in both skeletal muscle (16-week-old: $r=0.66$, $P=0.05$, n=9; 30-week-old:
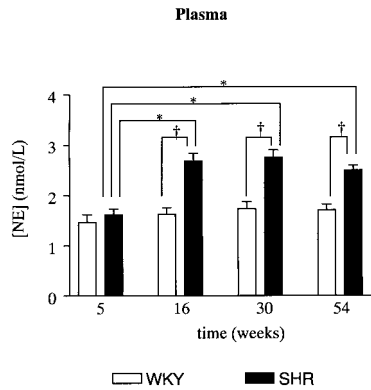
Plasma

Figure 1. Age-related changes of arterial plasma NE concentration during the natural course of hypertension in SHR and WKY at 5 (n=10 per group), 16 (SHR, n=9; WKY, n=8), 30 (n=8 per group), and 54 (SHR, n=6; WKY, n=7) weeks of age. Values are presented as mean±SEM. * $P<0.01$ compared with 5-week-old SHR; † $P<0.01$ compared with age-matched SHR.

$r=0.72$, $P=0.04$, n=8) and subcutaneous adipose tissue of SHR (16-week-old: $r=0.67$, $P=0.05$, n=9; 30-week-old: $r=0.75$, $P=0.03$, n=8).

Interstitial Norepinephrine and Tyrosine Hydroxylase Activity in Skeletal Muscle

Mean NE levels (average of 4 consecutive interstitial samples) in dialysates from skeletal muscle (adjusted for the in vivo recovery of DHBA) were greater in SHR as compared with WKY rats at all ages (ANOVA between age-matched SHR and WKY: F=97.3, $P<0.01$, 1 df; Figure 2A). At 16 weeks of age, a further significant increase in NE levels was observed in SHR compared with younger and with 54-week-old SHR (Figure 2A). No significant difference was observed between WKY of various ages. A strong positive correlation was found between interstitial NE in skeletal muscle and in the subcutaneous adipose tissue at 5 weeks of age in SHR ($r=0.821$, $P<0.0001$, n=29; Figure 3A) but not in WKY ($r=0.189$, $P=0.319$, n=29; Figure 4A). After 5 weeks of age, these relations were not statistically significant in older rats of both strains, despite the high correlation coefficients found in a smaller sample size of SHR.

TH activity was similar in 5-week-old rats from both strains and markedly increased after the development of hypertension in SHR (ANOVA over time: F=7.9, $P<0.01$, 3 df; ANOVA between age-matched SHR and WKY: F=52.9, $P<0.01$, 1 df) (Figure 2B). TH activities between the two

tissues were not correlated at the age of 5 weeks in both strains (SHR: $r=0.34$, $P=0.07$, n=29; WKY: $r=0.21$, $P=0.26$, n=29), whereas a strong correlation was found after the development of hypertension in SHR but during the lifespan of WKY (16-week-old SHR: $r=0.76$, $P=0.009$, n=10; 30-week-old SHR: $r=0.81$, $P=0.014$, n=8; 54-week-old SHR: $r=0.80$, $P=0.027$, n=8).

Although the correlation between TH activity and interstitial NE in skeletal muscle of prehypertensive SHR and age-matched WKY was not significant (5-week-old SHR: $r=0.322$, $P=0.081$, n=29, Figure 3B; 5-week-old WKY: $r=0.296$, $P=0.117$, n=29, Figure 4B), a strong positive correlation was observed in SHR after the development of hypertension (16-week-old SHR: $r=0.83$, $P=0.003$, n=10; 30-week-old SHR: $r=0.79$, $P=0.013$, n=8; 54-week-old SHR: $r=0.77$, $P=0.037$, n=8). No significant correlations were measured in WKY (16-week-old WKY: $r=0.51$, $P=0.13$, n=10; 30-week-old WKY: $r=0.61$, $P=0.11$, n=8; 54-week-old WKY: $r=0.60$, $P=0.12$, n=8).

Interstitial Norepinephrine and Tyrosine Hydroxylase Activity in Adipose Tissue

During their lifespan, marked increases in the levels of NE and TH activity were observed in SHR compared with age-matched WKY ($P<0.01$, t test; Figures 5A and 5B). Analysis of variance revealed an effect of the strain on NE interstitial levels (ANOVA, F=82.6, $P<0.01$, 1 df; Figure 5A) and on TH activity (ANOVA: F=48.0, $P<0.01$, 1 df) (Figure 5B), whereas aging had no effect.

TH activity and interstitial NE in subcutaneous adipose tissue were strongly correlated in SHR (5-week-old SHR: $r=0.445$, $P=0.015$, n=29, Figure 3C; 16-week-old SHR: $r=0.84$, $P=0.0017$, n=10; 30-week-old SHR: $r=0.76$, $P=0.025$, n=8; 54-week-old SHR: $r=0.82$, $P=0.021$, n=8) but not in WKY at all ages (5-week-old WKY: $r=-0.189$, $P=0.319$, n=29, Figure 4C; 16-week-old WKY: $r=0.43$, $P=0.21$, n=10; 30-week-old WKY: $r=-0.15$, $P=0.71$, n=8; 54-week-old WKY: $r=-0.44$, $P=0.25$, n=8).

Discussion

In the present study, we demonstrated, in in vivo studies and in a longitudinal study on the same awake and freely moving rats by using microdialysis techniques, greater interstitial NE concentrations in the skeletal muscle and in the subcutaneous adipose tissue of SHR as compared with WKY throughout their lifespans. The present findings extend peripheral sym-
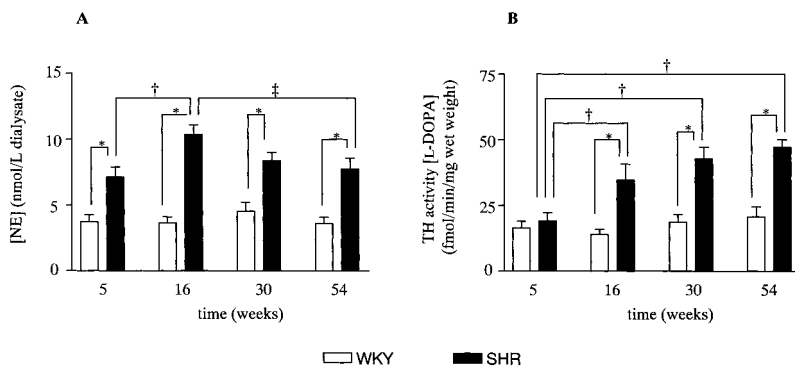

Figure 2. Age-related changes of NE interstitial concentrations (A) and of TH activity (B) in skeletal muscle during the natural course of hypertension in SHR and WKY at 5 (n=29 per group), 16 (n=10 per group), 30 (n=8 per group), and 54 (SHR, n=7; WKY, n=8) weeks of age. NE values were corrected for the in vivo recovery of DHBA. Values are presented as mean±SEM. After the differences between groups and/or over time were analyzed by 2-way ANOVA for repeated measures, a Student's t test was used if a significant effect of the factor (age or strain) was observed. * $P<0.01$ between age-matched SHR and WKY; † $P<0.01$ 5-week-old versus older SHR; ‡ $P<0.05$ between 16-week-old and 54-week-old SHR.
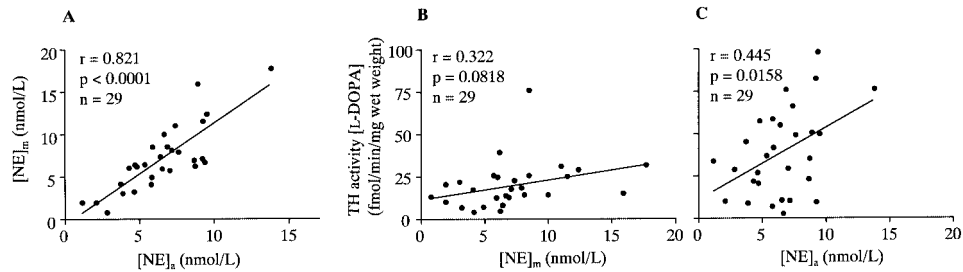
Figure 3. Relation of interstitial NE in skeletal muscle to subcutaneous adipose tissue (A), between TH activity and interstitial NE in skeletal muscle (B), and in subcutaneous adipose tissue (C) of 5-week-old SHR. NE_m and NE_a indicate, respectively, the interstitial concentration in skeletal muscle and in subcutaneous adipose tissue.

pathetic activation to the late phases of the natural history of hypertension, which our group and others already observed in the early developmental phase.[5–9,15,22,23] The activation of the peripheral sympathetic pathway involves baroreflex-dependent and -independent tissues in the SHR model, because in the subcutaneous adipose tissue, where the sympathetic outflow is mainly dependent on thermoregulatory and metabolic influences and weakly related to baroreflex function,[16–19] higher interstitial NE levels were measured. The increased NE interstitial levels in both tissues parallel the activation of TH in the subcutaneous adipose tissue of SHR at all ages, whereas the greater TH activity in the skeletal muscle was found only after the development of arterial hypertension. If we consider that the interstitial NE concentration should be the result of different processes at the synaptic level, including the release of NE from the nerve endings, neuronal uptake, and intra- and extraneuronal metabolic degradation, the present findings are consistent with an increased NE release in SHR tissues, which is maintained by a higher TH synthetic activity. In this fashion, NE vesicles do not become depleted especially when the increased sympathetic activation is sustained and stable throughout the SHR life span. The increased NE release may derive from the increased active exocytosis but also from the passive leaking of the neurotransmitter from the vesicles into the synaptic cleft as demonstrated by the increased deaminated metabolite 3,4-dihydroxyphenylethylene glycol (DHPG), suggesting an increased amount of NE in the vesicular stores of SHR tissues, especially in younger rats.[7,8,14,15] Neuronal uptake of NE, which represents the most important mechanism for removal of the neurotransmitter when released into the synaptic cleft, may participate in the elevation of interstitial NE in both tissues.[7] Its contribution appears to be different in

the various phases of the natural history of hypertension in the SHR model. In fact, we have recently demonstrated in in vivo studies a more active presynaptic reuptake of NE (5 times) in the skeletal muscle and in the subcutaneous adipose tissue of prehypertensive SHR as compared with age-matched WKY.[15] However, in the same animals, a blunted neuronal uptake (2 to 3 times instead of 5 times) was evident only in the skeletal muscle of SHR after the development of arterial hypertension at 16 weeks of age, thus explaining the further increase of interstitial NE observed in this tissue at this age.[15] Previous reports on neuronal uptake, obtained in in vitro or ex vivo preparations, have found a diversified pattern of activity in the different tissues and phases of hypertension development in the SHR model. Neuronal uptake has been found to be normal in the cardiac and vascular tissues[24] and increased in the skeletal muscle and the kidney[5] of young SHR, but it is reduced in the heart[10] and increased in the resistance arteries[25] in hypertensive SHR. Furthermore, it has been reported that aging progressively reduces neuronal uptake of NE,[24,26] suggesting that in older SHR the contribution of the reduced reuptake in the maintenance of greater interstitial NE levels may become more important. In human hypertension, a recent report showed an impairment of total and cardiac NE neuronal reuptake,[27] suggesting a functional reduction in NE presynaptic transporter activity linked to a possible gene mutation.[28] NE metabolism also includes its degradation by the intraneuronal enzyme monoamine oxidase, which has been reported to be unaltered in cardiac and vascular tissues of prehypertensive and hypertensive SHR. In contrast, the extraneuronal enzyme involved in the metabolism of NE, the catechol-O-methyltransferase, has been found to be normal in young but increased in the adult SHR.[24] An interesting hypothesis explaining this increased interstitial
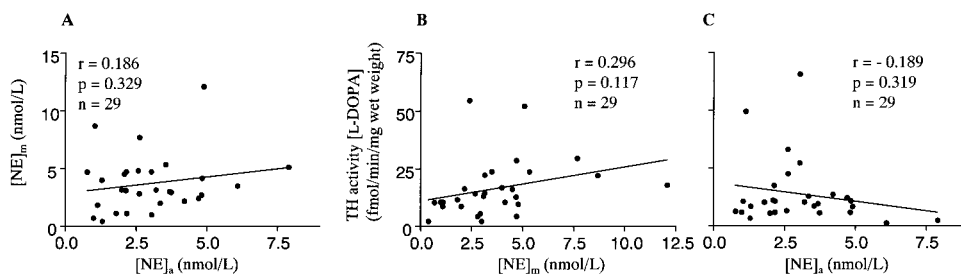

Figure 4. Relation of interstitial NE in skeletal muscle to subcutaneous adipose tissue (A), between TH activity and interstitial NE in skeletal muscle (B), and in subcutaneous adipose tissue (C) of 5-week-old WKY. NE_m and NE_a indicate respectively the interstitial concentration in skeletal muscle and in subcutaneous adipose tissue.
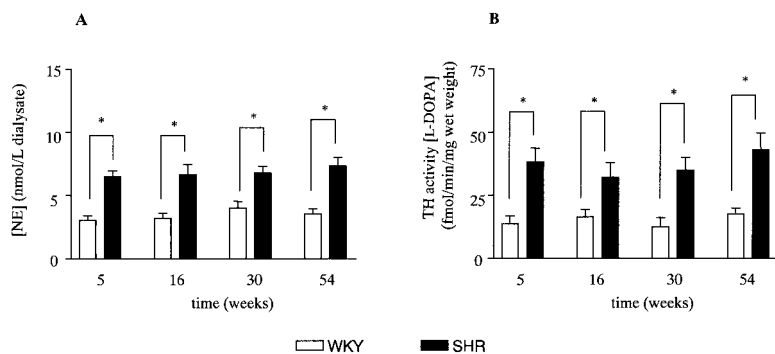
Figure 5. Age-related changes of NE interstitial concentrations (A) and of TH activity (B) in subcutaneous adipose tissue during the natural course of hypertension in SHR and WKY at 5 (n=29 per group), 16 (n=10 per group), 30 (n=8 per group), and 54 (SHR, n=7; WKY, n=8) weeks of age. The NE values were corrected for the in vivo recovery of DHBA. Values are presented as mean±SEM. After the differences between groups and/or over time were analyzed by 2-way ANOVA for repeated measures, a Student's t test was used if a significant effect of the factor (age or strain) was observed. * $P<0.01$ between age-matched SHR and WKY.

NE concentration may include the combined effect of angiotensin II on TH activity and on neuronal NE uptake, which are respectively activated[24] and inhibited even at very low concentrations.[29] Another possible explanation for the higher interstitial NE concentrations in SHR, even after having taken into account the synaptic uptake and metabolic processes, may arise from the observation of the high gradient between interstitial and plasma NE concentrations. The diffusion of the NE from the interstitium toward the bloodstream might be limited by some sort of biochemical or anatomical "barrier." Our data do not permit us to check this possibility. But in a previous report,[6] we showed that the gradient from interstitial space to plasma was much less evident for NE metabolites than for the parent molecule in young prehypertensive SHR. If anything, this observation argues against a mechanism of limited diffusion.

An apparent discrepancy in the skeletal muscle of young still normotensive SHR has been observed, where high NE interstitial level is associated with a normal TH activity. No significant correlation between the two variables was measured in the prehypertensive phase. In this context, a short-term inhibition of TH activity by the intraneuronal NE level[20] could be responsible for normal TH in the skeletal muscle of 5-week-old SHR. But when the noradrenergic neuronal firing remains high for prolonged periods, the rate of synthesis of TH increases,[20] thus explaining the high positive correlation between TH activity and interstitial NE after the development of hypertension in this tissue.

Plasma NE levels were higher in SHR only after the development of hypertension but not in the prehypertensive phase. No correlations were found between plasma and interstitial NE levels in WKY. Only a slight positive correlation was found in SHR in the intermediate ages (16 and 30 weeks of age). Therefore, conclusions about sympathetic activity drawn solely on the basis of NE plasma levels seem to be limited and not always representative of the higher levels found in interstitium.

The continuous sympathetic activation in skeletal muscle may play both short-term and long-term effects on cardiovascular and metabolic patterns. The increased sympathetic discharge to the striated muscle in young SHR was not sufficient to cause a rise in vascular tone and consequently in blood pressure because of the efficiency of other counterbalancing mechanisms. But those counterbalancing and vasodilatory factors become rapidly overwhelmed by the increased sympathetic activation leading to the rise in blood pressure.

The skeletal muscle sympathetic stimulation is associated not only with vasoconstriction but also with metabolic changes in the muscles and may contribute to the altered oxygen consumption, glucose uptake, and insulin resistance found in SHR[30] and in many hypertensive patients.[31]

The importance of the adrenergic innervation in regulating the metabolic activity of adipose tissue is supported by observations showing that the nerve fibers are present around vessels, but they are also abundantly distributed directly on parenchymal fat cells.[32] A strong positive correlation between subcutaneous adipose tissue TH activity and interstitial NE was always detectable during the lifespan. The increased sympathetic activation in the subcutaneous adipose tissue, which is less dependent on baroreflex control,[16–18] is mainly involved in lipolysis and thermogenesis regulation.[19,33] In our study we observed that the body weight growth curve in the SHR as compared with age-matched WKY revealed a stable averaged weight reduction of 20% to 25% after the development of hypertension in the former group. The increased sympathetic activity in subcutaneous adipose tissue and perhaps in other white adipose fat pads could be the underlining metabolic irregularity explaining the different body weight growth in the SHR model. Our preliminary data in the older SHR (30 and 54 weeks of age) as compared with age-matched WKY, showed a marked reduction of the retroperitoneal (−61% at 30 weeks; −57% at 54 weeks) and epididymal fat (−43% at 30 weeks; −63% at 54 weeks) pad weight associated with a lesser reduction in inguinal adipose pad weight (−16% at 30 weeks; −29% at 54 weeks).

In conclusion, sympathetic neuronal activation is increased in skeletal muscle and subcutaneous adipose tissue during all the natural course of arterial hypertension in awake and freely-moving SHR. The increased sympathetic peripheral activity seems to be not simply the result of a decreased baroreflex inhibition but reflects a more diffused peripheral activation, presumably the result of a heightened central nervous system sympathetic drive in this hypertensive model.

Acknowledgments

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