Contribution of nitric oxide to cutaneous microvascular dilation in individuals with type 2 diabetes mellitus

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Sokolnicki LA, Roberts SK, Wilkins BW, Basu A, Charkoudian N. Contribution of nitric oxide to cutaneous microvascular dilation in individuals with type 2 diabetes mellitus. Am J Physiol Endocrinol Metab 292: E314–E318, 2007. First published September 5, 2006; doi:10.1152/ajpendo.00365.2006.—Microvascular pathophysiology associated with type 2 diabetes mellitus (T2DM) contributes to several aspects of the morbidity associated with the disease. We quantified the contribution of nitric oxide (NO) to the cutaneous vasodilator response to nonpainful local warming in subjects with T2DM (average duration of diabetes mellitus 7 ± 1 yr) and in age-matched control subjects. We measured skin blood flow in conjunction with intradermal microdialysis of Nω-nitro-l-arginine methyl ester (l-NAME; NO synthase inhibitor) or vehicle during 35 min of local warming to 42°C. Microdialysis of sodium nitroprusside (SNP) was used for assessment of maximum cutaneous vascular conductance (CVC). Resting CVC was higher in T2DM subjects at vehicle sites (T2DM: 19 ± 2 vs. control: 11 ± 3%maxCVC; P < 0.05); this difference was abolished by l-NAME (T2DM: 10 ± 1 vs. control: 8 ± 1%maxCVC; P > 0.05). The relative contribution of NO to the vasodilator response to local warming was not different between groups (T2DM: 46 ± 4 vs. control: 44 ± 6%maxCVC; P > 0.05). However, absolute CVC during local warming was ∼25% lower in T2DM subjects (T2DM: 1.79 ± 0.15 AU/mmHg; controls: 2.42 ± 0.20 AU/mmHg; P < 0.01), and absolute CVC during SNP was ∼20% lower (T2DM: 1.91 ± 0.12 vs. control: 2.38 ± 0.13 AU/mmHg; P < 0.01). We conclude that the relative contribution of NO to vasodilation during local warming is similar between subjects with T2DM and control subjects, although T2DM was associated with a lower absolute maximum vasodilation.

During local warming, increases in temperature cause localized cutaneous vasodilation via two independent mechanisms: an initial rapid peak followed by a slower vasodilator response that reaches a plateau around 30 min of heating. Local sensory nerves are primarily responsible for the vasodilation causing the initial rapid peak, whereas the plateau phase includes a substantial NO component (11, 14). It has been proposed that this vasodilator response could be used as a noninvasive assessment of microvascular endothelial function (11).

In the present study, our goal was to further evaluate mechanisms for decreased microvascular dilation in the skin of subjects with T2DM. We used a standardized nonpainful local warming protocol in conjunction with intradermal microdialysis of the NO synthase inhibitor Nω-nitro-l-arginine methyl ester (l-NAME) or vehicle (Ringer solution). We tested the hypothesis that subjects with T2DM have a decreased contribution of NO to this cutaneous vasodilator response.

METHODS

Subjects. We studied 9 subjects with T2DM (age range 44–67 yr; 3 women) and 11 nondiabetic healthy subjects (age range 43–69 yr; 7 women). All subjects were screened by telephone and physical examination to rule out potential for confounding influences of several comorbidities associated with age and/or T2DM. Subjects with clinically relevant neuropathy and/or a history of cardiovascular disease were excluded. Of the individuals with T2DM, three were taking metformin, three were diet controlled, one took a combination of

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metformin and glipizide, one took a combination of metformin and insulin, and one took glipophage. If taking oral anti-hyperglycemic agents, subjects discontinued taking these medications for 2 wk before their study day. Subjects with T2DM had an average duration of seven years since the diagnosis. Subjects reported to the Mayo General Clinical Research Center (GCRC) for a screening visit and again on the day of the study. Informed consent was obtained before any procedures began. The protocol for this experiment was approved by the Institutional Review Board at the Mayo Clinic in Rochester (Rochester, MN).

Screening visit. Subjects reported to the GCRC for a screening visit, during which blood samples were taken for measurement of glucose, Hb, glycosylated Hb (Hb A1c), hematocrit, albumin, cholesterol, and triglycerides. T2DM subjects were seen by a neurologist to screen for neuropathy. The screening visit also consisted of a treadmill exercise test in the Mayo Cardiovascular Health Clinic using a standard Bruce protocol to rule out occult cardiovascular disease in both control subjects over 50 years of age and in all T2DM subjects. Body composition was measured using dual-energy X-ray absorptiometry. Subjects with T2DM tested their blood glucose with their personal glucose meter and the meter at the GCRC.

Study day. Subjects reported to the GCRC at 7:00 A.M. after fasting overnight. Blood glucose values for individuals with T2DM were obtained before any study procedures began. Two intradermal sites were obtained before any study procedures began. To place the probes, 25-gauge needles were inserted just under the surface of the skin with entry and exit points placed at least 5 cm apart and taped in place. We then waited 60–90 min for the hyperemia related to needle insertion to resolve. Skin blood flow was measured as cutaneous red blood cell flux using Periflux laser-Doppler flowmetry (LDF) over the two microdialysis sites. Two integrating LDF probes (Periflux System 5000; Perimed, Stockholm, Sweden) were attached to the left forearm to measure skin blood flow. The probes were attached to the skin in specialized holders that measured and controlled temperature over an area of 12 cm² (Perimed). Each probe was placed directly over a microdialysis membrane. Blood pressure was measured beat-to-beat using a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands) placed on the subjects’ right middle finger.

Protocol. Subjects rested supine for 1 h before data collection began. One microdialysis fiber was used as a control site, and the other was used for administration of L-NAME (10 mM) for local inhibition of NO synthase (14, 15). The control site was perfused with vehicle (Ringer solution), and the treated site was perfused with L-NAME both at a rate of 4 µl/min using a microperfusion pump (Harvard 22 syringe pump; Harvard Apparatus, South Natick, MA). The L-NAME infusion was begun 30–45 min after microdialysis fiber placement and was continued for an additional 30–45 min while steady-state baseline values were attained at both vehicle and L-NAME sites. We determined that a steady baseline had been reached when the LDF signal had decreased to a stable plateau for ≥10 min; at that time, we began data collection for the baseline period before local warming. Vehicle and L-NAME infusions were continued throughout the local warming protocol.

Baseline data were recorded for 7–10 min while local temperature was held constant at 32°C. For local heating of the skin, local temperature was increased at both LDF sites to 42°C at a rate of 1°C every 7 s. A local temperature of 42°C was held constant for 35 min. Subjects were asked whether they felt any pain or discomfort during the local warming; no subject reported feeling any pain or discomfort. The temperature was then increased to 43°C while sodium nitroprusside (SNP; 28 mM) was infused at both L-NAME and vehicle sites for 35–45 min to elicit maximum cutaneous vascular conductance (CVC). Previous work has shown that this dose of SNP delivered by microdialysis causes maximal vasodilation in human skin (11, 15).

Data analysis. Skin blood flow was divided by mean arterial pressure to derive an index of CVC. Baseline CVC was assessed as the average of the last 2 min during baseline, initial peak CVC as a 60-s average during the highest point of the initial peak of the vasodilator response, nadir CVC as a 60-s average during the short decline in blood flow after this initial peak, and plateau CVC as the average of the last 2 min during local warming before SNP microdialysis.

Maximum CVC was assessed as the average of the last 3 min during SNP microdialysis. CVC data are expressed as a percentage of this maximum (%maxCVC) or as absolute values (AU/mmHg). To quantify the relative contribution of NO to the vasodilator responses observed in control subjects and T2DM subjects, we compared vehicle-treated sites with L-NAME-treated sites in both groups. The NO contribution was assessed by subtracting CVC at L-NAME-treated sites from that at vehicle-treated sites at each point in the local warming protocol (initial peak, nadir, and plateau).

Statistics. Results are expressed as means ± SE. Comparisons between control and T2DM subjects were made using unpaired Student’s t-test or ANOVA with Bonferroni post hoc tests to identify individual differences between groups. Statistical significance was set at P < 0.05.

RESULTS

Subject demographics, including mean age, height, weight, %body fat, body mass index (BMI), and time since T2DM diagnosis are shown in Table 1. Subjects with T2DM and control subjects were of similar age, height, and %body fat; however, body weight and BMI were significantly higher in individuals with T2DM (P < 0.05). Screening visit blood test results and study day blood glucose values are shown in Table 2. Both glucose and glycosylated Hb were significantly higher in T2DM subjects (P < 0.05).

Table 1. Subject demographics

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (males)</td>
<td>11 (4)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>Years since diagnosis of T2DM</td>
<td>NA</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Age, yr</td>
<td>54 ± 3</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>Height, cm</td>
<td>167 ± 2</td>
<td>171 ± 2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>69 ± 2</td>
<td>84 ± 5*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25 ± 1</td>
<td>28 ± 2*</td>
</tr>
<tr>
<td>Fat, %</td>
<td>37 ± 4</td>
<td>35 ± 2</td>
</tr>
</tbody>
</table>

Data are means ± SE; n, no. of subjects. T2DM, type 2 diabetes mellitus; BMI, body mass index. *Significant difference compared with control subjects, P < 0.05. NA, not applicable.

Table 2. Blood screening results

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Screening visit, mg/dl</td>
<td>95 ± 2</td>
<td>157 ± 18*</td>
</tr>
<tr>
<td>Study day, mg/dl</td>
<td>NA</td>
<td>212 ± 24</td>
</tr>
<tr>
<td>Hemoglobin, g/dl</td>
<td>14.0 ± 0.2</td>
<td>14.2 ± 0.4</td>
</tr>
<tr>
<td>Hemoglobin A1c, %</td>
<td>5.2 ± 0.1</td>
<td>6.6 ± 0.3*</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>40.6 ± 0.7</td>
<td>41.1 ± 1.4</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>4.4 ± 0.1</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>209 ± 9</td>
<td>176 ± 15</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>100 ± 11</td>
<td>133 ± 26</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *Significant difference compared with control subjects, P < 0.05.
A representative tracing illustrating the cutaneous vasodilator responses to local warming, one in a control subject and one in an individual with type 2 diabetes mellitus (T2DM), showing initial peak, nadir, and plateau phases of the vasodilator response. Bottom: local temperature. Absolute values for cutaneous vascular conductance (CVC) were lower throughout local heating in this T2DM subject and were consistently lower for the plateau phase in the T2DM subjects as a group (see text for details).

Baseline values for CVC at vehicle and l-NAME sites are shown for both groups in Fig. 2. Baseline CVC at the vehicle site was significantly higher in T2DM subjects compared with controls (control 11 ± 3% maxCVC and T2DM 19 ± 2% maxCVC), consistent with previous findings from our laboratory (17). At the l-NAME site, baseline CVC values were similar between subject groups (control 8 ± 1% maxCVC and T2DM 10 ± 1% maxCVC).

Figure 3 shows the average local warming data, shown as % maximum CVC, for both subject groups during the initial peak, nadir, and plateau phase at both vehicle and l-NAME sites. Individuals with T2DM demonstrated a small decrease during the initial peak at the l-NAME-treated site compared with the vehicle site (\(P < 0.05\)). l-NAME decreased the initial peak slightly in the control subjects; however, this difference was not statistically significant (\(P > 0.05\)). In both groups, l-NAME decreased the nadir CVC as well (\(P < 0.05\)). As expected, plateau CVC during local warming was substantially lower at l-NAME-treated sites compared with vehicle sites in both subject groups, averaging a 44% maxCVC difference (\(P < 0.05\)). During local warming, neither initial peak nor nadir % maximum CVC values differed between groups at vehicle- or l-NAME-treated sites (\(P > 0.05\)).

Figure 4 shows absolute values for maximum CVC during SNP microdialysis at both sites. Values are expressed as means ± SE. \(*\) Significant difference between controls and T2DM subjects at l-NAME site (\(P < 0.05\)).
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T2DM subjects. Overall average values for maximum CVC were \( \sim 20\% \) lower in T2DM subjects compared with control subjects (T2DM subjects: \( 1.91 \pm 0.12 \) AU/mmHg; controls: \( 2.38 \pm 0.13 \) AU/mmHg; \( P < 0.01 \)).

Although \( \%\text{maxCVC} \) values during local warming were similar between groups (Fig. 3), this represented similar percentages of an overall lower maximum, as noted above (Fig. 4). Although absolute CVC values for the initial peak and nadir were not statistically different between the controls and T2DM subjects (\( P > 0.05 \)), the plateau phase was significantly different between subject groups. Absolute CVC values at vehicle-treated sites in T2DM subjects during the plateau phase of local warming vasodilation were \( \sim 25\% \) lower when compared with control subjects (T2DM: \( 1.79 \pm 0.15 \) AU/mmHg; controls: \( 2.42 \pm 0.20 \) AU/mmHg; \( P < 0.01 \)).

We used the difference between vehicle- and \( \text{-NAME} \)-treated sites in \( \%\text{maxCVC} \) to calculate the contribution of NO to the plateau phase of the local warming response in both groups. The relative contribution of NO was similar between T2DM subjects and control subjects, averaging \( 46 \pm 4\%\text{maxCVC} \) in T2DM subjects and \( 44 \pm 6\%\text{maxCVC} \) in controls (\( P > 0.05 \)). In addition to using the relative \( \%\text{maxCVC} \) values to calculate NO contribution, we examined the results using the absolute CVC data. With the use of absolute values, the NO contribution demonstrated a trend to be smaller in the T2DM group (\( 0.9 \pm 0.2 \) AU/mmHg (T2DM) vs. \( 1.3 \pm 0.2 \) AU/mmHg (control), \( P = 0.06 \)).

DISCUSSION

In the present study, we quantified for the first time the contribution of NO to the vasodilator response to nonpainful local warming in subjects with T2DM. Our main new finding was that the relative contribution of NO (expressed as \( \%\text{maxCVC} \)) to the vasodilation to local warming was not different between T2DM and control subjects, averaging \( \sim 45\% \) in both groups. An unexpected new finding in the present study was an apparent greater contribution of NO to baseline (preheating) CVC in T2DM subjects compared with control subjects.

Our present findings of a similar contribution of NO (as \( \%\text{maxCVC} \)), in combination with differences of \( 20\% - 25\% \) in absolute CVC during both local warming and SNP administration, suggest that the decreased vasodilation in T2DM could include both a decrease in NO “signal” (synthesis, release, and/or bioavailability) and/or impaired vasodilator mechanisms downstream from endothelial release of NO. Decreased responsiveness to SNP, shown in the present and previous work (2, 18), suggests a downstream decrease in responsiveness at the level of the vascular smooth muscle. For example, elevated oxidant levels associated with increased circulating glucose can impair vascular smooth muscle responsiveness to NO (4).

Overall, our present results are consistent with a decrease in NO-mediated cutaneous microvascular dilation in relatively healthy individuals with T2DM. It has been suggested previously that the nonpainful local warming paradigm used in the present study could be used to identify changes in endothelial function in pathophysiological conditions such as those associated with T2DM (11). Our results suggest that this paradigm may in fact identify impairments in NO-dependent vasodilation in this population, although the semiquantitative nature of the LDF measurements presents some limitations in this regard (see below).

Our present results do not provide specific insight into mechanisms of decreased maximal vasodilator responsiveness in the skin in our T2DM group. One possibility is that the total density of microvessels in the skin (and therefore the number under any given LDF probe) is decreased in our T2DM group compared with our control group. Data from obese Zucker rats suggest that the metabolic syndrome is associated with vascular rarefaction in skeletal muscle tissue, a phenomenon that appears to be mechanistically linked to decreased NO bioavailability in this model (5, 6). In healthy humans, an inverse relationship was found between fasting plasma glucose and capillary density in the skin (8), although specific implications for T2DM remain to be elucidated.

In our recent study (17), as well as in our present work, we found that baseline CVC was higher in T2DM subjects compared with control subjects. In the present study, \( \text{-NAME} \) caused an average nonsignificant decrease of \( \sim 27\% \) (of vehicle baseline) in control subjects and a statistically significant decrease of \( 50\% \) of baseline in T2DM subjects. This influence of \( \text{-NAME} \) is consistent with the \( \sim 35\% \) decrease in baseline CVC by \( \text{-NAME} \) in healthy individuals recently reported by Hodges et al. (7). The fact that baseline CVC was similar between groups after \( \text{-NAME} \) treatment is supportive of the idea that NO-mediated vasodilator tone contributes more to baseline CVC in T2DM subjects compared with controls. Additionally, the elevated baseline CVC may be related to the fact that our T2DM group was relatively healthy; in a group of subjects with more symptomatic T2DM, including peripheral neuropathy, this might not be the case.

The relative good health of our T2DM subjects might also explain the initial peak vasodilator responses of our T2DM subjects. The initial peak phase of local warming vasodilation relies primarily on local sensory nerve release of vasodilator substrates (14). This phase of the vasodilator response was not statistically different between groups in the present study at either vehicle- or \( \text{-NAME} \)-treated sites, suggesting that sensory nerve-mediated vasodilation was intact in our group of T2DM subjects. This preserved sensory nerve-mediated dilation may not be the case in individuals with more symptomatic T2DM.

In our comparison of skin blood flow mechanisms between control subjects and subjects with T2DM, it was important in the present study that we compared T2DM subjects with control subjects of similar age, since aging per se alters control mechanisms in the cutaneous circulation. For example, skin blood flow responses to both whole body and local heating of the skin are decreased in older subjects (12, 13). Because T2DM increases in prevalence with increasing age, it is important to compare T2DM subjects with controls of similar age to minimize potential confounding influences of the aging process itself. In this context, we report here that the relative contribution of NO to local warming vasodilation was \( \sim 45\% \) in T2DM subjects and control subjects. This is somewhat lower than the NO contributions of \( 60\% - 75\% \) reported in the literature (11, 14). This may be related to the fact that we were studying an older population, since it has been shown that the contribution of NO to local warming-induced vasodilation is decreased with aging.
Study Limitations

We were interested to note that baseline CVC was higher in our T2DM subjects and that this increase appeared to be mediated by NO, since it was reversed at L-NAME-treated sites. Because it was not a major goal of the present study to evaluate the role of NO in baseline CVC, we did not specifically compare baseline CVC before L-NAME to baseline CVC with L-NAME at the L-NAME site. Although our comparison between vehicle and L-NAME sites allowed us some insight into the control of skin blood flow in T2DM, further insight into the role of altered NO-mediated vasodilation is important to follow up on the present observations to gain information about absolute levels of blood flow (i.e., ml/min). Data derived from LDF measurements are best used for measurement of relative changes in blood flow in a given subject and experiment. However, given our and others’ repeated previous findings of lower absolute vasodilation with local warming or other local stimuli in subjects with T2DM (2, 16, 17), we think it is reasonable to consider that these findings represent an actual decrease in vasodilator responsiveness in the cutaneous microvasculature in these T2DM subjects.

With regard to our finding of decreased absolute vasodilator responses to both local warming and SNP microdialysis, it is important to note that LDF measurements of skin blood flow are limited in that they cannot accurately give information about absolute levels of blood flow (i.e., ml/min). Data derived from LDF measurements are best used for measurement of relative changes in blood flow in a given subject and experiment. However, given our and others’ repeated previous findings of lower absolute vasodilation with local warming or other local stimuli in subjects with T2DM (2, 16, 17), we think it is reasonable to consider that these findings represent an actual decrease in vasodilator responsiveness in the cutaneous microvasculature in these T2DM subjects.

In summary, we report here that the relative contribution of NO to local thermal vasodilation in subjects with T2DM was similar to that seen in control subjects of similar age. The absolute values of CVC responses to both local warming and SNP microdialysis were 20–25% lower in T2DM subjects compared with controls; this is consistent with our and others’ previous reports of decreased vasodilator responsiveness in the skin of subjects with T2DM (2, 16, 17). Taken together, these data suggest that the mechanisms of the cutaneous vasodilator response to local warming are similar in T2DM but that the overall ability to dilate (availability of, or responsiveness to, NO) is diminished in T2DM. Interestingly, it appears that the NO contribution to resting skin blood flow may be greater in subjects with T2DM compared with control subjects. It will be important to follow up on the present observations to gain further insight into the role of altered NO-mediated vasodilation in the control of skin blood flow in T2DM.

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GRANTS

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