Nitric oxide synthase activity and endothelial ultrastructure in ischaemic skin-flaps

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SUMMARY. The aim of the present study was to detect and quantify nitric oxide synthase (NOS) activity and to investigate morphological changes in the endothelium in two different ischaemic dorsal flap models in the rat, one based cranially and one based caudally. Intact skin from the dorsum was used as control. In both groups flaps were removed at 1, 4, 12, 24 and 72 h after surgery respectively. NOS-activity was measured by the conversion of L-arginine to L-citrulline and endothelial morphology was investigated using transmission electron microscopy.

Intact skin showed Ca²⁺-dependent but no Ca²⁺-independent NOS-activity. A time-dependent decrease in Ca²⁺-dependent NOS-activity was seen in the proximal and distal part of the flaps in both flap models and was most pronounced in the distal part. Ca²⁺-independent NOS-activity increased in the proximal and distal part of flaps based cranially and in the proximal part of flaps based caudally.

Morphological analysis of the endothelium showed signs of endothelial damage including blebbed membranes, swelling and endothelial loss.

These findings show that ischaemia caused by skin-flap surgery leads to endothelial damage and a decrease in Ca²⁺-dependent nitric oxide synthase activity. Furthermore, in the skin-flaps an induction of Ca²⁺-independent nitric oxide synthase (iNOS) activity was noted both in surviving flap tissue and in flap tissue destined to necrose.

The use of flaps is a common technique in reconstructive surgery. Despite advances in flap design, surgical techniques and new pharmacological and non-pharmacological strategies to improve flap survival, flap necrosis continues to be a clinical problem. The pathophysiological mechanisms in flap ischaemia and necrosis remain partly unclear.

In 1980 Furchgott and Zawadzki postulated the existence of an endothelial derived relaxing factor (EDRF). Seven years later EDRF was identified as nitric oxide (NO) and the enzyme responsible for its production was named nitric oxide synthase (NOS). Several different isoforms of NOS have been characterised, i.e. eNOS (endothelial), nNOS (neuronal) and iNOS (inducible), and have had their cDNA cloned. NOS produces NO by the conversion of L-arginine to L-citrulline and NO. The isoforms eNOS and nNOS are constitutive and require free ionic calcium in order to synthesise NO. eNOS has been shown to affect vascular tone and blood flow, platelet aggregation, and leukocyte adhesion whereas nNOS takes part in neurotransmission. The isoform iNOS is inducible, does not require ionic calcium and is synthesised de novo by immunocompetent cells, smooth muscle cells and endothelial cells when stimulated by various cytokines. Thus iNOS is involved in host defence mechanisms.

Besides the physiological roles of nitric oxide several pathophysiological roles have been found and in several studies NO has been shown to play an important role in ischaemia-reperfusion injury and septic shock. The use of NOS-inhibitors have been shown to influence the survival of ischaemic skin-flaps, ischaemic brain-tissue and ischaemic myocardium.

The aim of the present study was to investigate the levels of constitutive, Ca²⁺-dependent (eNOS and nNOS) and inducible, Ca²⁺-independent (iNOS) nitric oxide synthase in ischaemic skin-flaps and to correlate these with ultrastructural changes in the endothelium. Two different experimental dorsal flap-models in the rat were used, one based cranially in which a superficial necrosis was expected and one based caudally in which a full-thickness necrosis was expected.

Materials and methods

Animals

One hundred and nine male albino rats (Sprague-Dawley) weighing 300–380 g were used in the study; 91 for flap-surgery (46 with the flap based cranially and 45 with the flap based caudally) and 18 as controls. The rats were housed in environmentally controlled rooms with 12 h of light and dark and offered standard rat chow and water ad libitum.

Experimental skin-flap models

Two different ischaemic experimental flap-models were used in the study, both measuring 2 × 7 cm and both being modifications of the dorsal skin-flap model in the rat originally described by McFarlane et al. In 46 rats the base of the flap was located cranially and designed according to Kjartansson and Dalsgaard and in 45 rats the base of the flap was located caudally and designed according to Hammond et al.

Both flap models are ischaemic and the distal part of the flap is expected to necrose. The caudally based
flap-model is more ischaemic and the necrosis is expected to develop earlier. Previous studies have shown that after 3 days 51% of the caudally based flap is necrotic whereas the cranially based flap develops a 55% necrosis after 7 days. Furthermore, the necrosis seen after 3 days (72 h) in the caudally based flap is a full thickness necrosis whereas, according to our experience, the necrosis in the cranially based flap is superficial.

Animal preparation

On the day before surgery the rats were anaesthetised with chloral hydrate 0.4 g/kg i.p. and had their backs shaved. Remaining hair was removed using a cream hair remover (Veet, Reckitt & Colman, Massy, France) for 10 min. The rats were then left for 24 h.

Surgical procedure

On the day of surgery the rats were anaesthetised with chloral hydrate and a flap designed according to a standard pattern, 2 cm wide and 7 cm long. In the cranially based flap the base was located on the caudal part of the scapulae and in the caudally based flap the base was located on the point where the gluteus muscles come together in the midline. The flap was raised from the deep fascia of muscle and included the superficial fascia, panniculus carnosus, subcutaneous tissue and skin. After having been raised the cranially based flap was sutured back in position and in rats operated with a caudally based flap the wound was closed in the midline and the flap was positioned on top of the closed wound and sutured in place.

Flap and control tissue removal

The rats were again anaesthetised using chloral hydrate, the sutures cut and the flaps removed at 1, 4, 12, 24 or 72 h after surgery respectively.

Control tissue samples were obtained by anaesthetising 18 rats and quickly cutting out two segments respectively. The rats were again anaesthetised using chloral hydrate, flaps and control tissue removal closed in the midline and the flap was positioned on the top of the closed wound and sutured in place.

Measurement of nitric oxide synthase (NOS) activity

Control tissue samples and two parts of each flap, the proximal 10 mm and the distal 10 mm, were analysed for the presence of nitric oxide synthase activity by the conversion of L-[U-"C]arginine to L-[U-"C]citrulline.

Briefly, tissues were homogenised in ice cold homogenisation buffer containing 520 mmol/L sucrose, 10 mmol/L HEPES, 0.1 mmol/L ethylene glycoltetraacetic acid (EGTA), 1 mmol/L DL-dithiothreitol, 10 μg/ml trypsin inhibitor, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin (adjusted to pH 7.2 at 20°C with 1 mol/L HCl). The homogenate was centrifuged at 12 000 g for 30 min at +4°C, and the soluble fraction was used for the measurement of NOS activity. The tissue extract was added to tubes prewarmed to 37°C, containing 100 μL of a buffer consisting of 50 mmol/L potassium phosphate, pH 7.2, 50 mmol/L L-valine, 100 μmol/L reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 mmol/L L-citrulline, 20 μmol/L L-arginine and L-[U-"C]arginine (Amersham, 150 000 dpm), 1.2 mmol/L magnesium chloride (MgCl) and 0.24 mmol/L calcium chloride (CaCl). Duplicate incubations for 10 min at 37°C were performed for each sample in the presence or absence of either EGTA (2 mmol/L) or EGTA plus L-nomonomethyl-L-arginine (2 mmol/L each), to determine the level of Ca"- dependent and Ca"-independent NOS activity, respectively. The reaction was terminated by removal of substrate by addition of 1.5 ml 1:1 (v/v) water/Dowex AF 50W X8, pH 7.5. Then 5 ml of H2O were added to the incubation mix and 2 ml of the supernatant were removed and examined for the presence of L-[U-"C] citrulline by liquid scintillation counting. The level of citrulline is expressed as picomoles per gram of tissue (wet weight) per minute.

Transmission Electron Microscopy (TEM) studies

After removal of flaps based caudally (as described above) a thin strip (1 x 20 mm) was cut from the proximal part (10 mm from the base) and from the distal part (10 mm from the distal end) of the flaps. Equally thin strips were cut from proximal and distal control-tissue samples. The strips were immediately placed in 2.5% phosphate-buffered glutaraldehyde and fixed for 7 days. Samples from strips from controls and from flaps removed at 4, 12, 24 or 72 h after surgery were then post-fixed in 2% osmium tetroxide and stained en bloc with 1% uranyl acetate. After progressive dehydration in a graded series of ethanol washes, ending with propylene oxide, specimens were transferred to a 50 to 50 mix of propylene oxide and Agar Resin 100 in which the specimens were polymerised at 40°C (24 h) and 60°C (48 h). Semi-thin sections were cut, stained with 0.4% toluidine blue, and viewed under light microscopic. Sections suitable for electron microscopy were determined. Ultra-thin sections were cut and mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Carl Zeiss EM-109 transmission electron microscope operating at 50 kV.

As criteria for endothelial damage findings such as chromatin margination, cytoplasmic vacuolisation, disrupted membranes (blebbing), cellular swelling and endothelial loss were accepted.

Drugs and chemicals

Statistics

All values are expressed as the mean ± standard error for n observations. One-way analysis of variance (ANOVA) was used in the comparisons of multiple independent means and Tukey’s HSD (Honest Significant Difference) test was used as a post hoc test for the comparison of two independent means. P < 0.05 was considered statistically significant.

A power-calculation resulted in a more than 80% power when calculating on differences in nitric oxide synthase activity over time.

Results

At 72 hours after surgery no demarcation line was evident in the cranially based flap-model. However the distal part displayed a slightly darkened colour. In the caudally based flap-model a distal full-thickness necrosis, representing 48% (6%) of the flap, was evident at 72 h. The proximal part in both flap-models showed no macroscopic changes.

Calcium-dependent nitric oxide synthase activity

Proximal and distal control tissue samples (time 0 h) in cranially and caudally based flaps showed similar Ca²⁺-dependent NOS-activity ranging from 90.9 (10.1) to 101.4 (9.8) pmol/g/min (Figs 1A and 1B and Table 1).

Fig. 1—Ca²⁺-dependent nitric oxide synthase activity in the proximal and distal part of cranially (A) and caudally (B) based skin flaps expressed as pmol/g/min at different times after surgery. Values are mean ± standard error. *P < 0.05 and ***P < 0.001.

Table 1 Ca²⁺-dependent nitric oxide synthase activity in the proximal and distal part of cranially and caudally based skin flaps expressed as pmol/g/min at different times after surgery. Values are mean ± standard error.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cranially based flap model</th>
<th>Caudally based flap model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Distal</td>
</tr>
<tr>
<td>0</td>
<td>101.4 (9.8)</td>
<td>97.0 (16.8)</td>
</tr>
<tr>
<td>1</td>
<td>90.9 (16.0)</td>
<td>78.4 (13.1)</td>
</tr>
<tr>
<td>4</td>
<td>68.5 (16.5)</td>
<td>62.2 (28.5)</td>
</tr>
<tr>
<td>12</td>
<td>58.7 (18.9)</td>
<td>8.3 (4.8)</td>
</tr>
<tr>
<td>24</td>
<td>35.0 (9.7)</td>
<td>9.4 (4.1)</td>
</tr>
<tr>
<td>72</td>
<td>25.0 (11.9)</td>
<td>6.6 (3.5)</td>
</tr>
</tbody>
</table>

When comparing multiple means over time using ANOVA a significant decrease in the Ca²⁺-dependent NOS-activity was seen in the proximal (P < 0.05) and distal (P < 0.001) part of cranially based flaps and in the proximal (P < 0.001) and distal (P < 0.001) part of caudally based flaps.

In the distal part of cranially based flaps a significant decrease in Ca²⁺-dependent NOS-activity to 8.3 (4.8) pmol/g/min was seen at 12 h and was then maintained at 24 and 72 h (Figure 1A and Table 1). In the proximal part of cranially based flaps a significant decrease to 25.0 (11.9) pmol/g/min was seen at 72 h (Figure 1A and Table 1).

In caudally based flaps a significant decrease in Ca²⁺-dependent NOS-activity to 27.8 (6.9) pmol/g/min was noted at 4 h in the distal part and was then maintained at 12, 24 and 72 h whereas in the proximal part the decrease was significant at 24 h measuring 48.3 (13.6) pmol/g/min and continued at 72 h to 9.4 (5.3) pmol/g/min (Figure 1B and Table 1).

Calcium-independent nitric oxide synthase activity

In proximal and distal control-tissue samples (time 0 h) no Ca²⁺-independent NOS activity was detected in either caudally or cranially based skin-flaps (Figs 2A and 2B and Table 2).

Fig. 2—Ca²⁺-independent nitric oxide synthase activity in the proximal and distal part of cranially (A) and caudally (B) based skin flaps expressed as pmol/g/min at different times after surgery. Values are mean ± standard error. *P < 0.05 and ***P < 0.001.

Table 2 Ca²⁺-independent nitric oxide synthase activity in the proximal and distal part of cranially and caudally based skin flaps expressed as pmol/g/min at different times after surgery. Values are mean ± standard error.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Cranially based flap model</th>
<th>Caudally based flap model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proximal</td>
<td>Distal</td>
</tr>
<tr>
<td>0</td>
<td>0.5 (3.3)</td>
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</tr>
<tr>
<td>1</td>
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<td>7.3 (0.0)</td>
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<td>7.8 (4.0)</td>
<td>15.8 (8.4)</td>
</tr>
<tr>
<td>12</td>
<td>16.6 (6.2)</td>
<td>40.0 (14.3)</td>
</tr>
<tr>
<td>24</td>
<td>40.7 (12.2)</td>
<td>143.1 (28.0)</td>
</tr>
<tr>
<td>72</td>
<td>9.0 (3.9)</td>
<td>87.9 (13.8)</td>
</tr>
</tbody>
</table>
The distal end of the flaps morphological analysis was found a decrease in constitutive, Ca\(^{2+}\)-dependent and an in the proximal (P < 0.01) and distal (P < 0.001) part of cranially based flaps and in the proximal (P < 0.001) part of caudally based flaps. In cranially based skin-flaps an increase in Ca\(^{2+}\)-independent NOS-activity was seen at 24 h in the proximal part measuring 40.7 (12.2) pmol/g/min and the distal part measuring 143.1 (28.0) pmol/g/min (Fig. 2A and Table 2).

In the proximal part of caudally based flaps an increase in Ca\(^{2+}\)-independent NOS-activity was seen at 24 h measuring 268.9 (47.1) pmol/g/min and was then maintained at 72 h (Fig. 2B and Table 2). No significant change in Ca\(^{2+}\)-independent NOS-activity was seen in the distal part of caudally based flaps (Fig. 2B and Table 2) during the entire experiment.

**Morphological analysis of caudally based skin-flaps**

In the control-tissue samples arteries (Fig. 3, Panel A and B) and veins (Fig. 3, Panel C) with normal appearance were found. The proximal part of the flaps removed at 4 h after surgery showed no changes in blood vessel ultrastructure. At the same time arterial endothelial cells in the distal part of the flaps showed swelling of the nuclei and cytoplasm, chromatin margination, cytoplasmic membranes. At 12 h after surgery the endothelium in the proximal part of the flaps was still visually undamaged but an increase in the number of leukocytes such as neutrophil granulocytes in the vessels and in the connective tissue and macrophages in the connective tissue was seen (Fig. 4, Panel A). In the distal part of the flaps veins were enlarged due to stasis and the endothelium displayed chromatin margination and loss of contact with the basement membrane allowing erythrocytes to pass into the surrounding tissue (Fig. 4, Panel B). The arteries found were all small.

At 24 h after surgery both arteries and veins in the proximal part of the flaps showed blebbed membranes and an increase in the number of leukocytes in and around the vessels (Fig. 4, Panel C). In the distal part of the flaps arteries were small whereas veins displayed stasis, chromatin margination and endothelial loss of contact with the basement membrane.

The arteries in the proximal part of the flaps removed at 72 h after surgery showed endothelial swelling of both the nuclei and cytoplasm (Fig. 4, Panels D and E). Veins displayed thrombosis formation and in places loss of vessel integrity allowing erythrocytes to escape into the surrounding tissue (Fig. 4, Panel F). In the surrounding interstitium an increase in the number of leukocytes was noted at this time. In the distal end of the flaps morphological analysis was impossible due to severe tissue-damage.

**Discussion**

In the present study on experimental skin-flaps we found a decrease in constitutive, Ca\(^{2+}\)-dependent and an increase in inducible, Ca\(^{2+}\)-independent NOS-activity parallel to morphological findings of endothelial damage and increase in the number of leukocytes.

In control-tissue Ca\(^{2+}\)-dependent NOS-activity was found indicating the presence of a constitutive NOS in the skin. The source of this constitutive NOS is probably mainly vascular endothelial cells (eNOS) and to a lesser extent autonomic nerves (nNOS).\(^{17,20}\) Ca\(^{2+}\)-independent inducible NOS-activity (iNOS) on the other hand was not found in controls.

With increasing time after surgery a decrease in the constitutive, Ca\(^{2+}\)-dependent NOS activity was seen in both proximal and distal parts of both flap models. This decrease correlated with increasing signs of endothelial damage as seen in the caudally based flaps. Endothelial dysfunction and decreased constitutive NOS activity and NO-production during ischaemia and ischaemia-reperfusion has been seen in other tissues such as heart and brain.\(^{5,10}\) The decreased Ca\(^{2+}\)-dependent NOS activity in the present study could be caused by endothelial damage but in addition autonomic neuronal damage must be considered as a possible cause of the decrease.\(^{27,29}\) The decrease in Ca\(^{2+}\)-dependent NOS activity was more rapid in onset and also more profound in the distal than in the proximal part in both flap model. Furthermore the decrease was seen earlier in the caudally based flap model than in the cranially based flap model. This is likely due to more severe ischaemia and subsequently more tissue damage in the distal as compared to the proximal part of the flaps and in the caudally as compared to the cranially based flap model. This was supported by the electron microscopic findings.

In the proximal and distal part of flaps based cranially and in the proximal part of caudally based flaps a gradual increase in Ca\(^{2+}\)-independent, inducible, NOS activity (iNOS) was seen. A similar increase in inducible NOS activity has been reported during ischaemia and ischaemia-reperfusion in other tissues such as heart and brain, the source being immunocompetent cells and endothelial cells.\(^{17,29}\) In ischaemic skin-flaps leukocytes, which in the present study were seen to increase, could be the source of the inducible NOS-activity.\(^{17}\) However, the increase could also be caused by an induction in other cells such as endothelial cells and smooth muscle cells.\(^{16}\) The reason no increase in Ca\(^{2+}\)-independent NOS activity was seen in the distal part of cranially based flaps is likely an early stasis leading to no blood-flow and severe hypoxia. This was supported by the electron microscopic findings.

Nitric oxide has been shown to play a dual role during ischaemic injury. First, constitutive nitric oxide production from the endothelium has been shown to be tissue-protective, maintaining a basal blood flow and inhibiting the aggregation and adherence of platelets and neutrophils.\(^{12,23}\) A decrease in this basal release of nitric oxide has been reported during ischaemia in the brain and heart.\(^{18,24}\) Second, ischaemia in the brain and heart has been shown to lead to an induction of iNOS and to the production of high levels of NO which may be cytotoxic.\(^{17,18}\) The present observations of changes in NOS activity in ischaemic skin-flaps are in line with these findings and equivalent.
Nitric oxide synthase activity in ischaemic flaps

Figure 3  Transmission electron microscopic photographs of blood vessels in control tissue samples and in caudally based skin flaps at 4 h after surgery. Panel A: Overview of control artery. Bar 10 μm. Panel B: Detailed view of endothelial cells in control artery. Bar 1 μm. Panel C: Overview of control vein. Bar 10 μm. Panels D-F: Artery in the distal end of a flap at 4 h after surgery showing endothelial swelling and nuclear chromatin margination (arrows, Panel D), cytoplasmic membrane blebbing (arrow, Panel E), vacuole formation (arrows, Panel F). Bars 1 μm.
Figure 4 — Transmission electron microscopic photographs of blood vessels in caudally based skin flaps at different times after surgery. Panel A: Vein in the base of flap at 12 h after surgery showing neutrophile granulocytes (arrows) inside the vessel and in the surrounding interstitium. Bar 10 μm. Panel B: Vein in the distal end of flap at 12 h after surgery showing stasis and endothelial loss (arrows). Bar 6.36 μm. Panel C: Vein in the base of flap at 24 h after surgery showing blebbed membranes (small arrow) and neutrophile granulocytes (large arrow) moving into the interstitium. Bar 2.33 μm. Panel D: Artery in the base of flap at 72 h after surgery showing endothelial swelling. Bar 10 μm. Panel E: Detailed view of artery in the base of flap at 72 h after surgery showing endothelial swelling (arrow). Bar 2.33 μm. Panel F: Vein in the base of flap at 72 h after surgery showing thrombosis-formation (large arrow) and loss of vessel integrity (small arrows). Bar 10 μm.
mechanism leading to tissue damage and necrosis could therefore be possible. A decrease in constitutive Ca^{2+}-dependent NOS activity could lead to constriction of blood vessels and impaired blood-flow as NO is involved in the maintenance of normal skin circulation.\textsuperscript{27,28} In addition a decrease in constitutive NOS activity could lead to an increase in the aggregation of platelets\textsuperscript{11,12} and increase in the adhesion of leukocytes.\textsuperscript{12,13} Induction of iNOS has been shown to lead to high levels of nitric oxide and to the formation of tissue-damaging free radicals such as peroxynitrite.\textsuperscript{11,13} The production of free radicals has been shown during ischemia and reperfusion of skin-flaps.\textsuperscript{14} The use of NOS-inhibitors has been shown to attenuate the induction of iNOS, counteract the formation of free radicals and to increase the survival of ischaemic tissues.\textsuperscript{15,16} In a study on ischaemic skin-flaps Knox et al showed that NOS inhibitors increased flap-survival possibly through the same mechanism.\textsuperscript{15} The decrease in constitutive and induction of inducible NOS activity seen in the present study could therefore aggravate an initial skin-flap ischemia, leading to further tissue damage and increasing the risk for flap failure and necrosis.

In conclusion, the findings show that ischemia in experimental skin-flaps is associated with endothelial injury and a decrease in constitutive NOS activity and an induction of inducible NOS activity.

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