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Effect of L-NMMA on microvascular blood flow and glucose metabolism after an oral glucose load

Alexandra Högstedt¹, Fredrik Iredahl¹, Erik Tesselaar^{1,2}, Simon Farnebo^{1,3}

1. Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden
2. Department of Medical Radiation Physics, Linköping University, Linköping Sweden
3. Department of Hand Surgery, Plastic Surgery and Burns, Linköping University, Linköping, Sweden

Corresponding author:

Alexandra Högstedt, M.D.

Department of Clinical and Experimental Medicine

Faculty of Health Sciences

Linköping University

SE-58185 Linköping, Sweden

E-mail: alexandra.hogstedt@liu.se

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Conceived and designed the experiments: AH, FI, ET, SF

Performed the experiments: AH

Analyzed/interpreted the data: AH, FI, ET, SF

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Effect of L-NMMA on local blood flow

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Abstract

Objective

The aim of this study was to investigate whether the effects on local blood flow and metabolic changes observed in the skin after an endogenous systemic increase in insulin are mediated by the endothelial nitric oxide pathway, by administering the nitric oxide synthase inhibitor N^G-monomethyl L-arginine (L-NMMA) using microdialysis.

Methods

Microdialysis catheters, perfused with L-NMMA and with a control solution, were inserted intracutaneously in 12 human subjects, who received an oral glucose load to induce a systemic hyperinsulinemia. During microdialysis the local blood flow was measured by urea clearance and by laser speckle contrast imaging (LSCI), and glucose metabolites were measured.

Results

After oral glucose intake, microvascular blood flow and glucose metabolism were both significantly suppressed in the L-NMMA catheter compared to the control catheter (urea clearance: $p < 0.006$, glucose dialysate concentration: $p < 0.035$). No significant effect of L-NMMA on microvascular blood flow was observed with LSCI ($p = 0.81$).

Conclusion

Local delivery of L-NMMA to the skin by microdialysis reduces microvascular blood flow and glucose delivery in the skin after oral glucose intake, presumably by decreasing local insulin-mediated vasodilation.

Keywords:

L-NMMA, Insulin, Metabolism, Microcirculation, Microdialysis

List of abbreviations

NO = nitric oxide

eNOS = endothelial nitric oxide synthase

L-NMMA = N^G-monomethyl L-arginine

HOMA = homeostatic model assessment

IR = insulin resistance

LSCI = laser speckle contrast imaging

OGTT = oral glucose tolerance test

ROI = region of interest

SEM = standard error of the mean

SD = standard deviation

PU = perfusion units

Introduction

The metabolic effects of insulin are well-studied. During the last decade, there has been an increasing interest in studying the actions of insulin on the microvasculature, and today, it is acknowledged that insulin has direct vasoactive properties [1-5]. The general view is that insulin has the ability to exert both vasoconstriction and vasodilation in various tissues [6]. The effects of insulin on the vessel tonus are believed to be mediated by the local metabolic state, local endothelial and neuronal function as well as external factors [7]. Under normal homeostatic conditions, the net effect of the vascular actions of insulin usually favors vasodilation [8]. Insulin induces vasodilation by upregulation of endothelial nitric oxide synthase (eNOS) and a subsequent increase in the production of nitric oxide (NO), which then exerts a vasodilatory effect [5, 8, 9]. Many studies have proven this by using N^G-monomethyl L-arginine (L-NMMA), a NO synthase inhibitor, to blunt the vasodilator effect of insulin [5, 10, 11].

Insulin has also been suggested to redirect blood flow from non-nutritive vessels to nutritive capillaries, through a process known as capillary recruitment [6, 7, 12], which increases the endothelial surface available for transportation of insulin, glucose and other nutrients to nearby cells. Impaired endothelium-dependent vascular recruitment reduces the available endothelial surface [13]. A dysfunction in the vasoactive and metabolic actions of insulin has been proposed to be an important factor in microvascular dysfunction and insulin resistance [6, 8, 13], and is also associated with obesity and hypertension [13-15].

We have previously found that the vasodilatory actions of insulin in the skin are dependent on nitric oxide (NO) [6]. We have also previously studied the microvascular response to insulin using the microdialysis technique, and we have observed an increase in cutaneous local blood

flow simultaneously with changes in local glucose concentrations [16]. In these previous studies, however, insulin was administered locally to the skin. Whether the vasoactive actions of endogenous insulin are dependent on the endothelial NO pathway, and how they affect local metabolism has however not been studied in the skin using microdialysis.

The aim of this study was therefore to investigate whether the effects on local blood flow and metabolic changes observed in the skin after an endogenous systemic increase in insulin are mediated by the endothelial NO pathway. We hypothesized that local administration of L-NMMA would decrease the local microvascular and metabolic response in the skin after an oral glucose load.

Methods

Subjects

Twelve subjects (six women) with a mean age of 24 ± 2 years (range 21-28), consecutively recruited through advertising on social media, were included in the study (Table 1). All subjects were healthy non-smokers and used no regular medication, except for oral contraceptives. On the day of the experiment the subjects arrived in the morning after an overnight fast and were only allowed to drink water during the experiments. All subjects gave their written consent before they participated.

The study was carried out according to the Helsinki declaration and was approved by the regional ethical committee of Linköping (application ID: DNR 2011/362-31 and DNR 2016/122-32).

Drugs

Two different perfusates were used to perfuse the microdialysis catheters. In one of the two catheters, Ringer's acetate with addition of 2.5 % albumin and 30 mmol/L urea (APL AB, Stockholm, Sweden) was used as a control solution. In the other catheter, L-NMMA (Bachem Distribution Services, Weil am Rhein, Germany), a NO synthase inhibitor, was added to the control solution to inhibit any nitric oxide mediated vasodilation caused by endogenously released insulin. The L-NMMA was prepared freshly on the day of the experiment by dissolving 2.8 mg L-NMMA in 1.5 ml control solution, resulting in a perfusate with a concentration of 1.87 mg/mL L-NMMA.

Study protocol

The test subjects maintained in a reclined position during the experiment. The volar skin of the non-dominant forearm was disinfected (Chlorhexidine 5 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) and then a 0.1 mL xylocaine injection (Xylocaine 20 mg/mL, AstraZeneca, Södertälje, Sweden) was given at two sites, at least three cm from each other. When the local anesthesia had taken effect, the skin was punctured with an 18-Gauge cannula (BD, Venflon™ Pro, Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden) which was then advanced along the skin surface at a depth of approximately one mm, placing the catheters intracutaneously. The steel mandarin was removed and the cannula cut off, leaving only the plastic cannula as a guide to insert the microdialysis catheters (CMA 71, M dialysis AB, Stockholm, Sweden). One catheter was inserted in each cannula. The catheters were positioned parallel to each other, at least three cm apart and at least 2.5 cm from the xylocaine injection site. Finally, the plastic cannula was removed and the insertion site was covered with a thin transparent adhesive film (Tegaderm, 3M Healthcare, St. Paul, MN, US). A microinjection pump (CMA 107, CMA AB, Solna, Sweden) was connected to each catheter and

perfused with the control solution, Ringer's acetate solution with addition of 2.5 % albumin and 30 mmol/L urea. To ensure adequate function of the catheters, all catheters were perfused for 90 minutes before they were inserted in the skin.

A timeline and an overview of the protocol is shown in Figure 1. After the catheters had been inserted, a 90-minute period was allowed to recover from the insertion trauma in the tissue [17, 18]. No samples were analyzed during this period. Then, a 60-minutes baseline period was observed. At the end of this baseline period, one of the perfusate syringes (106 Syringe, M dialysis AB, Stockholm, Sweden) was changed to a syringe with L-NMMA added to the control solution. Because the opening and closing of a pump initiates an automatic flush sequence, both pumps were opened and closed although the perfusate syringe was not changed for the control catheter. Because of the flush sequence, the microvial collecting the first 15 minutes after the syringe exchange was discarded for both catheters. After the addition of L-NMMA, dialysate samples were collected for 60 minutes. Thereafter, an oral glucose tolerance test (OGTT) was performed according to the WHO standard to induce an increase of endogenous insulin concentration. The test subjects ingested 75 g glucose (APL AB, Stockholm, Sweden) diluted in 2 dL water within 5 minutes. Samples were then collected during an additional four hours. During the whole experiment, skin temperature was measured on the surface of the volar part of the same forearm that the catheters were inserted in, using a thermometer equipped with a K Type thermocouple (TES 1300, TES Electrical Electronic Corporation, Taipei, Taiwan).

All microvials were labelled and weighed (CPA225D, Sartorius Weighing Technology GmbH, Goettingen, Germany) before and after sampling, to determine the volume of the recovered dialysate in each vial. Flow rate was set to 1.0 $\mu\text{L}/\text{min}$ and vials were swapped every 15

minutes. The lag time, which is the time it takes for the microdialysate to flow from the membrane to the microvial, was 5.1 minutes. To compensate for this, the oral glucose load was therefore ingested 5 minutes before the exchange of the last vial in the phase “addition of L-NMMA” and the first vial in the phase “post glucose load”. Hence, no compensation for lag time was needed during data analysis.

Analyses for the metabolic markers glucose, lactate and pyruvate, and the blood flow marker urea, were made directly after sampling using a microdialysis analyzer (CMA 600, CMA AB, Solna, Sweden). The microvials were then frozen to -20°C awaiting analysis of insulin. Within a few weeks, insulin was analyzed using an enzyme-linked immunosorbent assay (Ultrasensitive ELISA, Mercodia AB, Uppsala, Sweden). The dialysate of four microvials were pooled, giving hourly insulin concentrations, to obtain sufficient volumes for the insulin assay (25 µL).

Capillary glucose was collected eight times during the experiment; at baseline, when the syringe in the L-NMMA catheter was changed to perfusate containing L-NMMA, right before giving the glucose load and then every hour for 4 hours after the oral glucose load. Before collecting the samples, the skin of the fingertip was disinfected (Chlorhexidine 5 mg/ml, Fresenius Kabi AB, Uppsala Sweden) and a lancet with 1.8 mm puncture depth (Haemolance Plus, HaeMedic, Ozorków, Poland) was used to puncture the skin. Capillary samples were collected alternately from the index, middle and ring finger of the hand opposite the arm where the microdialysis catheters were inserted. Analysis of the capillary samples were performed directly using a handheld spectrophotometer (Accu Chek Inform II, Cobas, Switzerland), which was calibrated according to manufacturer’s recommendations.

Venous blood samples were collected during fasting and 2 hours after the ingestion of the oral glucose load from the arm opposite the arm where the microdialysis catheters were inserted. After disinfection (Chlorhexidine 5 mg/ml, Fresenius Kabi AB, Uppsala Sweden), the median cubital vein was punctured using a butterfly needle (Vacurette®, Greiner bio-one, Kremsmünster, Austria). Blood samples were analyzed directly by the clinical laboratory at Linköping University Hospital. Serum insulin was collected in serum gel tubes (VWR, Vacutest KIMA, Pieve di Sacco, Italy) and analyzed using electrochemiluminescence. Plasma glucose was collected in citrate/fluoride/EDTA tubes (Venosafe, Terumo, Västra Frölunda, Sweden) and analyzed using the hexokinase enzymatic method. The HOMA model ($\text{HOMA} = [\text{fasting insulin } (\mu\text{U/mL}) \times \text{fasting glucose (mmol/L)}] / 22.5$) was used to quantify insulin resistance and beta-cell function [19].

Skin perfusion was measured every 15 minutes using the microdialysis urea clearance technique and by laser speckle contrast imaging (LSCI, PeriCam PSI NR System, Perimed AB, Järfälla, Sweden). The microdialysis urea clearance has previously been described in detail [20, 21]. The rate of diffusion of added urea from the perfusate to the surrounding tissue is dependent on local blood flow. With increasing blood flow, urea diffuses at a higher rate because of an increased gradient between the perfusate in the catheter and the interstitium. LSCI is a camera-based imaging technique for measuring microvascular blood flow. The technique has previously been described in detail [22, 23]. A divergent laser beam with a wavelength of 785 nm is used to illuminate an area of the skin. A part of the laser light is scattered by the moving red blood cells of the microvasculature and is directed towards an image sensor. Skin perfusion is estimated based on the reduction of the local speckle contrast in the resulting image and is expressed as perfusion units (PU). For our laser speckle instrument, the speckle size is 7.6 μm , while the camera pixel size is 6.45 μm . Perfusion

images were acquired by averaging data from 42 images taken in rapid succession (acquisition time 2 seconds) with a measurement distance of 23 cm and a spatial resolution of 0.2 mm/pixel. Mean perfusion levels were then calculated in regions of interest (ROI) using PIMsoft 1.3 (Perimed AB, Järfälla, Sweden). The ROI size was set to correspond to the area surrounding each catheter membrane, 0.4 cm × 1.0 cm, and placed over the membrane of the L-NMMA catheter (ROI 1) and control catheter (ROI 2). The system was calibrated according to the manufacturer's recommendations.

Statistical analysis

Data in the text and tables are presented as means and SD, while the data in the figures are presented as means and SEM. Paired Student's t tests were used to compare blood pressure before and after the experiment and differences in volume recovery between the catheters. Change in skin temperature during the experiment was analyzed using one-way analysis of variance for repeated measures. Changes in glucose, lactate, pyruvate, urea and insulin in the dialysate, and skin perfusion measured using LSCI, (i) between the control and L-NMMA catheters and (ii) between baseline and different time points during the experiment, were analyzed using two-way analysis of variance (ANOVA) for repeated measures followed by multiple comparisons using Sidak's correction.

Due to technical issues with the microdialysis analyzer, the dialysate of three test subjects was pooled hourly for pyruvate and urea. Because of missing data, data from one subject for glucose and lactate, and from four subjects for pyruvate and urea had to be excluded from the two-way ANOVA for glucose and lactate. However, the graphs are based on data from all test subjects. When analyzing insulin concentration with ELISA, all analysis results under the detection limit were set to zero to enable statistical analysis.

GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, California, USA) was used for all statistical calculations. The alpha level for statistical significance was set to 0.05.

Results

All catheters were successfully inserted in the skin. An adequate volume recovery was obtained in all microvials ($0.98 \pm 0.02\%$), although the volume recovery in the L-NMMA catheter was slightly higher than in the control catheter (L-NMMA: $0.97 \pm 0.02\%$, control: $0.99 \pm 0.03\%$, $p=0.058$).

Quantifying beta cell function and insulin resistance using the HOMA model and by measuring insulin and glucose in blood samples showed that none of the test subjects were insulin resistant (Table 1). All test subjects were normotensive and blood pressure did not change between the beginning and the end of the experiment (systolic: $p=0.88$, diastolic: $p=0.64$). After acclimatization the skin temperature was $30.8 \pm 1.9^\circ\text{C}$. An increase in skin temperature was noted over the course of the experiment but there was no significant difference compared to baseline ($p=0.16$). Detailed numeric data can be found in Supplementary files.

Metabolic markers

The change in the concentration of glucose in the dialysate is presented in Figure 2. At baseline, there was no significant difference between the catheters (L-NMMA: 1.25 ± 0.26 mmol/L, control 1.30 ± 0.58 mmol/L, $p=0.98$). After the oral glucose intake, the concentration of interstitial glucose increased in both catheters with peak concentrations at 195 minutes into the experiment (L-NMMA: 1.97 ± 0.50 mmol/L, control 2.23 ± 0.77 mmol/L). In both catheters, the concentration was significantly increased from 150 minutes to 315 minutes into

the experiment compared to baseline ($p<0.0001$). From 15 minutes after the addition of L-NMMA in the perfusate (at 90 minutes) until 270 minutes into the experiment, a significantly higher increase in glucose concentration was observed in the control catheter compared to the L-NMMA catheter ($p<0.035$).

A increase in lactate and pyruvate was seen after the oral glucose intake in the dialysate of both catheters (Figure 3 and Figure 4, respectively). Compared to baseline (L-NMMA 0.44 ± 0.11 mmol/L, control 0.44 ± 0.17 mmol/L), lactate concentration was increased from 165 minutes into the experiment, and this increase lasted during the rest of the experiment (L-NMMA catheter: peak at 0.72 ± 0.12 at 240 minutes, $p<0.001$; control catheter: peak at 0.76 ± 0.31 at 225 minutes, $p<0.001$). The concentration of pyruvate was increased compared to baseline (L-NMMA 36.24 ± 7.62 $\mu\text{mol/L}$, control 31.41 ± 16.18 $\mu\text{mol/L}$), from 165 to 345 minutes into the experiment in the control catheter (peak at 60.33 ± 34.30 $\mu\text{mol/L}$ at 225 minutes, $p<0.001$), and from 210 to 300 minutes into the experiment in the L-NMMA catheter (peak at 56.08 ± 17.13 $\mu\text{mol/L}$ at 255 minutes, $p<0.043$). No difference in the concentration of pyruvate or lactate was observed between the L-NMMA catheter and the control catheter (pyruvate: $p>0.16$, lactate: $p>0.13$), except for the first 30 minutes after baseline (at 75-90 minutes into the experiment) where a significant decrease in lactate concentration was seen in the control catheter compared to the L-NMMA catheter ($p<0.02$).

Skin perfusion

Figure 5A presents the change in dialysate concentration of urea during the experiment. In the L-NMMA catheter, the urea concentration was stable during baseline (mean 18.86 ± 2.24 mmol/L). A slight decrease, indicating gradually increasing local blood flow in the surrounding tissue, was thereafter observed, but only with significantly decreased urea concentration

compared to baseline at 300 respectively 345 minutes into the experiment (17.21 ± 1.90 mmol/L $p < 0.02$ respectively 17.46 ± 2.65 mmol/L $p < 0.008$). At the end of the baseline period ($t = 75$), when the perfusate syringes were changed and the catheters automatically flushed, a sudden peak in urea concentration was observed in both the L-NMMA catheter and the control catheter. In the control catheter, the urea concentration decreased 30 minutes after glucose intake (at 165 minutes into the experiment) and remained significantly decreased compared to baseline for the rest of the experiment (< 0.005), indicating an increase in local blood flow. Compared to the L-NMMA catheter, the urea concentration in the control catheter was significantly lower from 165 to 285 minutes into the experiment ($p < 0.006$), indicating a higher local blood flow in the tissue surrounding the control catheter than around the L-NMMA catheter.

The change in perfusion in the skin was measured by LSCI every 15th minute, but in Figure 5B presented as mean from baseline, initially every 30th minute and after oral glucose intake every 60th minute. Baseline perfusion was stable in both the control catheter (45.06 ± 10.11 PU) and the L-NMMA catheter (41.94 ± 6.75 PU). After the glucose intake, an increase in perfusion was seen in the area surrounding the control catheter, whereas it remained stable during the rest of the experiment in the L-NMMA catheter, although the difference was not statistically significant ($p = 0.61$).

Insulin

An increase in dialysate insulin concentration after the oral glucose intake was observed in both catheters (Figure 6). Compared to baseline (L-NMMA: 0.072 ± 0.095 , control: 0.16 ± 0.26), insulin concentration was significantly increased in both catheters at 1 and 2 hours after OGTT (L-NMMA catheter: 0.68 ± 0.61 at 210 minutes, 1.02 ± 1.37 at 270 minutes, $p = 0.01$; control

catheter: 1.02 ± 1.37 at 210 minutes and 1.02 ± 1.97 at 270 minutes, $p < 0.0001$). The increase in insulin concentration in the dialysate was higher in the control catheter, compared to the catheter to which L-NMMA was added, however the limit for statistical significance was not reached ($p = 0.30$).

Discussion

In this study, we have shown that local L-NMMA administration reduces the blood flow and glucose delivery in the skin associated with oral glucose intake, presumably by decreasing a local insulin-mediated vasodilation. Conclusions on possible secondary effects with suppressed local glucose metabolism, can however not be drawn based on this study. The microvascular effects of insulin have been studied before with the microdialysis technique in other tissues, such as the brain [24], skeletal muscle and subcutaneous adipose tissue [25, 26]. The effects from endogenously produced insulin on skin metabolism after an oral glucose load have also been studied in the skin [16], in muscle and in adipose tissue [27]. There have, however, not been many studies on the interplay between local blood flow and metabolic effects in the skin, and to our best knowledge, this is the first time the nitric oxide dependence of the vasoactive actions of insulin, and its effects on local metabolism has been studied in the skin using the microdialysis technique.

Samuelsson et al [28] have previously studied the microcirculation in patients with severe burns in the intensive care unit, and found a discrepancy in the local metabolism in the skin compared to the systemic circulation, indicating that there might be a local trauma induced insulin resistance in the skin in this group of patients affecting skin survival and morbidity. The interest to monitor both microvascular changes and metabolic changes in the skin

therefore arises. Microdialysis is a suitable technique because of its ability to simultaneously deliver substances and measure changes in the concentration of metabolites [29] and blood flow [30, 31] in the very same tissue.

The physiological mechanisms that provide the foundation of this study are basic. The oral glucose load causes hyperglycemia and increase endogenous insulin production. It is generally believed that insulin, by endothelial NO production, causes vasodilation that increases the tissue blood flow. Insulin is thereby boosting its own delivery to target tissues [10, 32]. When the ability of insulin to increase tissue blood flow is impaired as a result of endothelial dysfunction, both its own delivery as well as the supply of glucose to the surrounding tissue is reduced [6, 33]. This is the case in various disease states, including hypertension [34] and obesity [2, 13, 32]. In our study, we inhibited endothelial function in healthy subjects locally in the skin by blocking vascular NO production using L-NMMA. We observed that, after oral glucose intake, more urea was cleared from the tissue surrounding the control catheter than the L-NMMA catheter. This indicates that the delivery of L-NMMA to the skin inhibits the local vascular effects of the endogenously released insulin. As a result, the expected increase in local tissue blood flow does not occur to the same extent, resulting in a reduced delivery of glucose and insulin to the tissue. Although we observed an increase in both glucose and insulin in the tissue surrounding the L-NMMA catheter, the dialysate concentration was significantly lower than for the control catheter. At the same time, a lower concentration of pyruvate was observed in the L-NMMA catheter compared to the control catheter. Although not statistically significant, this indicates reduced glucose metabolism. Altogether, these findings suggest that L-NMMA suppresses the insulin dependent increase in skin blood flow seen after oral glucose intake, which in turn may have impaired local glucose metabolism.

The relationship between the blood flow changes observed and altered metabolism however needs further attention, with similar experiments at larger sample size.

It is known that insertion of microdialysis catheters into the skin causes a local inflammatory response, lasting for 24 hours [35] and it is important to consider any trauma dependent increases in tissue blood flow in studies using microdialysis. This local inflammatory process affects the local blood flow. We have previously monitored microvascular blood flow changes with urea clearance and LSCI on control subjects who did not receive an oral glucose load, using otherwise the same experimental protocol used in the current study [16]. These control subjects presented a slight increase in urea clearance, indicating a trauma dependent increase in tissue blood flow surrounding the insertion area. However, the increase in tissue blood flow was significantly higher in the control catheter of the test subjects who received an oral glucose load compared to those who did not. This suggests that the increase in local tissue blood flow seen after oral glucose intake is insulin dependent and not only a result of tissue trauma. This is consistent with the results in the current study, where the level of urea clearance in the control catheter was similar to the levels of urea in our previous study. Furthermore, the level of urea clearance in the L-NMMA catheter in this study corresponded to the level observed in control subjects not receiving an oral glucose load in our previous study. These results taken together indicate that L-NMMA suppresses the insulin dependent, but not the trauma dependent, increase in tissue blood flow. Altogether, the similarity between these two studies indicates good reproducibility and reinforces our belief that the experimental model is adequate.

Measurements of local blood flow using LSCI showed a clear trend of reduced local blood flow in the tissue surrounding the L-NMMA catheter, however not statistically significant. There are

several possible explanations for the fact that LSCI did not show a significant difference in blood flow between the catheter sites. One explanation may be the difference in measurement volumes between the LSCI and urea clearance. Because the catheters are located near the subdermis, it is likely that the effects on urea clearance are related to changes in subdermal blood flow, whereas LSCI is sensitive to changes in perfusion in the superficial dermis. The second explanation is that our sample size was too small for the LSCI to detect the difference in perfusion. Although LSCI was not able to measure a significant difference in local blood flow, the two methods (LSCI and urea clearance) showed the same trend, i.e. a reduced vasodilatation surrounding the catheter to which L-NMMA was added.

We observed a tendency towards decreased blood flow in the tissue surrounding the L-NMMA catheter, already before giving the oral glucose load. This may be caused by an increase in vessel tone due to the inhibition of NOS, which in turn may restrict the delivery of other vasoactive agents, not necessarily related to the glucose-insulin pathway. For example, the closely related NOS inhibitor L-NAME (N^G -nitro-L-arginine methyl ester) is able to directly constrict myogenically active resistance arteries independent of the endothelium [36]. These effects have however not been validated in the skin nor for the inhibitor used in this experiment.

There are other possible indirect mechanisms in which the presence of L-NMMA affects the metabolism, including vascular permeability and the effects of NO on mitochondrial metabolism. These effects are not taken into account in this study. Also, hypothetically urea clearance is affected by microvascular permeability, which in turn may be affected by NOS. Urea is regarded as a very stable compound that is often used as reference in microdialysis

studies [20, 21]. We are not aware of any studies that indicate that there is an interference between NOS and urea permeability, but hypothetically we cannot exclude this interference.

In this study, the microvascular and metabolic responses after an oral glucose load have been investigated in healthy test subjects, with no underlying diseases. The response in glucose metabolism and blood flow changes are probably different in the presence of insulin resistance, for example in type 2 diabetics. A decrease in glucose, on the basis of decreased insulin sensitivity, can be expected. To inhibit NOS production in such a patient group would be an interesting approach to a future study.

Limitations

This study has several limitations. First, the microdialysis catheters are inserted manually in the skin, aiming for a final membrane position at a depth as superficial as possible but at a maximum depth of 1 mm. The depth is not verified in these experiments, however, our group has previously measured the depth of microdialysis catheters inserted with the same insertion technique using ultrasound device [37]. Our previous data indicate that the catheters in this model are reliably placed intradermally with a mean depth of 0.78 ± 0.23 mm. Second, insulin is a large molecule that is known to be adsorbed by different materials, such as glass and polyacrylamide (i.e. the microdialysis membrane) [38]. This results in low recovery of insulin from the interstitial compartment to the microdialysate and might thereby not reflect the true insulin concentration in the skin. In future studies we may look into including other markers related to insulin, such as C-peptide. Finally, we know the dose of L-NMMA added to the perfusate but we do not know the quantity of the delivered L-NMMA to the skin. An improvement for future studies could be to measure the concentration of L-NMMA in the dialysate to calculate the dosage of L-NMMA delivered to the target tissue.

Perspectives

This paper shows how a local delivery of L-NMMA by microdialysis suppresses the local blood flow in the skin after an oral glucose load. These results further increase the understanding of how insulin affects the healthy microvascular physiology as well as pathophysiology processes in the skin in states when the endothelial function is impaired. This experimental model paves the way for future studies on the effects of insulin on local blood flow, but also inhibition of other specific pathways in the microvasculature in the skin. Also, this model opens for tissue specific studies on skin morbidity in, for example, the severely sick patient where insulin resistance may affect outcome.

Conclusion

We conclude that local delivery of L-NMMA to the skin by microdialysis reduces the blood flow and glucose delivery in the skin associated with oral glucose intake, presumably by decreasing a local insulin-mediated vasodilation. Conclusions on possible secondary effects with suppressed local glucose metabolism, can however not be drawn based on this study.

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Figure legends

Figure 1. Experimental protocol and setup. C-glucose = capillary glucose. P-glucose = plasma glucose. S-insulin = serum insulin. LSCI ROI = Laser Speckle Contrast Imaging, Region of Interest.

Figure 2. Changes in dialysate concentration of glucose in the skin during continuous intradermal delivery of L-NMMA (black markers) or a control substance (open circles), and after oral intake of 75 g glucose load in healthy subjects (N=12).

* indicates a significant difference between the L-NMMA catheter and the control catheter.

Figure 3. Changes in dialysate concentration of lactate in the skin during continuous intradermal delivery of L-NMMA (black markers) or a control substance (open circles), and after oral intake of 75 g glucose load in healthy subjects (N=12).

* indicates a significant difference between the L-NMMA catheter and the control catheter.

Figure 4. Changes in dialysate concentration of pyruvate in the skin during continuous intradermal delivery of L-NMMA (black markers) or a control substance (open circles), and after oral intake of 75 g glucose load in healthy subjects (N=12).

Figure 5. Changes in skin perfusion during continuous intradermal delivery of L-NMMA (black markers) or a control substance (open circles), and after oral intake of 75 g glucose load in healthy subjects (N=12). A) Intradermal blood flow around the catheters measured using the urea clearance technique. B) Skin perfusion around the microdialysis catheters measured using laser speckle contrast imaging. PU = perfusion units.

* indicates a significant difference between the L-NMMA catheter and the control catheter.

Figure 6. Changes in dialysate concentration of insulin in the skin during continuous intradermal delivery of L-NMMA (black markers) or a control substance (open circles), and after oral intake of 75 g glucose load in healthy subjects (N=12).

Table legends

Table 1. Demographics of the subjects enrolled in the study. Data presented in mean (SD).

A

B

Table 1

Table 1. Demographics of the subjects enrolled in the study. Data presented in mean (SD).

	Experimental Subjects
N	12
Sex (female/male)	6/6
Age (years)	23.6 (2.0)
BMI (kg/m²)	23.3 (3.5)
Blood pressure (mmHg)	
- Before experiment	110/70 (10/7)
- After experiment	109/68 (11/9)
Serum insulin (mU/L)	
- Fasting	6.0 (3.2)
- 2 hours after oral glucose	39.5 (33.7)
Capillary glucose (mmol/L)	
- Fasting	4.6 (0.3)
- 30 min after oral glucose	7.2 (1.1)
- 1 hours after oral glucose	7.5 (1.4)
- 2 hours after oral glucose	6.4 (0.8)
- 3 hours after oral glucose	4.9 (1.0)
- 4 hours after oral glucose	4.1 (0.4)

HOMA IR	1.3 (0.8)
Skin temperature (°C)	
- Fasting	30.8 (1.9)
- 30 min after oral glucose	30.7 (1.4)
- 1 hours after oral glucose	31.0 (1.2)
- 2 hours after oral glucose	31.6 (1.4)
- 3 hours after oral glucose	32.0 (1.4)
- 4 hours after oral glucose	31.7 (1.4)

Demographic data

Study ID	1	2	3	4	5	6	7	8	9	10	11	12
Date	160405	160406	160407	160408	160411	160616	160619	160704	161103	161104	161106	161108
Age (y)	22	21	23	23	24	24	22	23	22	26	25	28
Length (m)	1,86	1,85	1,87	1,69	1,68	1,65	1,64	1,9	1,66	1,84	1,9	1,6
Weight (kg)	66	90	74	90	75	58	55	78	64	81	85	53
BMI	19,1	26,3	21,2	31,5	26,6	21,3	20,4	21,6	23,2	23,9	23,5	20,7
Fitzpatrick scale	2	4	2	2	2	2	4	2	2	2	2	2
Smoking	No	No	No	No	No	No	No	No	No	No	No	No
Physical activity	No	No	No	No	No	No	No	No	No	No	No	No
Fasting status	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Coffee/Tea	No	No	No	No	No	No	No	No	No	No	No	No
Alcohol	No	No	No	No	No	No	No	No	No	No	No	No
Medications	None	None	None	None	None	None	None	None	None	None	None	Oral contra- ceptives
Skin disease	No	No	No	No	No	No	No	No	No	No	No	No
Blood pressure T=0 (mmHg)	111/66	118/69	100/73	114/78	110/69	113/83	110/70	105/61	88/58	105/64	126/68	116/76
Blood pressure T=360 (mmHg)	105/56	129/71	109/78	x	109/77	106/71	103/57	106/56	88/56	123/76	116/76	103/69

Volume recovery		L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	Control	Control
Vial	Time (min)	1	2	3	4	5	6	7	8	9	10	11	12	1	2
A1	0	94%	95%	87%	100%	94%	94%	98%	91%	101%	98%	98%	97%	94%	93%
A2	15	96%	100%	88%	99%	101%	90%	99%	93%	98%	94%	96%	94%	96%	93%
A3	30	94%	95%	84%	99%	98%	92%	97%	95%	95%	97%	96%	90%	99%	95%
A4	45	93%	96%	81%	96%	96%	94%	97%	93%	99%	96%	99%	99%	99%	95%
A5	75	87%	100%	90%	90%	76%	98%	92%	100%	93%	104%	99%	102%	114%	107%
A6	90	97%	97%	100%	96%	90%	96%	91%	100%	91%	99%	98%	88%	101%	102%
A7	105	93%	96%	98%	87%	90%	98%	94%	93%	98%	97%	97%	96%	100%	95%
A8	120	94%	101%	98%	101%	92%	92%	97%	94%	97%	94%	92%	93%	95%	94%
A9	135	94%	89%	96%	93%	90%	97%	96%	96%	97%	98%	98%	95%	96%	99%
A10	150	103%	95%	99%	96%	99%	98%	95%	95%	101%	107%	95%	96%	102%	95%
A11	165	102%	100%	97%	104%	103%	99%	97%	88%	99%	100%	102%	98%	96%	96%
A12	180	95%	86%	98%	101%	102%	91%	100%	95%	101%	92%	89%	94%	100%	92%
A13	195	99%	95%	98%	99%	100%	107%	97%	100%	97%	100%	99%	97%	95%	98%
A14	210	110%	101%	98%	97%	104%	102%	100%	93%	102%	100%	102%	97%	101%	108%
A15	225	101%	103%	94%	100%	100%	90%	100%	95%	105%	95%	91%	93%	99%	99%
A16	240	92%	102%	100%	102%	94%	98%	101%	101%	97%	98%	93%	102%	96%	105%
A17	255	98%	91%	106%	104%	102%	95%	98%	103%	102%	101%	x	94%	99%	90%
A18	270	103%	102%	99%	101%	100%	95%	99%	85%	101%	101%	102%	100%	96%	97%
A19	285	98%	96%	94%	101%	103%	96%	102%	92%	105%	93%	94%	97%	100%	101%
A20	300	104%	97%	100%	99%	100%	101%	100%	95%	93%	106%	98%	98%	99%	98%
A21	315	101%	97%	97%	102%	98%	95%	100%	96%	102%	105%	103%	101%	98%	100%
A22	330	99%	99%	100%	105%	99%	96%	102%	92%	105%	98%	97%	104%	95%	98%
A23	345	97%	98%	98%	100%	98%	98%	102%	94%	97%	97%	93%	99%	100%	98%
A24	360	97%	97%	99%	101%	101%	95%	99%	97%	98%	101%	102%	114%	98%	99%

Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
3	4	5	6	7	8	9	10	11	12
92%	98%	92%	95%	99%	90%	102%	84%	98%	98%
94%	99%	98%	87%	97%	93%	97%	83%	98%	100%
97%	101%	95%	97%	93%	99%	97%	91%	98%	94%
102%	102%	95%	92%	98%	89%	103%	92%	99%	102%
115%	108%	108%	97%	140%	105%	112%	104%	103%	115%
102%	108%	96%	102%	158%	99%	100%	98%	91%	108%
98%	102%	103%	106%	99%	97%	109%	105%	95%	111%
102%	101%	100%	99%	96%	98%	98%	97%	96%	104%
93%	97%	98%	92%	102%	97%	97%	100%	94%	102%
95%	99%	100%	91%	99%	96%	101%	104%	100%	105%
92%	102%	104%	92%	95%	96%	101%	102%	98%	106%
95%	98%	98%	93%	100%	98%	103%	94%	95%	99%
95%	101%	93%	108%	102%	95%	98%	97%	95%	100%
96%	100%	97%	101%	100%	95%	103%	99%	101%	106%
92%	94%	98%	94%	94%	95%	101%	97%	98%	98%
94%	102%	97%	99%	100%	101%	99%	100%	97%	107%
106%	105%	96%	96%	101%	96%	103%	97%	x	102%
92%	98%	100%	93%	97%	94%	102%	104%	99%	107%
98%	99%	100%	107%	97%	96%	103%	102%	99%	100%
95%	99%	97%	101%	102%	95%	97%	100%	98%	103%
103%	102%	94%	90%	101%	96%	102%	106%	101%	107%
98%	104%	93%	93%	97%	96%	102%	95%	97%	105%
96%	100%	101%	95%	99%	98%	98%	95%	97%	101%
100%	97%	93%	94%	100%	96%	99%	94%	96%	110%

Glucose (mmol/L)		L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	Control	Control
Vial	Time (min)	1	2	3	4	5	6	7	8	9	10	11	12	1	2
A1	0	1,465	0,922	1,073	1,331	1,21	0,893	x	0,994	1,591	1,598	1,235	1,483	1,43	1,148
A2	15	1,489	0,935	0,934	1,282	1,232	0,958	x	1,031	1,554	1,515	1,285	1,215	1,654	1,077
A3	30	1,614	0,918	0,903	1,392	1,248	0,894	x	1,085	1,545	1,55	1,296	1,286	1,614	1,092
A4	45	1,627	0,951	0,926	1,437	1,236	0,874	x	1,054	1,487	1,612	1,312	1,447	1,578	1,07
A5	75	1,634	0,848	0,882	1,399	1,522	0,758	x	0,96	1,337	1,38	1,163	1,194	1,484	0,902
A6	90	1,423	0,889	0,8	1,398	1,382	0,775	x	0,936	1,506	1,573	1,213	1,131	1,474	0,906
A7	105	1,439	0,922	0,849	1,36	1,271	0,772	x	0,999	1,467	1,482	1,198	1,11	1,709	1,057
A8	120	1,426	0,915	0,834	1,332	1,269	0,864	1,562	1,005	1,477	1,596	1,215	1,098	1,671	1,063
A9	135	1,439	0,94	0,87	1,418	1,31	0,866	1,513	0,939	1,465	1,643	1,283	1,267	1,833	1,04
A10	150	1,687	1,086	1,143	1,556	1,571	0,963	1,615	1,284	1,666	1,736	1,52	1,466	1,862	1,292
A11	165	2,033	1,164	1,297	1,967	1,757	1,172	1,674	1,656	2,081	2,257	1,629	1,512	2,107	1,575
A12	180	2,506	1,312	1,263	2,22	1,9	1,395	1,828	1,889	2,408	2,561	1,7	1,39	2,923	1,749
A13	195	2,744	1,285	1,18	2,247	2,063	1,751	1,774	1,91	2,616	2,457	2,065	1,489	3,317	1,737
A14	210	2,719	1,308	1,305	2,26	2,098	1,459	1,668	1,977	2,575	2,264	1,895	1,602	3,446	1,693
A15	225	2,664	1,307	1,49	2,33	2,047	1,562	1,722	1,957	2,44	2,412	1,984	1,479	3,139	1,778
A16	240	2,63	1,426	1,483	2,409	2,098	1,736	1,779	1,532	2,325	2,409	2,056	1,632	2,733	1,802
A17	255	2,209	1,509	1,255	2,331	1,888	1,901	1,846	1,353	2,314	2,455	2,03	1,555	2,531	1,766
A18	270	1,927	1,351	1,36	2,037	1,815	1,688	1,739	1,223	2,391	2,245	1,74	1,4	2,24	1,631
A19	285	1,91	1,213	1,335	1,88	1,964	1,537	1,64	1,464	2,381	2,255	1,865	1,455	2,175	1,403
A20	300	1,849	1,151	1,248	1,759	2,051	1,568	1,636	1,638	2,439	1,945	1,646	1,047	2,07	1,243
A21	315	1,678	0,912	1,04	1,641	1,913	1,526	1,699	1,582	2,48	1,724	1,473	1,272	1,771	1,005
A22	330	1,451	0,909	0,974	1,511	1,692	1,491	1,583	1,395	2,368	1,561	1,441	0,783	1,553	0,973
A23	345	1,182	0,847	1,045	1,442	1,715	1,528	1,462	1,166	2,147	1,549	1,433	0,874	1,288	0,893
A24	360	1,065	0,837	1,021	1,353	1,642	1,437	1,442	1,06	1,654	1,499	1,464	0,781	1,146	0,936

Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
3	4	5	6	7	8	9	10	11	12
1,247	1,206	0,925	0,847	0,97	1,323	1,226	1,492	0,635	3,156
1,341	1,211	0,944	0,863	0,976	1,268	1,151	1,603	0,605	2,675
1,263	1,244	0,911	0,818	1,043	1,495	1,144	1,604	0,592	2,782
1,314	1,261	0,965	0,799	1,013	1,41	1,092	1,727	0,562	2,968
1,126	1,194	0,912	0,761	0,652	1,184	1,024	1,353	0,637	3,24
1,349	1,378	0,966	0,849	0,932	1,171	1,237	1,603	0,76	3,345
1,385	1,349	0,964	0,814	1,374	1,15	1,239	1,515	0,744	3,02
1,375	1,318	1,014	0,904	1,344	1,21	1,418	1,522	0,77	2,895
1,461	1,387	1,114	0,927	1,308	1,267	1,576	1,531	0,816	3,353
1,764	1,703	1,376	1,196	1,597	1,763	1,85	1,736	0,933	3,698
2,082	1,984	1,524	1,501	1,804	2,384	2,352	2,059	1,023	3,825
1,901	2,207	1,614	1,789	1,938	2,586	2,755	2,38	1,153	3,464
1,792	2,227	1,762	1,775	1,578	2,375	2,915	2,141	1,313	3,854
2,046	2,28	1,794	1,754	1,584	2,555	2,861	2,059	1,314	3,183
2,093	2,251	1,748	1,836	1,606	2,011	2,723	2,423	1,256	3,691
2,204	2,32	1,742	1,851	1,797	1,956	2,375	2,264	1,232	3,495
1,964	2,157	1,506	1,991	1,536	1,464	2,147	2,57	1,197	3,757
2,107	1,883	1,449	1,856	1,362	1,473	2,205	2,432	1,158	3,528
1,767	1,616	1,5	1,744	1,36	1,669	2,172	2,102	1,011	3,384
1,8	1,539	1,667	1,671	1,27	1,739	2,206	1,973	0,931	3,226
1,485	1,272	1,565	1,693	1,191	1,523	2,218	1,756	0,782	2,831
1,384	1,16	1,423	1,636	1,162	1,316	2,049	1,587	0,759	1,758
1,495	1,206	1,445	1,616	1,038	1,147	1,881	1,418	0,798	1,488
1,537	1,079	1,376	1,491	0,886	1,087	1,333	1,456	0,85	1,61

Lactate (mmol/L)		L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	Control	Control
Vial	Time (min)	1	2	3	4	5	6	7	8	9	10	11	12	1	2
A1	0	0,467	0,742	0,498	0,392	0,379	0,28	x	0,388	0,251	0,488	0,364	0,439	0,419	0,757
A2	15	0,537	0,508	0,56	0,405	0,357	0,247	x	0,395	0,295	0,465	0,367	0,405	0,454	0,772
A3	30	0,503	0,621	0,63	0,443	0,423	0,358	x	0,437	0,308	0,495	0,367	0,441	0,472	0,711
A4	45	0,526	0,654	0,677	0,461	0,397	0,317	x	0,413	0,287	0,518	0,398	0,459	0,423	0,722
A5	75	0,566	0,527	0,645	0,549	0,563	0,312	x	0,414	0,29	0,537	0,365	0,432	0,326	0,523
A6	90	0,622	0,578	0,666	0,489	0,543	0,337	x	0,445	0,412	0,611	0,468	0,509	0,391	0,592
A7	105	0,65	0,569	0,676	0,446	0,5	0,29	x	0,462	0,311	0,612	0,434	0,543	0,409	0,544
A8	120	0,613	0,579	0,678	0,494	0,518	0,35	0,515	0,428	0,258	0,686	0,422	0,5	0,422	0,566
A9	135	0,625	0,598	0,712	0,534	0,504	0,389	0,573	0,442	0,247	0,73	0,48	0,616	0,417	0,566
A10	150	0,643	0,659	0,658	0,528	0,492	0,276	0,624	0,464	0,251	0,675	0,427	0,634	0,374	0,598
A11	165	0,585	0,624	0,663	0,602	0,558	0,455	0,704	0,548	0,282	0,682	0,477	0,784	0,372	0,556
A12	180	0,609	0,669	0,747	0,677	0,495	0,459	0,847	0,558	0,378	0,633	0,494	0,805	0,493	0,637
A13	195	0,712	0,644	0,667	0,671	0,548	0,641	0,758	0,584	0,503	0,604	0,591	0,922	0,567	0,515
A14	210	0,752	0,674	0,696	0,723	0,563	0,587	0,755	0,621	0,499	0,512	0,545	0,934	0,687	0,626
A15	225	0,814	0,592	0,79	0,735	0,616	0,677	0,74	0,672	0,613	0,584	0,587	0,798	0,697	0,618
A16	240	0,929	0,623	0,77	0,778	0,643	0,789	0,799	0,608	0,613	0,565	0,666	0,891	0,785	0,633
A17	255	0,9	0,627	0,717	0,719	0,606	0,772	0,813	0,613	0,635	0,614	0,664	0,904	0,94	0,639
A18	270	0,835	0,655	0,69	0,686	0,531	0,734	0,774	0,629	0,597	0,511	0,61	0,743	0,909	0,679
A19	285	0,806	0,623	0,838	0,649	0,526	0,755	0,679	0,665	0,589	0,635	0,7	0,732	0,848	0,541
A20	300	0,794	0,609	0,828	0,599	0,511	0,749	0,661	0,582	0,602	0,649	0,662	0,838	0,757	0,561
A21	315	0,75	0,534	0,682	0,572	0,491	0,713	0,625	0,562	0,556	0,647	0,64	0,793	0,722	0,51
A22	330	0,678	0,56	0,725	0,567	0,456	0,614	0,559	0,539	0,563	0,679	0,604	0,606	0,608	0,552
A23	345	0,621	0,552	0,689	0,623	0,482	0,56	0,517	0,512	0,551	0,65	0,585	0,618	0,538	0,557
A24	360	0,612	0,593	0,678	0,565	0,459	0,527	0,499	0,487	0,493	0,488	0,625	0,406	0,517	0,581

Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
3	4	5	6	7	8	9	10	11	12
0,501	0,395	0,313	0,286	0,647	0,271	0,278	0,22	0,294	0,584
0,54	0,46	0,314	0,324	0,689	0,351	0,278	0,196	0,319	0,539
0,533	0,46	0,317	0,282	0,737	0,362	0,307	0,181	0,395	0,56
0,537	0,461	0,321	0,283	0,734	0,537	0,293	0,307	0,356	0,612
0,413	0,433	0,285	0,325	0,351	0,334	0,24	0,176	0,335	0,722
0,513	0,443	0,302	0,334	0,403	0,472	0,333	0,199	0,409	0,67
0,5	0,431	0,399	0,333	0,71	0,504	0,349	0,212	0,411	0,651
0,532	0,452	0,323	0,355	0,748	0,66	0,333	0,191	0,437	0,699
0,595	0,511	0,375	0,377	0,719	0,623	0,307	0,185	0,423	0,672
0,588	0,522	0,345	0,373	0,725	0,71	0,34	0,192	0,444	0,816
0,614	0,566	0,367	0,47	0,797	0,663	0,363	0,187	0,455	1,354
0,665	0,642	0,451	0,545	1,051	0,725	0,519	0,253	0,499	1,308
0,707	0,707	0,47	0,668	0,842	0,813	0,651	0,178	0,59	1,606
0,754	0,78	0,543	0,646	0,92	1,012	0,737	0,178	0,612	1,317
0,795	0,806	0,543	0,725	0,902	0,875	0,823	0,205	0,606	1,527
0,778	0,79	0,555	0,722	0,826	0,815	0,785	0,206	0,699	1,24
0,695	0,794	0,583	0,71	0,844	0,774	0,732	0,23	0,693	1,454
0,515	0,839	0,526	0,669	0,857	0,854	0,767	0,25	0,709	1,279
0,685	0,804	0,461	0,637	0,792	0,713	0,72	0,272	0,753	1,33
0,75	0,788	0,496	0,594	0,709	0,559	0,76	0,264	0,727	1,06
0,593	0,72	0,425	0,598	0,659	0,533	0,731	0,25	0,758	1,004
0,614	0,662	0,421	0,534	0,641	0,546	0,678	0,272	0,721	0,761
0,558	0,706	0,387	0,476	0,558	0,527	0,707	0,277	0,707	0,684
0,568	0,678	0,362	0,472	0,543	0,491	0,705	0,293	0,71	0,777

Pyruvate (μmol/L)		L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	Control	Control
Vial	Time (min)	1	2	3	4	5	6	7	8	9	10	11	12	1	2
A1	0	37,4	29,3	x	28,4	29,3	28,9	x	32,8	38,8	48,1	32,9	53,4	32,3	33,9
A2	15	37,4	29,3	x	27,9	25,7	32,8	x	36,6	37,8	46,9	37,1	46,4	32,3	33,9
A3	30	37,4	29,3	x	31	28,1	30,6	x	39,4	38,2	48	38,5	46,9	32,3	33,9
A4	45	37,4	29,3	x	30,9	27,4	27,9	x	36,1	36,4	47,9	39,5	52	32,3	33,9
A5	75	44,8	24,9	41	29,3	27,5	24,5	x	32,3	33,4	45,5	35	14,5	35,6	26,4
A6	90	44,8	24,9	41	30,6	22,5	28,8	x	35,1	39,5	51,4	40,5	10,7	35,6	26,4
A7	105	44,8	24,9	41	25,7	23,9	28	x	36,9	36,5	48,3	39,5	9,6	35,6	26,4
A8	120	44,8	24,9	41	27,7	26	32,7	45,1	38,9	35,8	51,3	40,3	7,3	35,6	26,4
A9	135	48,2	28,3	45,5	30,7	29,9	33,1	49	35	35,2	51,9	40,1	7,7	39,1	29,1
A10	150	48,2	28,3	45,5	19,8	25,2	31	48,4	38,2	33,7	49,5	39,6	10,7	39,1	29,1
A11	165	48,2	28,3	45,5	36,6	28,9	36	51,4	45,7	41,9	49,4	39,7	14,3	39,1	29,1
A12	180	48,2	28,3	45,5	46,4	35,1	46,3	53,5	55,1	50,3	51,9	43,7	18,7	39,1	29,1
A13	195	72,1	x	61,1	55,9	43,5	53,2	50,9	55,4	57,7	48,9	54,2	19,3	68,3	40,8
A14	210	72,1	x	61,1	62,2	50,1	49,6	52,5	58,1	61,5	48,2	52,6	20,6	68,3	40,8
A15	225	72,1	x	61,1	59,6	47,8	55,4	51,5	63,8	64	50,5	59,5	16,5	68,3	40,8
A16	240	72,1	x	61,1	65,3	50,1	62,9	53,9	50	63,1	53,5	64,9	18	68,3	40,8
A17	255	83,5	x	55,7	61,8	48,2	55,4	52,5	54,4	67,2	57,1	67,6	13,5	83,1	35,4
A18	270	83,5	x	55,7	59,8	43,6	54,5	49,3	54,1	64,7	54,2	63,2	12,2	83,1	35,4
A19	285	83,5	x	55,7	58,3	42,3	50,5	46,3	55,2	63,4	59,4	68,3	12	83,1	35,4
A20	300	83,5	x	55,7	51,4	42,7	48,5	43,8	50,3	63,1	59,3	65,7	11,2	83,1	35,4
A21	315	69,7	27,8	x	46,3	41,5	48,9	47,2	46,8	62	60,8	62,4	11,3	72,8	31
A22	330	69,7	27,8	x	48,2	40,7	48,1	40,7	47,3	62,6	60,8	58,7	9,3	72,8	31
A23	345	69,7	27,8	40,8	49,5	35,6	49	37,8	45,3	59,8	60,3	57,3	9,3	72,8	31
A24	360	69,7	27,8	44,4	48	31,7	45,2	36,7	42,5	54,9	54,9	57,6	6,8	72,8	31

Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
3	4	5	6	7	8	9	10	11	12
38,5	24,4	20,9	11,7	59,5	20,3	35,9	7,9	23,3	64,1
38,5	27	21,6	10,1	61,2	22,3	36,3	7,5	24,9	54,5
38,5	23,1	20,4	x	64,1	30,5	37,1	7,6	26,5	54,9
38,5	22,4	21,3	x	61,7	35,9	33,7	9,4	26,2	58,9
41,8	19,1	18,4	x	32,6	26,7	29,1	6,9	26,6	64,7
41,8	23,8	18,3	10,8	41,5	31,8	34,6	10	32,4	66,3
41,8	26,1	19,1	x	67,7	29,7	35,9	9,9	31,3	61,8
41,8	27,1	21,1	10,6	67	39,1	37,6	8,6	31,5	64,4
48,7	26,3	19,1	10,5	62,3	40,8	37,8	10	32,2	62,9
48,7	25,3	21,2	12,9	63,1	53	35,4	10,5	31,2	90,5
48,7	31,6	23,9	20,3	66,9	53,4	38,8	11,1	32,9	148,8
48,7	42,1	28,4	25,2	76	58,5	52,5	16	37,3	168,6
62,9	51,4	37,6	26,4	64,8	64,5	64	12,5	42,3	172,2
62,9	57,6	41,6	25,9	68,2	72,4	66,9	13,9	46,7	139,8
62,9	59,4	42,5	25,4	64,2	68,4	68,7	19,6	48,6	155,2
62,9	59,3	41,9	27,5	58,4	64	67,2	21,6	48,3	133,4
49,9	61,7	40,6	28,4	64,4	55,7	64,1	28,4	47,3	136
49,9	58,5	39,4	28,4	56,8	65,1	61,3	31,4	50,2	115,7
49,9	57,9	30,3	26,7	54,9	51,5	61,3	29,8	50,5	119,7
49,9	47,7	31,5	28	45,6	42,1	60,8	29,5	48,4	105,1
50,8	44,6	33,6	26	41,4	39,6	57,9	32,8	44,1	102,4
50,8	38,5	30,1	25,8	40,9	39,5	55,8	27,3	41,2	82
47,6	38,7	27,9	23,1	36	37,4	58,5	21,7	42,4	74
50,1	36,9	25,6	22,7	33,3	36,5	52,4	20,4	43	65,5

Urea (mmol/L)		L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	L-NMMA	Control	Control
Vial	Time (min)	1	2	3	4	5	6	7	8	9	10	11	12	1	2
A1	0	17,48	23,45	x	18,48	21,37	20,39	x	18,06	15,91	16,68	20,92	17,42	17,54	21,53
A2	15	17,48	23,45	x	18,45	20,83	20,7	x	18,85	15,84	16,66	20,13	17,92	17,54	21,53
A3	30	17,48	23,45	x	18,62	20,45	19,61	x	16,96	16,24	16,58	19,84	18	17,54	21,53
A4	45	17,48	23,45	x	18,04	20,3	19,88	x	17,41	16,51	16,35	19,74	17,48	17,54	21,53
A5	75	18,64	23,56	21,79	18,02	19,4	21,52	x	17,88	18,15	17,85	20,78	19,38	17,37	22,51
A6	90	18,64	23,56	21,79	17,37	20,25	21,17	x	17,65	16,42	16,87	20,15	19,11	17,37	22,51
A7	105	18,64	23,56	21,79	17,47	20,19	21,01	x	17,66	15,96	17,07	20,42	19,58	17,37	22,51
A8	120	18,64	23,56	21,79	16,98	20,48	19,66	15,46	17,1	16,18	15,88	19,77	19,54	17,37	22,51
A9	135	19,06	23,24	21,79	17,56	19,34	20,36	15,88	18,2	16,16	16,07	19,93	19,54	16,19	21,86
A10	150	19,06	23,24	21,79	18,59	19,67	20,53	17,45	17,48	15,97	17,19	19,17	15,67	16,19	21,86
A11	165	19,06	23,24	21,79	17,81	20,37	20,76	17,06	17,02	15,11	16,31	19,37	18,72	16,19	21,86
A12	180	19,06	23,24	21,79	16,9	20,49	20,19	17,42	17,21	15,22	16,34	19,46	18,75	16,19	21,86
A13	195	20	x	22,66	17,63	19,66	18,53	17,37	18,07	16,24	16,93	19,01	18,11	18,13	22,79
A14	210	20	x	22,66	16,75	19,33	20,13	17,13	17,53	16,6	16,71	19,77	18,26	18,13	22,79
A15	225	20	x	22,66	16,08	19,51	18,92	16,81	16,52	17	16,34	19,22	19,22	18,13	22,79
A16	240	20	x	22,66	16,05	18,9	18,65	17,16	19,47	16,76	16,21	17,68	18,01	18,13	22,79
A17	255	19,21	x	21,23	16,76	18,47	18,44	16,33	18,95	16,12	16,11	17,44	18,67	18,03	21,99
A18	270	19,21	x	21,23	17,02	18,69	18,52	16,42	18,66	16,31	16,41	18,46	18,87	18,03	21,99
A19	285	19,21	x	21,23	16,74	18,82	17,78	16,18	16,17	15,87	15,83	17,68	18,8	18,03	21,99
A20	300	19,21	x	21,23	15,85	17,68	17,34	16,01	16,17	14,83	15,26	17,17	18,51	18,03	21,99
A21	315	19,49	24,52	x	15,56	18,53	17,66	16,04	17,04	14,84	15,59	17,14	18,23	19,74	21,74
A22	330	19,49	24,52	x	15,76	17,75	17,49	15,41	16,11	14,97	16,18	16,69	19,45	19,74	21,74
A23	345	19,49	24,52	18,29	15,87	17,73	17,24	15,36	15,71	14,38	15,92	17	18,04	19,74	21,74
A24	360	19,49	24,52	17,9	16,57	17,69	16,51	15,57	16,21	15,09	14,9	16,76	20,14	19,74	21,74

Control	Control	Control	Control	Control	Control	Control	Control	Control	Control
3	4	5	6	7	8	9	10	11	12
19,29	20,09	22,43	22,16	19,1	17,09	17,92	20,11	24,22	9,62
19,29	18,9	22,41	21,97	19,22	17,35	18,14	18,85	x	11,11
19,29	19,09	22,47	21,62	18,5	15,89	18,38	18,39	24,48	10,92
19,29	19,41	22,08	21,71	18,36	16,43	18,82	18,22	23,88	9,99
22,27	19,81	22,89	21,81	23,96	17,22	19,35	20,4	24,22	8,86
22,27	18,15	22,42	20,61	22,63	16,39	17,91	19	22,78	7,73
22,27	17,72	21,42	21,76	16,89	16,94	16,9	19,21	23,36	9,61
22,27	18,15	20,95	20,49	16,16	16,25	16,01	19,38	22,85	9,06
19,64	17,49	21,65	20,4	16,69	16,43	15,23	19,38	21,92	8,92
19,64	18,03	21,11	19,86	16,89	16,17	14,94	19,76	21,96	10,61
19,64	17,55	21,58	19,26	16,63	15,68	14,22	19,98	22,05	9,67
19,64	18,19	21,45	18,94	16,39	16,09	13,4	18,57	21,78	8,82
18,82	16,93	21,1	18,43	17,69	16,18	14,91	19,76	20,91	7,66
18,82	16,28	20,6	18,69	16,81	14,94	14,26	20,41	20,84	11,03
18,82	15,89	21,42	18	16,81	16,44	14,78	17,95	22,2	9,16
18,82	15,41	20,54	17,82	16,84	16,48	13,92	18,05	21,22	10,22
x	15,58	20,05	17,21	16,81	16,94	14,68	16,98	21,14	8,3
x	16,13	20,68	17,2	16,8	16,45	15,17	16,86	20,52	9,3
x	15,34	20,35	16,96	16,57	15,71	14,74	16,64	20,49	7,72
x	15,6	20,26	16,49	17,05	16,51	14,71	16,3	19,77	8,54
18,01	16,03	19,89	16,09	17,49	16,85	14,68	15,45	19,65	7,41
18,01	16,98	19,12	15,79	16,42	16,4	14,81	17,46	20,03	9,95
16,59	16,83	19,13	15,67	17,13	16,21	14,05	17,82	19,83	10,25
16,11	16,69	19,14	15,7	17,17	16,9	14,45	17,4	19,07	10,66

C-glucose (mmol/L)

	Baseline	Addition of L-NMMA	Pre-OGTT start	30 min after OGTT	1 h after OGTT	2 h after OGTT	3 h after OGTT	4 h after OGTT
Time (min)	0	60	120	150	180	240	300	360
Study ID 1	4,4	4,7	4,3	7,4	10,5	6,4	5,0	4,3
Study ID 2	4,9	4,7	4,6	8,2	7,4	6,6	3,2	4,5
Study ID 3	4,7	4,6	4,3	7,2	5,4	5,6	3,5	3,9
Study ID 4	4,7	4,7	4,8	8,4	8,4	7,0	5,1	3,8
Study ID 5	4,2	4,2	4,6	6,3	7,5	5,9	6,3	4,6
Study ID 6	4,3	4,1	4,7	7,9	8,8	8,2	5,3	4,2
Study ID 7	5,1	5,2	5,3	8,3	7,2	6,4	5,3	4,6
Study ID 8	4,6	4,2	4,3	8,0	7,3	5,3	5,7	3,8
Study ID 9	4,4	4,5	4,6	4,7	7,3	6	6,3	3,8
Study ID 10	4,7	4,4	4,4	7	7,2	6,7	4,4	x
Study ID 11	4,6	5,1	4,9	6,7	7,9	5,9	3,9	4,3
Study ID 12	4,4	4,4	4,3	5,9	5,6	6,3	4,6	3,3

P-glucose (mmol/L)

	Baseline	2 h after OGTT
Time (min)	120	240
Study ID 1	5,1	5,3
Study ID 2	5,3	6,5
Study ID 3	4,8	4,8
Study ID 4	5,4	6,8
Study ID 5	4,8	6,1
Study ID 6	4,5	8,1
Study ID 7	5,3	6,8
Study ID 8	4,8	4,5
Study ID 9	4,7	5
Study ID 10	5,3	8
Study ID 11	5,1	6,3
Study ID 12	4,8	5,8

Dialysate Insulin (mU/L)

	Baseline	Addition of L-NMMA	OGTT start - 1 h after OGTT	1-2 h after OGTT	2-3 h after OGTT	3-4 h after OGTT
Time (min)	30	90	150	210	270	330
L-NMMA 1	0,111	0,098	0,142	1,276	1,291	0,327
L-NMMA 2	0,047	0,02	0,065	0,311	0,341	0,06
L-NMMA 3	0,063	x	0,076	0,179	0,118	0,016
L-NMMA 4	0,099	0,158	0,51	2,059	1,778	0,691
L-NMMA 5	0,086	0,093	0,252	0,994	0,866	0,483
L-NMMA 6	0,052	0,109	0,14	0,315	0,529	0,17
L-NMMA 7	0,032	0,29	0,239	0,235	0,369	0,105
L-NMMA 8	0,025	0,037	0,158	0,217	0,218	0,319
L-NMMA 9	0,35	x	0,426	0,853	0,474	0,324
L-NMMA 10	x	x	0,219	0,135	x	x
L-NMMA 11	x	x	x	0,363	0,333	0,296
L-NMMA 12	x	x	0,787	1,251	1,237	0,574
Control 1	0,036	0,044	0,119	1,381	1,241	0,389
Control 2	0,146	0,029	0,245	0,651	0,426	0,075
Control 3	0,053	x	0,175	0,217	0,099	x
Control 4	0,119	0,177	0,425	1,905	0,949	0,457
Control 5	0,034	0,029	0,228	0,697	0,487	0,37
Control 6	0,149	0,018	0,14	0,222	0,496	0,345
Control 7	0,083	0,243	0,186	0,379	0,219	0,119
Control 8	0,149	0,002	0,24	0,719	0,26	0,211
Control 9	x	x	0,244	0,607	0,491	0,474
Control 10	x	x	0,237	0,394	0,416	x
Control 11	0,225	x	x	x	x	x
Control 12	0,962	1,133	4,927	5,041	7,191	2,585

S-insulin (mU/L)

	Pre-OGTT	2 h after OGTT	HOMA-IR
Time (min)	120	240	-
Study ID 1	4,7	40	1,07
Study ID 2	4,7	40	1,11
Study ID 3	3,4	17	0,73
Study ID 4	14	131	3,36
Study ID 5	7,8	26	1,66
Study ID 6	5,8	63	1,16
Study ID 7	6,7	22	1,58
Study ID 8	2,7	11	0,58
Study ID 9	4,3	18,0	0,90
Study ID 10	3,4	23,0	0,80
Study ID 11	4,4	19,0	1,00
Study ID 12	9,5	64,0	2,03

Skin temperature (°C)

	Baseline	Addition of L-NMMA	Pre-OGTT start	30 min after OGTT	1 h after OGTT	2 h after OGTT
Time (min)	0	60	120	150	180	240
Study ID 1	30,5	30,7	30,1	30,9	30,5	31,0
Study ID 2	31,6	32,2	31,8	31,2	31,1	31,4
Study ID 3	30,7	30,4	29,2	29,1	28,8	28,3
Study ID 4	26,8	27,7	28,1	27,7	29,1	33,4
Study ID 5	27,6	26,5	30,1	30,0	30,6	30,2
Study ID 6	31,4	32,6	32,6	32,7	32,7	32,7
Study ID 7	32,5	33,1	32,2	32,1	32,7	33,6
Study ID 8	32,4	32,5	32,0	31,8	31,6	31,7
Study ID 9	32,0	31,9	31,7	31,3	31,5	31,3
Study ID 10	32,5	31,8	30,9	30,5	30,6	31,8
Study ID 11	31,7	31,8	30,1	31,0	31,2	31,5
Study ID 12	29,8	32,6	31,3	30,3	31,5	31,9

3 h after OGTT	4 h after OGTT
300	360
30,6	30,6
31,8	33,1
28,6	29,1
33,7	33,0
32,4	32,2
33,7	33,7
32,6	32,2
32,6	32,5
31,6	31,5
32,1	29,7
32,9	32,0
31,0	31,0

Skin perfusion

LSCI (Perfusion Units)

	Baseline 1	Baseline 2	Addition of L-NMMA 1	Addition of L-NMMA 1	OGTT start to 1 h after OGTT	1-2 h after OGTT	2-3 h after OGTT	3-4 h after OGTT
Time (min)	15	45	75	105	150	210	270	330
L-NMMA 1	74,38	69,78	63,78	53,98	54,11	46,57	53,56	59,82
L-NMMA 2	44,69	53,14	46,79	32,21	40,39	41,44	47,93	50,29
L-NMMA 3	41,96	33,94	24,94	29,93	30,35	36,78	37,00	42,50
L-NMMA 4	45,28	54,01	47,94	50,76	53,39	68,54	55,62	62,56
L-NMMA 5	39,55	41,65	37,83	46,44	42,03	37,65	36,80	38,09
L-NMMA 6	46,81	57,57	48,50	55,63	44,51	45,94	49,27	42,15
L-NMMA 7	43,60	36,27	45,68	49,36	35,05	34,92	41,26	39,26
L-NMMA 8	39,79	37,90	34,44	38,14	35,04	36,41	35,87	37,95
L-NMMA 9	40,74	37,97	56,27	47,96	47,33	45,26	45,94	52,42
L-NMMA 10	44,89	43,79	30,10	30,00	34,30	37,86	33,43	33,99
L-NMMA 11	33,82	32,98	31,89	26,45	28,33	27,76	25,65	24,16
L-NMMA 12	44,31	42,65	40,93	41,13	47,84	41,73	38,16	41,05
Control 1	50,77	50,57	51,03	52,01	49,64	46,62	47,69	55,86
Control 2	48,98	42,35	35,25	26,93	34,40	34,18	45,99	47,18
Control 3	45,54	35,02	32,72	40,46	36,60	46,24	39,07	49,53
Control 4	30,92	43,53	36,29	45,78	50,55	63,04	59,83	62,39
Control 5	31,90	38,76	36,03	42,07	37,09	35,90	37,24	38,67
Control 6	35,07	45,47	37,85	41,37	41,63	44,60	47,42	44,52
Control 7	40,52	34,695	46,11	43,685	40,8925	41,705	51,36	50,8575
Control 8	39,98	47,06	44,20	35,77	44,10	45,18	33,83	34,24
Control 9	35,74	37,92	47,08	56,85	65,71	74,40	66,14	66,95
Control 10	60,215	55,58	32,815	40,295	34,41	42,445	37,1325	32,7775
Control 11	42,96	41,215	36,89	31,03	32,3	34,825	33,3325	32,605
Control 12	34,845	36,935	41,295	35,56	35,525	42,5775	32,935	32,8925