Lack of flow mediated dilation and enhanced angiotensin II-induced constriction in skeletal muscle arterioles of lupus-prone autoimmune mice

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Systemic lupus erythematosus (SLE) is associated with disturbances in the microcirculation of various tissues, yet the nature of arteriolar dysfunction has not been characterized. Thus, changes in diameter of isolated, pressurized skeletal muscle arterioles of mice with systemic autoimmune disease (lupus prone, MRL/lpr four-month old female) and control (MRL) mice were investigated by videomicroscopy. Arteriolar responses to changes in intraluminal pressure, flow, and to vasoactive agents with known mechanisms of action were compared. The active and passive (in Ca2+/H11001 free solution) diameter of MRL/lpr arterioles were not significantly different compared to MRL and morphometric changes were not apparent. Compared to MRL mice the endothelium-dependent dilations to increase in flow, acetylcholine and bradykinin were markedly reduced in arterioles of MRL/lpr mice. Endothelium-independent dilations to sodium-nitroprusside and adenosine were similar in MRL and MRL/lpr arterioles. Furthermore, angiotensin II elicited greater constrictions in MRL/lpr arterioles, whereas serotonin-induced constrictions were similar in both groups. Thus, in arterioles of MRL/lpr mice endothelium-dependent dilator mechanisms are impaired and constriction to angiotensin II is enhanced, suggesting specific alterations in the vasomotor function of microvessels that are likely contribute to the disturbance of skeletal muscle blood flow observed in systemic lupus erythematosus. Lupus (2006) 15, 1–9.

Key words: angiotensin II; endothelium; flow-mediated dilation; isolated arteriole; systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is frequently associated with cardiovascular diseases which may contribute substantially to the morbidity and mortality of patients. In a multicentre study involving seven countries, and 10 000 SLE patients, 17% of the patients developed hypertension during the five years follow-up period. In a cohort, prospective study of the SLE patients hypertension and peripheral vasculitits were found as the most common manifestations. It is of note, that in spite of the adequate anti-inflammatory therapy or a commonly observed remission of systemic autoinflammation, vascular complications are characteristically sustained and become prominent in SLE.

Previously, premature atherosclerosis and atherothrombosis has been suggested to play a key role in the development of SLE-associated peripheral occlusive vascular disease. Recent reports have demonstrated that in SLE patients, perfusion of cardiac and skeletal muscle is significantly reduced without the signs and symptoms of macrovascular obstruction, suggesting functional impairment of microvessels. It is known that arterioles are primary responsible for regulation of circulatory resistance, however the nature of arteriolar dysfunction in SLE remains obscure. The clinical observations suggest that SLE induces multiple alterations in vascular function however; these alterations have not yet been characterized in microvessels.

We hypothesized that mechanisms intrinsic to the arteriolar wall are affected by SLE. In order to assess...
these alterations we have utilized several vasoactive substances with known mechanisms of action to characterize arteriolar function. These vasoactive mediators produced and released from surrounding tissues, such as acetylcholine, bradykinin, adenosine, angiotensin II, can all affect arteriolar diameter via activating their specific receptors. Also, it has been demonstrated that mechanisms intrinsic to vascular wall that are sensitive to changes in intraluminal pressure and flow contribute importantly to the regulation of arteriolar diameter. However, whether or not these arteriolar responses and responsible mechanisms are altered in SLE is not well known. Thus, in the present study we aimed to characterize the vasomotor function of isolated skeletal muscle arterioles of lupus prone (MRL/lpr) mice, which are homozygous for the Fas\(^{pr}\) autosomal recessive mutation and in which systemic autoimmune disease develops similar to that of human SLE.

**Materials and methods**

**Experimental animals**

A mouse model of the human SLE was used throughout the experiments (MRL/MpJ-Faslpr, MRL lpr/lpr \(^{-/-}\); \(n = 11\), and wild type MRL/MpJ (MRL +/-/+MRL, \(n = 11\), The Jackson Laboratory /JAX/ Bar Harbor, ME, USA). Four-month old female mice were housed under specified pathogen free conditions in an individually ventilated cage system (Charles River). Mice had free access to standard animal chow and tap water. All experimental procedures were in accordance with guidelines set by the Institutional Animal Care and Use Committee of Institutions involved and in compliance with the NIH ‘Guide for the Care and Use of Laboratory Animals’ (DHEW Publication No. [NIH] 85-23, Revised 1985).

**Measuring the level of anti-dsDNA**

The levels of anti-dsDNA antibodies were evaluated in plasma samples using anti-dsDNA ELISA, as described elsewhere. Briefly, 96-well ELISA plates (Greiner, Budapest) were coated with 25 μg/mL double-stranded calf thymus DNA (Sigma, Sigma-Aldrich) in 0.1 M ammonium acetate and incubated overnight at 4°C. After washing with PBS-1% Tween 20 (PBS-T) following distilled water, sera diluted 1:200 in serum diluent (PBS-T) were added in duplicate and incubated for one hour at 37°C. After washing, horseradish peroxidase (HRP)-conjugated goat antimouse IgG (Sigma) was used as a conjugate antibody (1:1000 in PBS-T, one hour at 37°C), followed by o-phenilenediamine dihydrochloride (Sigma) as substrate. The OD was measured photometrically after 20 minutes at 450 nm (ref. = 690 nm).

**Isolation of arterioles**

Experiments were conducted on isolated arterioles (inside diameter: \(-100 \mu m\)) of mouse gracilis muscle, as described previously. Briefly, gracilis muscle of diethyl ether anesthetized mice was excised and isolated from surrounding tissues. Blood was then collected from the femoral artery, centrifuged and serum urea was measured with Reflotron IV automated analyzer (Boehringer Mannheim). From the gracilis muscle, a segment, \(-1 \text{ mm}\) in length of an arteriole running intramuscularly was isolated with microsurgical instruments and an operating microscope and transferred into an organ chamber containing two glass micropipettes filled with physiological salt solution (PSS). Arterioles were then cannulated on both ends in an organ chamber and were continuously superfused with PSS (in mmol/L: 110 NaCl, 5.0 KCl, 2.5 CaCl\(_2\), 1.0 MgSO\(_4\), 1.0 KH\(_2\)PO\(_4\), 10.0 dextrose and 24.0 NaHCO\(_3\); equilibrated with 10% O\(_2\), 5% CO\(_2\), 85% N\(_2\), at pH 7.4). Inflow and outflow pressures were measured by an electromanometer. The temperature was set at 37°C by a temperature controller and the vessel was allowed to develop spontaneous tone in response to intraluminal pressure (80 mmHg) under no flow conditions (equilibration period one hour). The inside diameter of arterioles was measured by videomicroscopy and recorded on a chart recorder (Cole-Parmer, Vernon Hills, IL, USA).

**Morphometric analysis of vessel walls**

Gracilis muscle including large segment of the gracilis arteriole was isolated from MRL and MRL/lpr mice (\(n = 4-4\)) and fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin-eosin (HE), or orcein to visualize the lamina elastica interna and externa of arteriole. This staining enabled us to determine the thickness of the intima and the media. Pictures were taken with a camera (Olympus U-TVO.5XC-2, Japan) attached to a microscope (Olympus BX50F-3, Japan) at a high power magnification (400×). The ratio of the media to the total vessel wall thickness (intima + media) was determined by Scion image analysis software (Scion Corporation/NIH/Bethesda ML, USA). HE stained sections were used to estimate inflammatory infiltration or necrosis of the vessel wall.

**Pressure-induced arteriolar responses**

After the equilibration period changes in diameter of arterioles in response to stepwise increases in
intraluminal pressure from 20 to 120 mmHg were measured under zero-flow condition. Each pressure step was maintained for five to ten minutes to allow the vessel to reach a steady-state diameter. At the end of the experiment, the superfusion solution was changed to a Ca\(^{2+}\)-free PSS containing EGTA (ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 10\(^{-3}\) mol/L) and 10\(^{-4}\) mol/L SNP, then pressure steps were repeated to obtain the maximum passive diameter at each pressure.

**Flow-induced arteriolar responses**

Changes in diameter of arterioles in response to stepwise increases in intraluminal flow (0 to 38 \(\mu\)L/minute) were also observed at 80 mmHg constant intraluminal pressure. In this protocol, each flow rate was maintained for five minutes to allow the vessel to reach a steady state diameter and then the maximal diameter changes were measured.

**Agonist-induced arteriolar responses**

At 80 mmHg constant intraluminal pressure and zero flow condition changes in diameter of arterioles were obtained to cumulative doses of endothelium-dependent dilators acetylcholine (ACh, 10\(^{-9}\)–10\(^{-6}\) mol/L) and bradykinin (BK) (10\(^{-10}\)–10\(^{-7}\) mol/L). Then, responses to endothelium-independent dilators sodium-nitroprusside (SNP) (10\(^{-9}\)–10\(^{-6}\) mol/L) and adenosine (ADO) (10\(^{-6}\)–10\(^{-4}\) mol/L), were obtained. Following that, arteriolar responses to angiotensin II (AT II) (10\(^{-10}\)–10\(^{-8}\) mol/L) and serotonin (5-HT) (10\(^{-8}\)–3 \times 10\(^{-7}\) mol/L) were also assessed.

**Data analysis**

Pressure-induced responses are expressed as changes in arteriolar diameter (in absolute values). Myogenic tone of arterioles was calculated from the active (AD, in Ca\(^{2+}\) containing PSS) and passive (PD, in Ca\(^{2+}\) free PSS) diameters at 80 mmHg pressure as follows: [(PD – AD)/PD]×100, expressed as a percent. Flow and agonist-induced dilations were expressed as a percentage of the maximal dilation in Ca\(^{2+}\) free PSS. Agonist-induced constrictions were expressed as percentage changes to the basal diameter. Data are expressed as means ± SEM. Statistical analyses were performed by two-way analysis of variance for repeated measures (ANOVA) followed by the Tukey post hoc test or Student’s t-test by using GraphPad Prism Software (version 4.00 for Windows, San Diego, California, USA). The P-value of less than 0.05 was considered to be statistically significant.

**Results**

**Animals**

There were no significant differences between the body weight of MRL and MRL/lpr mice (Table 1). In four-month old MRL/lpr mice the average serum urea was significantly higher compared to MRL (17.1 ± 11.1 versus 6.4 ± 0.8 mmol/L, \(P < 0.05\)) indicating that in MRL/lpr mice the autoimmune disease is already apparent, but kidney failure is not yet advanced (Table 1). Anti-dsDNA antibody levels constantly rose during the observation period, and represented moderate autoimmune activity in four-month old mice [median OD\(_{450}\) was 0.07 (at two months); 0.19 (at three months); and 0.27 (at four months) \(n = 10\)].

**Morphometric analysis of the gracilis muscle arteriole**

There were no differences in the morphology of vessel wall of MRL/lpr and MRL mice and no significant lymphocyte infiltration of MRL/lpr vessels could be observed in HE stained sections (Figure 1, B and D Panels). The thickness of the media relative to total vessel wall thickness in the orcein stained sections was 70.5 ± 2.3% in MRL/lpr and 71.3 ± 5.7% in MRL vessels \([P > 0.05\) (not significant), Figure 1, A and C Panels].

**Pressure-induced arteriolar response**

Arterioles isolated from gracilis muscles of MRL and MRL/lpr mice developed an active myogenic tone in response to 80 mmHg intraluminal pressure without the use of any vasoactive agent. The basal diameters (at 80 mmHg) of arterioles were not significantly different between MRL and MRL/lpr mice (158 ± 14 m, respectively). Also, there was no significant difference between the passive diameters (in Ca\(^{2+}\) free PSS) of arterioles isolated from MRL and MRL/lpr animals (158 ± 12 versus 167 ± 16 \(\mu\)m, respectively). The pressure-diameter curves (increasing

| Table 1 Basic characteristics of animals and diameters of isolated skeletal muscle arterioles of MRL and MRL/lpr mice |
|---------------------------------------------------------------|---------------|
| **Body weight (g)** | 41 ± 4 | 35 ± 9 |
| **Serum urea (mmol/L)** | 6.4 ± 0.8 | 17.1 ± 11.1* |
| **Active arteriolar diameter (m)** | 101 ± 12 | 113 ± 14 |
| **Passive arteriolar diameter (\(\mu\)m)** | 158 ± 12 | 167 ± 17 |

* \(P < 0.05\), significantly different, \(n = 6 – 16\).
intraluminal pressure from 20 to 120) of arterioles were not significantly different in MRL and MRL/lpr mice (Figure 2a). Under similar conditions, but in Ca\(^{2+}\)-free solution passive dilations of arterioles were not significantly different between the two groups (Figure 2a). In order to indicate the magnitude of pressure-induced constriction, the myogenic tone of arterioles was calculated at each pressure step. Figure 2b shows, that arterioles from MRL and MRL/lpr mice exhibited similar myogenic tone between pressure values of 20 and 120 mmHg.

**Flow-induced arteriolar dilation**

Increases in intraluminal flow (from 0 to 38 μL/minute) resulted in substantial dilations of arterioles isolated from MRL mice, but these responses were significantly reduced in arterioles of MRL/lpr compared to MRL mice (Figure 2c).

**Agonist-induced arteriolar dilations**

In separate group of experiments the function of arterioles were assessed by responses to vasoactive agents with known mechanisms of actions. Dilations to acetylcholine (Ach) (10\(^{-9}\)–10\(^{-6}\) mol/L) were significantly decreased in arterioles of MRL/lpr compared to MRL mice (Figure 3a). BK-induced (10\(^{-10}\)–10\(^{-8}\) mol/L) dilations were also significantly reduced in arterioles of MRL/lpr compared to MRL mice (Figure 3b). Dilations to the nitric oxide (NO) donor, SNP (10\(^{-9}\)–10\(^{-6}\) mol/L) and ADO (10\(^{-6}\)–10\(^{-4}\) mol/L) were not different in arterioles MRL and MRL/lpr mice (Figure 4a and 4b).

**Agonist-induced arteriolar constrictions**

In MRL/lpr arterioles angiotensin II (10\(^{-10}\)–10\(^{-8}\) mol/L) elicited significantly greater constrictions compared to that of MRL arterioles (Figure 5a). In contrast, constrictions to serotonin (5-HT, 10\(^{-8}\)–3 × 10\(^{-7}\) mol/L) were not significantly different in MRL and MRL/lpr arterioles (Figure 5b).

**Discussion**

The salient findings of the present study are that in the mouse model of SLE (MRL/lpr mice) flow, acetylcholine or bradykinin induced endothelium-dependent arteriolar dilations were reduced, whereas angiotensin II induced constrictions were enhanced. Arteriolar responses to the nitric oxide (NO)-donor sodium nitroprusside, adenosine and serotonin were not significantly different in the two groups of animals. These findings suggest that – before apparent vascular morphological changes – multiple abnormalities develop.
in the vasomotor responses of arterioles, primarily in those that are mediated by the endothelium. These alterations may contribute to the impaired local regulation of tissue blood flow in SLE.

Vascular complications are common manifestation during the development of SLE, which likely contribute significantly to the morbidity and mortality of patients. Several previous studies have demonstrated that in chronic SLE the vascular integrity is disrupted by immune complex deposition and subsequent complement activation intrinsic to the vascular wall. A recent study proposed that muscle perfusion in the...
lower extremities of SLE patients without symptoms of obliterator macrovascular disease is significantly decreased, although the nature of functional changes remains obscure. On the basis of previous observations we hypothesized that SLE impairs the regulation of arteriolar resistance by affecting mechanisms intrinsic to the vascular wall.

To reveal the early functional changes gracilis skeletal muscle arterioles of four-month old, female MRL/lpr mice were utilized. In the histological sections of gracilis muscle of MRL/lpr mice we did not find significant morphological changes and did not detect cellular infiltration as indicators of vascular inflammation (Figure 1). Moreover, in isolated gracilis muscle arterioles in response to increases in intraluminal pressure a spontaneous myogenic tone developed, which was not significantly different in MRL and MRL/lpr mice (Figure 2). Also, the pressure-passive diameter (measured in Ca^{2+} free solution) curves of arterioles of MRL/lpr and MRL mice were not different (Figure 2). We interpreted these findings to mean that altered mechanical characteristics of the arterial wall is unlikely to affect dilator and constrictor properties of arterioles and pressure sensitive, smooth muscle-dependent mechanisms are still preserved at that age in arterioles of MRL/lpr mice. To further characterize whether or not changes in vasoregulatory mechanisms of arteriolar smooth muscle contribute to the reduced arteriolar dilations in SLE mice, responses to increasing concentrations of SNP and adenosine were obtained. Arterioles from MRL/lpr and MRL

Figure 3 Dilations of skeletal muscle arterioles isolated from MRL (n = 9) and MRL/lpr (n = 9) mice in response to cumulative doses of acetylcholine (Panel A) and bradykinin (Panel B). Data are mean ± SEM. Asterisks indicate significant differences (P < 0.05).

Figure 4 Dilations of skeletal muscle arterioles isolated from MRL (n = 9) and MRL/lpr (n = 9) mice in response to cumulative doses of sodium nitroprusside (SNP, Panel A) and adenosine (Panel B). Data are mean ± SEM.
mice exhibited similar dilations in response to the NO donor, SNP and ADO, suggesting that mechanisms intrinsic to smooth muscle cells are not substantially altered in this stage of the autoimmune disease.

Endothelial function of arterioles was characterized by utilizing physiological stimuli to elic the impairment of signaling pathways located in the arteriolar endothelium. These findings are in accordance with recent results showing that occlusion-induced, endothelium-dependent brachial artery relaxation is reduced in patients with SLE.\textsuperscript{20,21} Several mechanisms could be responsible for the reduced endothelium-dependent dilations, since endothelium-dependent dilations are known to be mediated by the release of NO, dilator prostaglandins and/or endothelium derived hyperpolarizing factor (EDHF). Because our previous findings showed that ACh, BK and flow-induced dilations are mediated by NO and prostaglandins it is safe to assume that these two pathways are impaired in SLE. However, it is of note, that during the development of autoimmune disease alterations in the adhesion molecule expression in endothelial cells, such as platelet endothelial cell adhesion molecule 1 (PECAM-1) may also contribute to the reduction of flow/shear-induced arteriolar dilation in MRL/lpr mice.\textsuperscript{22,23}

In the present study we have also found that constrictions in response to angiotensin II were significantly enhanced in arterioles of SLE mice compared to those of controls. In contrast, there were no differences in the constrictor responses to serotonin in MRL/lpr and control MRL groups (Figure 5). These findings, together with the reduced dilations to BK suggest a specific alteration of the vasomotor activity of arterioles of MRL/lpr mice that might be related to the vascular renin angiotensin system (RAS). One can speculate that increased constrictions to angiotensin II may be due to upregulation of AT1 receptors, whereas the reduced dilations to BK may due to the enhanced activity of angiotensin converting enzyme (ACE) known to be responsible for breakdown of BK. Indeed, the vascular RAS plays a central role in the maintenance of vascular homeostasis, among others, determining vascular resistance\textsuperscript{24} and controlling vascular growth,\textsuperscript{25} although the specific role of RAS in the disease development in SLE are not known. In this context, it has been recently demonstrated that angiotensin II inhibition has protective effects on the development of immune-mediated renal damage in MRL/lpr mice, an effect that was involved in both hemodynamic and immune-modulatory effects.\textsuperscript{26} Previously it has been demonstrated that angiotensin II could elicit an enhanced formation of superoxide anion.\textsuperscript{24,27} Thus, one can speculate that in MRL/lpr mice upregulation of RAS may also contribute to the impaired arteriolar vasoregulatory mechanisms. Thus, superoxide produced due to upregulation of RAS, by interacting with NO released in response to increases in flow, could reduce the bioavailability of NO, leading to reduced flow and/or agonist-induced NO-dependent

![Figure 5](image-url) Constriction of skeletal muscle arterioles isolated from MRL (n = 9) and MRL/lpr (n = 9) mice in response to cumulative doses of angiotensin II (Panel A) and to serotonin (5-HT, Panel B). Data are mean ± SEM. Asterisks indicate significant differences (P < 0.05).
arteriolar dilations. In line with our present findings, recent clinical observations suggest a role for oxidative stress in the initiation of vascular dysfunction in SLE,28,29 however further studies are needed to substantiate this hypothesis.

It is also known that endothelial cells represent a key target for systemic auto-inflammation. An early involvement of certain endothelial mechanisms is frequently observed in different pathological conditions. In this context our recent studies have shown that chronic, low-grade inflammation of the vascular wall leads to impaired endothelium-dependent vasomotor mechanisms in aging and hyperhomocysteinemia.30,31 In aging and hyperhomocysteinemia, increases in the expression of pro-inflammatory cytokines, such as TNFa, IL-1, IL-6 and IL-17 were found, which may induce specific alterations intrinsic to vascular wall thereby interfering with endothelium and smooth muscle dependent signalling pathways.30,32 Interestingly, recent clinical studies have demonstrated that patient with SLE have significant elevations of plasma homocysteine levels,33 suggesting that hyperhomocysteinemia contributes to the impaired regulation of arteriolar vasomotor responses in systemic auto-inflammation of MRL/lpr mice. On the basis of the present functional findings further studies can be initiated to elucidate the nature of cellular mechanisms activated by systemic auto-inflammation in SLE, which will help to develop novel therapeutic approaches.

In summary, we have characterized the vasomotor dysfunction of skeletal muscle arterioles of MRL/lpr mice and found severe impairment of endothelium-dependent dilator responses, such as ACh-, BK- and flow-induced dilations and enhancement of angiotensin II-induced contractions. Such alterations in the endothelium-dependent regulation of arteriolar functions likely represent an early step in the pathogenesis of autoimmune vascular disease and inflammation observed in systemic lupus erythematosus.

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References


Dzau VJ. Significance of the vascular renin-angiotensin pathway. Hypertension 1986; 8: 553–559.
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