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Increased Asymmetric Dimethylarginine and Endothelin 1 Levels in Secondary Raynaud's Phenomenon

Implications for Vascular Dysfunction and Progression of Disease

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Objective. **To compare microvascular and macrovascular functions in a cohort of patients with primary and secondary Raynaud's phenomenon (RP) who were matched for demographic, risk factor, and severity profiles.**

Methods. **Forty patients with primary or secondary RP matched for vascular risk factors and severity scores underwent testing of endothelial function and cold pressor responsiveness of the brachial artery. Microvascular perfusion of the digital vasculature was assessed using laser Doppler fluxmetry in response to reactive hyperemia. Plasma was assayed for endothelin 1 (ET-1), asymmetric dimethylarginine (ADMA), intercellular adhesion molecule 1, vascular cell adhesion molecule 1 (VCAM-1), and monocyte chemoattractant protein 1 (MCP-1).**

Results. **Patients with RP had abnormal vasoconstrictor responses to cold pressor tests (CPT) that were similar in primary and secondary RP. There were no differences in median flow-mediated and nitroglycerin-mediated dilation or CPT of the brachial artery in the 2 populations. Patients with secondary RP were characterized by abnormalities in microvascular responses to reactive hyperemia, with a reduction in area under the curve adjusted for baseline perfusion, but not in time to peak response or peak perfusion ratio. Plasma ET-1, ADMA, VCAM-1, and MCP-1 levels were significantly elevated in secondary RP compared with primary RP. There was a significant negative correlation between ET-1 and ADMA values and measures of microvascular perfusion but not macrovascular endothelial function.**

Conclusion. **Secondary RP is characterized by elevations in plasma ET-1 and ADMA levels that may contribute to alterations in cutaneous microvascular function.**

Raynaud's phenomenon (RP) is a poorly understood disorder characterized by recurrent episodes of protracted vasospasm on cold exposure (1). The disease may occur as a primary manifestation (primary RP) or as part of a constellation of findings in patients with diverse rheumatologic conditions (secondary RP) (2). Although abnormalities in endothelial function in conduit and resistance vessels and elevations in circulating vasoconstrictor mediators such as endothelin 1 (ET-1) have been described (3–5), these findings have not been consistent (6,7). In addition, the precise etiologic role of these abnormalities in the pathogenesis of primary RP versus secondary forms of this disorder is not clear. Circulating ET-1 levels do not seem to correlate with the intensity of vasospasm and are not consistently elevated in RP (8). These differences may stem in part from studies comparing patient populations with different levels of disease severity, presence of underlying connective tissue disease, and concomitant vascular risk factors.

The loss of nitric oxide (NO), the hallmark of endothelial dysfunction, is an attractive possibility as an

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overall mechanism for the pathogenesis of RP, since perturbations in the NO pathway may have pleiotropic effects on multiple effector pathways that may be relevant in RP, including ET-1 (9). In this regard, although abnormalities of both conduit and microvascular endothelial function have been demonstrated in RP, the contribution of these 2 levels of circulation in explaining the differential features in primary and secondary RP requires further definition (3,4,10,11). Recently, a novel circulating endogenous inhibitor of endothelial nitric oxide synthase (eNOS), called asymmetric dimethylarginine (ADMA), has been described (12). ADMA is synthesized in human endothelial cells and is believed to alter eNOS function in many ways, including inhibition of function, increases in endothelial oxidative stress, and alterations in substrate binding (13).

We hypothesized that individuals with RP secondary to connective tissue diseases, when compared with subjects with primary RP, have heightened sensitivity of upper extremity conduit and resistance vessels, and that these changes are correlated with increases in ET-1 and ADMA. The present study was undertaken to test this hypothesis.

PATIENTS AND METHODS

Study subjects. The Institutional Review Board at the University of Michigan approved the study protocol. Subjects were screened and recruited in a prospective manner through advertisements in the University of Michigan rheumatology outpatient clinic and in the Ann Arbor/Detroit area. The study was performed during winter months to minimize seasonal effects on the severity and frequency of episodes. Diagnosis of RP involved a structured interview assisted by color charts, as previously described (14), to improve the reliability of the diagnosis. The following information was collected during the screening visit: demographic variables, including sex, age, occupational history, ethnicity, exposure to vibrating tools, chemicals, and dusts, use of medications, and association with other vasospastic syndromes (migraine, variant angina). In addition, information on history of cold injury, polyvinyl chloride exposure, thoracic outlet syndrome, or crutch pressure was obtained. Characteristics of RP, including the frequency and duration of attacks, number of fingers involved, associated subjective symptoms (numbness, tingling, pain), triggering factors, and age at onset, were collected. A clinical evaluation was performed that included a medical history with an emphasis on detecting RP-associated diseases. Capillary microscopy was performed to detect nailfold capillary abnormalities related to underlying rheumatologic conditions. Finally, screening laboratory tests were performed, including determinations of the levels of serum creatinine, blood urea nitrogen, and serum lipids (total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein [LDL]).

Patients with a diagnosis of RP, either primary or secondary, were included. Diagnosis of primary RP was performed using the following criteria: definite history of episodic attacks of digital pallor or cyanosis, no evidence of peripheral vascular disease, no evidence of digital ulcerations, pitting, or gangrene, normal nailfold capillaries as determined using the method described by Maricq and Weinrich (14), and negative results on antinuclear antibody and anti–topoisomerase I tests.

Patients with secondary RP met criteria for other rheumatologic conditions, including progressive systemic sclerosis (SSc) (15), systemic lupus erythematosus (SLE) (16), primary Sjögren's syndrome (primary SS) (17), polymyositis/dermatomyositis (18), rheumatoid arthritis (RA) (19), mixed connective tissue disease (MCTD) (20), and undifferentiated connective tissue disease (UCTD) (21). No patients were in clinical remission. UCTD was diagnosed in patients presenting with autoimmunity features but who did not fulfill the classification criteria for a definite connective tissue disease (21). Patients were excluded from participating in the study if they had congestive heart failure or documented ejection fraction $\leq$40%, uncontrolled hypertension (blood pressure $>$160/100), uncontrolled hyperlipidemia (cholesterol $>$200 mg/dl), diabetes (fasting glucose $>$100 mg/dl or glycosylated hemoglobin $>$8%), renal failure (creatinine $>$2.0 mg/dl), or hepatic failure. Patients were also excluded if they were current smokers, if they were taking $>$2 medications for hypertension, if they were receiving lipid-lowering therapy, or had recently (within the previous 4 months) begun treatment with an angiotensin-converting enzyme inhibitor or angiotensin-receptor blocker. Postmenopausal women, pregnant women, or women who were breastfeeding were excluded, as were patients with active cardiac, central nervous system, or pulmonary disease.

Estimation of attack severity and frequency of episodes. We attempted to recruit subjects with attacks of roughly similar severity (22). After vascular responses had been tested and blood had been obtained as described below, subjects were followed up in a prospective manner over a 6-week period during which they were given biweekly symptom diaries, color charts, and a small pocket notebook with daily attack cards that had been used in other RP studies, which summarized the number, severity, and duration of attacks, and the coping strategies undertaken by the subject (23). Participants were instructed to keep attack cards at all times, together with color charts to identify exact color changes of the fingers experienced daily, throughout the 6 weeks of followup. The attack incidence was calculated as the number of attacks divided by the number of days of followup. Severity of RP was assessed by a scoring system. Lack of an attack was scored as 0. If an attack occurred, the severity was assessed using a previously described and validated ordinal system (23) that ranked the severity on a scale of 1–9. The intrasubject mean for this parameter over the duration of the study incorporating multiple attacks was calculated. To integrate the frequency and severity of attacks into a single summary measure, a composite score was calculated for each subject, based on an area under the curve (AUC) approach. For each subject, the elapsed time (in hours) between attacks was multiplied by the severity score corresponding to the attack at the end of the given time interval. The intrasubject mean for this composite score was then determined.

Vascular studies. All studies were conducted between 8:00 AM and 12:00 noon in a darkened, temperature-controlled room, with the temperature set at 68°F. Studies were performed after an overnight fast.

Brachial artery vasoreactivity studies. Flow-mediated dilation (FMD%) of the brachial artery and nitroglycerin-mediated dilation (NMD%) were determined from 2-dimensional ultrasound images according to established and validated methodology (24,25). Images were obtained with a 10-mHz linear array transducer and an Image Point ultrasound system (Hewlett Packard, Andover, MA). Triggered events (occurring after the peak of the R wave on the electrocardiogram) were recorded and acquired through a frame grabber attached to a computer. Each triggered event consisted of 6 sequential frames. The media–adventitia interface in a linear portion of the vessel was chosen for analysis.

Laser Doppler studies. Laser Doppler studies were performed with the Lisca laser Doppler imager (PIM II system; Lisca, Linköping, Sweden) on the nondominant hand 10 minutes after FMD% studies. Briefly, a cuff was applied to the subject's nondominant forearm. The hand and middle finger were taped flat in the prone position to an examination table. After positioning the laser beam 15 cm above the center of the proximal phalanx of the middle digit, baseline measurements were obtained over 2 minutes (Doppler wavelength 670 nm). Baseline perfusion was expressed in perfusion units as the mean of 6 measurements. Each measurement was performed over 960 msec in an area measuring 16 mm^2. The cuff was then inflated to a pressure that was 50 mm Hg above the subject's resting systolic blood pressure and held for 4 minutes, at the end of which the pressure was released and serial laser Doppler scans were obtained immediately upon release for a total of 6 minutes following release. Reactive hyperemia measures were expressed as AUC of perfusion measurements (adjusted for baseline) obtained over a 4-minute period, time to peak response (the time to attain maximal cutaneous perfusion in seconds), peak perfusion ratio, which is the peak perfusion value obtained with reactive hyperemia divided by baseline perfusion units (arbitrary units), and percent reactive hyperemia, which was calculated as ([peak perfusion − baseline perfusion]/[baseline perfusion]) × 100. Reproducibility of our brachial artery studies and laser Doppler studies has been reported previously (26–28).

Cold pressor test (CPT). The CPT was performed by immersing the nondominant hand in a jacket surrounded by crushed ice for 45 seconds (29). Imaging of the brachial artery was obtained 30 seconds before cold exposure and at 30-second intervals following cold exposure for 4 minutes. The responses were expressed as the percentage change in baseline diameter and graphed at 30-second intervals for 3.5 minutes (29).

Laboratory measurements and serum and plasma markers. Blood samples were collected before vascular studies were performed. Serum and plasma were obtained by centrifugation of whole blood at 3,000*g* for 10 minutes, and aliquots were stored at −20°C.

ET-1 determination. The assay for ET-1 was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, a quantitative sandwich enzyme immunoassay technique was used. A monoclonal antibody to ET-1 was precoated onto a microplate. Standards and samples were pipetted into the wells and any ET-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for ET-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution was added to the wells. A microplate luminometer was used to measure the intensity of the light emitted. For ET-1, duplicate readings were averaged for each standard, control, and sample and subtracted from the average zero standard relative light units.

Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and monocyte chemoattractant protein 1 (MCP-1) determinations. Human ICAM-1 and VCAM-1 were measured from plasma using enzyme-linked immunosorbent assay kits (R&D Systems), according to the manufacturer's directions. MCP-1 levels were detected using a kit obtained from Endogen (Woburn, MA). The absorbance was read using a microplate reader (Molecular Dynamics, Sunnyvale, CA), and a standard curve was generated using a computer program (SoftPro; Molecular Dynamics). The concentration of adhesion molecules and chemokines in each sample was calculated from the standard curve equation.

Quantification of dimethylarginine derivatives. ADMA and symmetric dimethylarginine (SDMA) in human plasma samples were quantified by reverse-phase liquid chromatography (Waters, Milford, MA). A 2-pump gradient system (high-performance liquid chromatography model 510 solvent delivery system; Waters) delivered 82%–85% (at 27 minutes) to 0% 10 m*M* sodium acetate trihydrate (pH 4.76) with balance methanol at 1 ml/minute for 43 minutes. Following precolumn fluorescent derivatization (AccQ·Fluor; Waters), analyte separation was performed on a 4.6 mm × 250 mm, 3.5 μm column (Xterra MS C18; Waters) with an identical 3.9 mm × 20 mm guard column, both controlled at 36°C (integrated heater-controller CH-500; Eppendorf, Madison, WI). Standards, blanks, and samples (10 μl) were automatically injected (intelligent sample processor model 712; Waters), and fluorescent peak height and area were evaluated at an excitation of 250 nm and an emission of 395 nm (scanning fluorescence detector model 474; Waters) to original concentrations of 0.1 μM.

Statistical analysis. Data management and analysis were performed using Stata version 6.0 software (Stata Corporation, College Station, TX) and GraphPad Prizm version 3.0 (GraphPad Software, San Diego, CA). Baseline values were expressed as the mean ± SD in the 2 groups of subjects (primary RP and secondary RP) unless otherwise indicated. Values for each group were compared using paired 2-tailed *t*-tests ($\alpha = 0.05$). CPT results were graphed for each group at 30-second intervals from 0 to 3.5 minutes. An aggregate CPT response using the AUC approach was calculated for subjects with primary RP and secondary RP, and this was used for correlation analysis. Correlation analysis was performed between log-transformed circulating ET-1 levels, ADMA levels, or an aggregate score of ET-1/ADMA and measures of microvascular and macrovascular responses and reported as Spearman's rank correlations (r_s). Univariate and multivariate analyses using baseline subject characteristics as covariates were performed. *P* values less than 0.05 were considered significant.

RESULTS

Clinical and demographic characteristics. Forty patients with primary RP or secondary RP were studied.

Table 1. Baseline characteristics*

	Primary RP (n = 20)	Secondary RP (n = 20)
Age, years	47 ± 12	42 ± 13
Female, no. (%)	14 (70)	15 (75)
White, no. (%)	17 (85)	20 (100)
BMI, kg/m^2	24 ± 4	24 ± 6
Systolic blood pressure, mm Hg	122 ± 21	120 ± 14
Diastolic blood pressure	70 ± 14	72 ± 13
Total cholesterol, mg/dl	205 ± 45	188 ± 41
LDL, mg/dl	120 ± 42	103 ± 30
Duration of RP, years	10 ± 6	12 ± 8
Attack frequency/day	2.1 ± 1	2.4 ± 2
Composite severity score	100 ± 70	108 ± 83
Mean brachial artery diameter, mm	3.58 ± 0.70	3.50 ± 0.73

* Except where indicated otherwise, values are the mean ± SD. RP = Raynaud's phenomenon; BMI = body mass index; LDL = low-density lipoprotein.

The baseline demographics of these patients are included in Table 1. The majority of patients in both the primary and secondary RP cohorts were women. The mean (±SD) duration of RP was 10 ± 6 years in the primary RP population and 12 ± 8 years in the secondary RP population. Symptoms in the patients were moderate to severe, with patients in the primary RP population reporting a mean (±SD) of 2.1 ± 1 vasospastic attacks per day and patients in the secondary RP population reporting a mean (±SD) of 2.4 ± 2 vasospastic attacks per day. Severity scores were comparable. No patients with RP secondary to occupational hazards or medications were included in this study. No patients had evidence of ischemic ulcerations when the study was performed. There were no significant differences in the groups of patients with primary and secondary RP with regard to blood pressure, LDL cholesterol, or adiposity.

The underlying diagnoses in patients with secondary RP were as follows: SLE (25%), SSc/CREST syndrome (35%), primary SS (15%), UCTD (10%), polymyositis (5%), rheumatoid arthritis (5%), and MCTD (5%). None of the patients with secondary RP had evidence of clinical remission. No patients had evidence of ischemic ulceration or pitting at the time this study was performed. Of the 2 individuals diagnosed as having UCTD, 1 had polyarthralgias, RP, and autoantibodies, and the other had hypocomplementemia, arthralgias, and RP.

Vascular responses in primary and secondary RP. Baseline brachial artery diameters in patients with primary and secondary RP did not differ (mean ± SD 3.51 ± 0.8 versus 3.68 ± 0.08 mm, respectively; $P > 0.05$). Figure 1 shows flow-mediated dilation in brachial arteries in response to a reactive hyperemia stimulus. Median (25th, 75th percentiles) FMD% response at baseline in patients with primary RP was 3.95 (2.22, 7.36), compared with 2.55 (0.72, 5.60) in the cohort of patients with secondary RP ($P \geq 0.05$). Similarly, NMD% in both groups was comparable (16.22 [13.14, 26.20] in patients with primary RP and 19.55 [12.79, 24.77] in patients with secondary RP) (Figure 2). CPT responses of conduit vessels in patients with primary and secondary RP were determined, and results are shown in Figure 3. The responses in primary and secondary RP

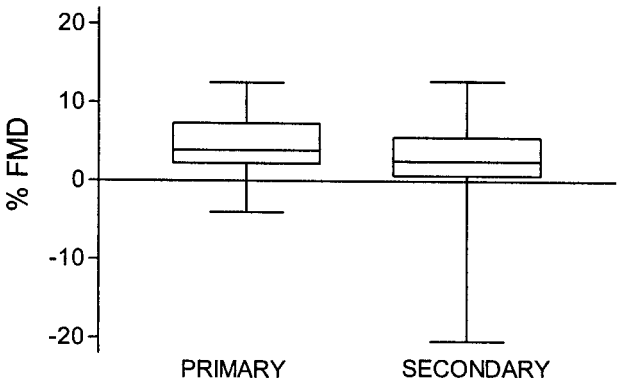


Figure 1. Flow-mediated dilation (FMD%) in brachial arteries in patients with primary and secondary Raynaud's phenomenon. $P \geq 0.05$ at all time points. Boxes show the 25th and 75th percentiles; horizontal lines in boxes show the medians; vertical bars show the ranges.

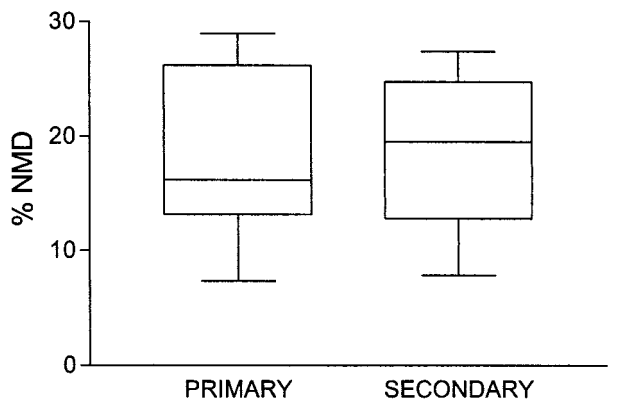


Figure 2. Nitroglycerin-mediated dilation (NMD%) in brachial arteries in patients with primary and secondary Raynaud's phenomenon. $P \geq 0.05$ at all time points. Boxes show the 25th and 75th percentiles; horizontal lines in boxes show the medians; vertical bars show the ranges.

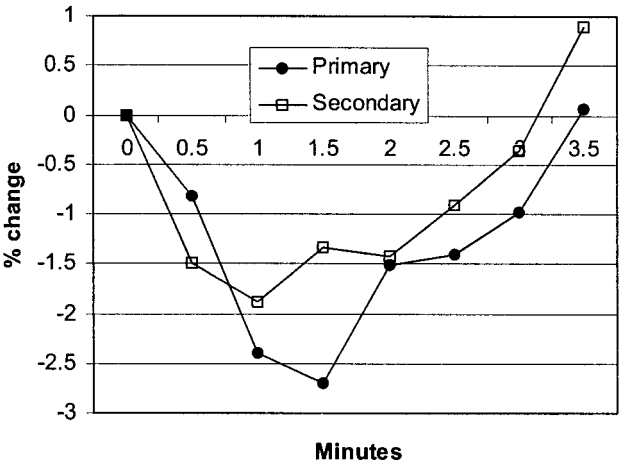


Figure 3. Cold pressor test responses in patients with primary and secondary Raynaud's phenomenon. Shown is the percentage change in brachial artery diameter for 3.5 minutes, at 30-second intervals, following 2 minutes of ice exposure. Values are the means. $P \geq 0.05$ at all time points.

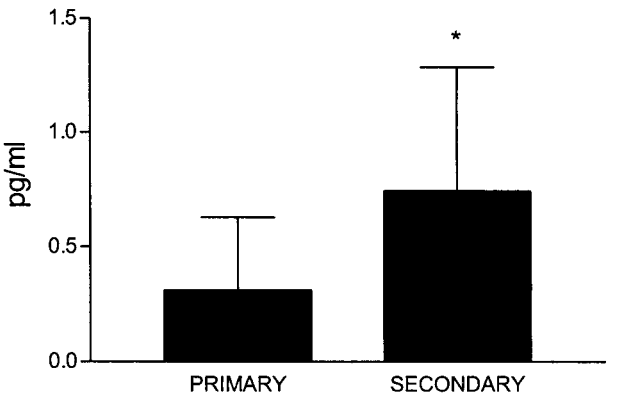


Figure 4. Plasma endothelin 1 levels in patients with primary and secondary Raynaud's phenomenon. Values are the mean and SD. * = $P \leq 0.02$, by Mann-Whitney U test.

were characterized by predominant vasoconstriction. No significant differences were observed at individual time points or in the aggregate response of conduit vessels to cold in either group.

We then evaluated cutaneous resistance vessel function in the 2 cohorts. The responses were quantified as a cumulative index of perfusion (AUC >4 minutes), peak perfusion ratio, percent reactive hyperemia, or as time to peak perfusion. As seen in Table 2, in the group of patients with secondary RP (but not the group with primary RP), laser Doppler–derived skin perfusion values in response to reactive hyperemia were characterized by a reduction in AUC responses adjusted for baseline perfusion, but not in percent reactive hyperemia, time to peak response, or peak perfusion ratios (AUC 183 ± 120 perfusion units in patients with secondary RP and 815 ± 1,342 perfusion units in patients with primary RP).

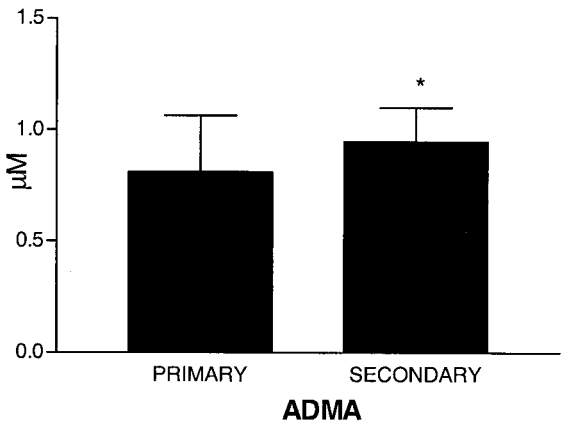
Table 2. Laser Doppler–derived skin perfusion values in patients with primary and secondary RP*

Index	Primary RP	Secondary RP
Microvascular: baseline perfusion	0.08 ± 0.1	0.14 ± 0.2
Reactive hyperemia		
Peak perfusion ratio	38 ± 65	10 ± 7
Percent reactive hyperemia	3,743 ± 6,474	878 ± 712†
Reactive hyperemia AUC	815 ± 1,342	183 ± 120
Time to peak response, seconds	30 ± 18	20 ± 15

* Values are the mean ± SD. RP = Raynaud's phenomenon; AUC = area under the curve; reactive hyperemia AUC = AUC over 4 minutes of reactive hyperemia response corrected for baseline perfusion.
† $P = 0.0048$ for AUC versus primary RP group, by unpaired t-test.

ET-1 and ADMA levels in RP. Circulating ET-1 in patients with secondary RP was elevated compared with that in patients with primary RP (mean ± SD 0.75 ± 0.12 and 0.31 ± 0.07 pg/ml, respectively; $P = 0.0183$ by Mann-Whitney U test) (Figure 4). Figure 5 shows the levels of ADMA, an endogenous inhibitor of nitric oxide synthase, in RP. ADMA, but not SDMA, levels were higher in patients with secondary RP when compared with patients with primary RP (mean ± SD 0.95 ± 0.03 and 0.81 ± 0.06, respectively; $P = 0.0275$ by Mann-Whitney U test). Finally, as seen in Figure 6, patients with secondary RP had higher levels of VCAM-1, ICAM-1, and MCP-1 when compared with patients with primary RP ($P < 0.05$ for all 3). Correlation analyses were performed for ET-1 and ADMA against measures of microvascular function and conduit vascular function in the entire RP population as well as in the primary and secondary RP groups. ET-1 and ADMA, when analyzed individually, predicted impairment in microvascular function as measured by the AUC in both primary and secondary RP. ET-1 levels, when combined with ADMA levels, correlated significantly with the AUC measure ($r_s = -0.54$, $P = 0.017$). There was no correlation between increases in ADMA and brachial artery endothelial function in either primary or secondary RP. Similarly, when the cumulative response to CPT in conduit vessels over 3.5 minutes was compared with ADMA and ET-1 levels, there did not appear to be a significant correlation in either the primary or secondary RP group.

Compared with RP in other autoimmune dis-



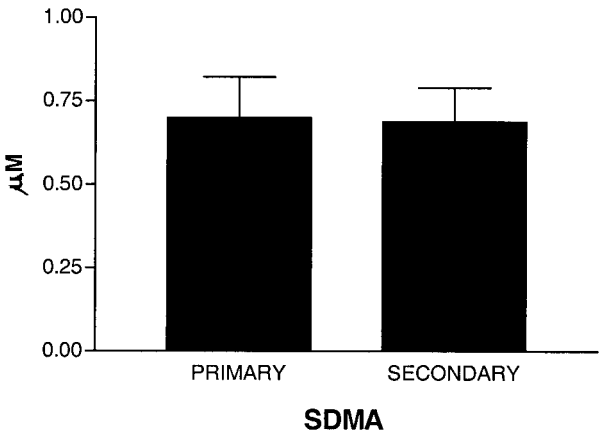


Figure 5. Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels in primary and secondary Raynaud's phenomenon. Values are the mean and SD. * = $P = 0.03$, by Mann-Whitney U test.

eases, RP attacks tend to be more severe in patients with SSc/CREST. These more frequently result in digital tip ischemic ulcers, infarcts, and amputations. To exclude the possibility that the abnormalities found in secondary RP were attributed only to the SSc/CREST subgroup, we additionally analyzed patients with SSc/CREST and individuals with other autoimmune diseases separately. We found that the differences between primary and

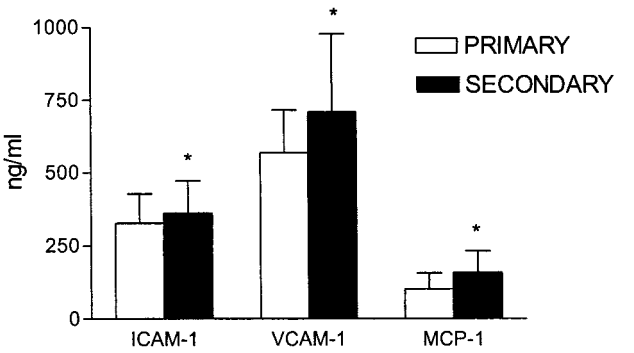


Figure 6. Adhesion molecule and chemokine expression in patients with primary and secondary Raynaud's phenomenon. ICAM-1 = intercellular adhesion molecule 1; VCAM-1 = vascular cell adhesion molecule 1; MCP-1 = monocyte chemoattractant protein 1. Values are the mean and SD. * = $P \leq 0.05$, by Mann-Whitney U test.

secondary RP in ET-1 and ADMA, as well as AUC measurements, persisted even after exclusion of the patients with SSc, who were a small percentage of the overall secondary cohort.

DISCUSSION

RP as a manifestation of connective tissue disease is frequent and often precedes other symptoms and signs (30,31). The manifestations, severity, and prognosis of secondary RP are very different from those of primary RP and include progression to cutaneous ulcerations and tissue necrosis (30,32). Accurate differentiation of secondary RP from primary RP is partly dependent on identification of pathophysiologic mechanisms and vascular abnormalities that are unique to these two disease states. To achieve this, comparisons of primary and secondary RP at relatively similar degrees of severity is important, because variations of disease severity and concomitant vascular risk factors may complicate the interpretation of observations (33).

Accordingly, the main objective of this study was to compare and contrast microvascular and macrovascular abnormalities in a cohort of patients with primary and secondary RP who were carefully matched for demographic, LDL, hemodynamic, and severity profiles. The main findings of this study were the following: no differences in endothelium-dependent or -independent function in conduit vessels between patients with primary and secondary RP; abnormal vasoconstrictor response of brachial arteries to cold in both patients with primary and those with secondary RP, with no differences in the intensity of vasoconstriction between patients with primary and secondary RP; abnormal micro-

vascular function in patients with secondary RP, but not patients with primary RP, as evidenced by a blunted cutaneous response to a reactive hyperemic stimulus; and increases in circulating ET-1 and ADMA in patients with secondary RP compared with patients with primary RP, accompanied by increases in VCAM-1 and MCP-1 levels.

To our knowledge, this is the first study to compare and contrast vasodilator mechanisms at both the resistance and conduit levels in primary and secondary RP. Our patient population with secondary RP was a heterogeneous group composed of patients with different connective tissue diseases that can present with RP symptoms. These individuals were compared with a cohort of patients with primary RP who were otherwise matched for disease severity and risk factors known to modulate vascular function. Indeed, blood pressure, LDL cholesterol, and adiposity were identical in the 2 groups. Conduit endothelial function and endothelium-independent function in our study, as determined by reactive hyperemia and responsiveness to cold in the brachial artery, did not differ between the 2 cohorts. However, both primary and secondary RP were characterized by abnormal vasoconstrictor responses to cold as opposed to the normal vasodilator response, reiterating abnormal sympathetic outflow at the vessel wall in this disease (34).

In contrast to the homogeneity of responses of conduit vessels in patients with primary and secondary RP, there was an impairment of microvascular responses in patients with secondary RP compared with patients with primary RP, as evidenced by a diminution in reactive hyperemic flow over time, adjusted for baseline perfusion. In our experience, AUC measurements, which represent a composite measurement over time, were superior to single-point measurements such as peak perfusion ratio and percent reactive hyperemia or time to peak. Consistent with prior studies, we demonstrated an increase in circulating ET-1 levels in patients with secondary RP compared with patients with primary RP (5,35).

A novel finding in this study was the demonstration of elevation of a circulating endogenous inhibitor of NOS, ADMA, in patients with secondary RP compared with patients with primary RP. ADMA has been previously reported to be elevated in a variety of conditions associated with attenuation of arterial vasodilator reserve, including atherosclerosis and hypercholesterolemia (12,36). In these conditions, ADMA levels are only elevated 0.5–3-fold above the normal concentrations. Thus, the range of values noted in these conditions is fairly small. Previous studies have shown that exogenous concentrations between 1 and 10 μM/liter affect endothelial-dependent vasodilation in rat mesenteric and cerebral vessels (37,38). Similarly, incubation of cultured endothelial cells with oxidized LDL results in increases of ADMA from 0.6 μM/liter to 1.1 μM/liter at the end of a 48-hour period (39). Thus, ADMA levels are tightly regulated and the levels reported are likely to be of pathophysiologic significance. Both ADMA and ET-1, either alone or potentially together, can modulate resistance vessel function (40,41).

In this regard, previous studies in experimental heart failure have shown that ET-1 stimulates ADMA (42), and our studies confirm a connection between the ET-1 axis and ADMA in the RP population. Inhibition of NOS in the skin with inhibitors such as L-N^G-nitroarginine methyl ester can potentiate the effect of ET-1–mediated vasoconstriction, and thus these two mediators can potentially act together (43). Increases in ET-1 are well known to interact negatively with the NOS pathway and, together with elevations in ADMA, could represent a potent vasoconstrictor combination that sets the stage for progression of vascular disease and its complications. Indeed, ET-1 and ADMA levels taken together significantly correlated negatively with microvascular perfusion. Thus, it may be argued that the vascular abnormalities observed in patients with RP are unlikely to be affected by targeting one pathway alone.

Since ADMA has been reported to be increased in patients with renal failure and hypercholesterolemia, it is important to mention that none of the patients studied had renal dysfunction (creatinine >1.4 mg/dl) or elevations in LDL cholesterol (44). As expected, an inactive structural isomer of ADMA, SDMA, was not elevated in patients with secondary RP, supporting the accuracy of our ADMA analysis by high-performance liquid chromatography. Although increases in ET-1 and ADMA were negatively correlated with changes in microvascular flow, they did not correlate with conduit endothelial function (FMD%) or responses to cold in patients with either primary or secondary RP. This finding may relate to the fact that once other traditional risk factors that more directly correlate with conduit endothelial function (such as LDL cholesterol) are accounted for, conduit responses in RP are comparable, despite differential elevations of ADMA and ET-1. Thus, differences in microvascular flow may represent an inherent susceptibility of the cutaneous resistance circulation to these mediators in patients with secondary RP.

Previous studies have shown that ET-1 release

and expression are linked to alterations in chemokine and adhesion molecule expression (45,46). Our findings reaffirm and extend these observations linking the inflammatory cytokine network with the neurohormonal system, which may promote peripheral inflammation and oxidative stress in the cardiovascular system of patients with connective tissue disease and RP. The elevation of MCP-1 and VCAM-1 in patients with secondary RP may reflect the unfavorable effects of dysfunctional NOS and ET-1 pathways in patients with RP and indicate early activation of a proinflammatory process in the vasculature of patients with secondary RP. These findings add to evidence from prior studies demonstrating that patients with different connective tissue diseases associated with secondary RP have increased VCAM-1, ICAM-1, and MCP-1 levels, and that their levels in plasma correlate with their in situ expression (47).

In conclusion, both primary and secondary RP are characterized by abnormal conduit vessel vasoconstriction to cold. Secondary RP is also associated with attenuation of microvascular responses to reactive hyperemia and elevations in ADMA and ET-1. Increases in these vasoactive mediators acting together in patients with secondary RP may influence alterations in vascular function and proinflammatory pathways, and therefore, disease progression.

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