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Deficiency of inducible and endothelial nitric oxide synthase results in diminished bone formation and delayed union and nonunion development

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ABSTRACT

Background: Between 5% and 10% of all fractures fail to heal adequately resulting in nonunion of the fracture fragments. This can significantly decrease a patient's quality of life and create associated psychosocial and socio-economic problems.
Nitric oxide (NO) and nitric oxide synthases (NOS) have been found to be involved in fracture healing, but until now it is not known if disturbances in these mechanisms play a role in nonunion and delayed union development. In this study, we explored the role of endothelial and inducible NOS deficiency in a delayed union model in mice.
Materials and methods: A 0.45 mm femur osteotomy with periosteal cauterization followed by plate-screw osteosynthesis was performed in the left leg of 20–24 week old wild type, *Nos2*$^{-/-}$ and *Nos3*$^{-/-}$ mice. Contralateral unfractured legs were used as a control. Callus volume was measured using micro-computed tomography (μCT) after 28 and 42 days of fracture healing. Immuno histochemical myeloperoxidase (MPO) staining was performed on paraffin embedded sections to assess neutrophil influx in callus tissue and surrounding proximal and distal marrow cavities of the femur. After 7 and 28 days of fracture healing, femurs were collected for amino acid and RNA analysis to study arginine-NO metabolism.
Results: With μCT, delayed union was observed in wild type animals, whereas in both *Nos2*$^{-/-}$ and *Nos3*$^{-/-}$ mice nonunion development was evident. Both knock-out strains also showed a significantly increased influx of MPO when compared with wild type mice. Concentrations of amino acids and expression of enzymes related to the arginine-NO metabolism were aberrant in NOS deficient mice when compared to contralateral control femurs and wild type samples.
Discussion and conclusion: In the present study we show for the first time that the absence of nitric oxide synthases results in a disturbed arginine-NO metabolism and inadequate fracture healing with the transition of delayed union into a nonunion in mice after a femur osteotomy. Based on these data we suggest that the arginine-NO metabolism may play a role in the prevention of delayed unions and nonunions.

1. Introduction

Normal fracture healing is a process of partially overlapping phases of inflammation, callus formation and bone remodeling in which there is an interplay between various cells, growth factors and extracellular matrix [1]. However, five to ten percent of all patients experience difficulties during the healing process [2] resulting in delayed union or nonunion of the fracture, indicated by persisting fracture lines and presence of a hypertrophic or atrophic callus [3]. Malnutrition, drug therapy, inadequate stabilization of the fracture and/or inadequate blood supply (*i.e.* periosteal injury) contribute to nonunion development [4,5].

Adequate production of NO (nitric oxide), a free radical produced during the conversion of arginine into citrulline by nitric oxide synthases (NOSs) stimulates bone cells to regulate bone remodeling and influences vascular reactivity [6–8]. Furthermore, NO is suggested to stimulate polyamine production through the formation of ornithine, as precursors of collagen synthesis [9,10]. An intricate interplay exists between the substrate availability of arginine and citrulline and the NOS enzyme complex (Fig. 1). Disturbances in arginine and citrulline have already been associated with an impaired fracture healing resulting in nonunion in humans [11]. However, the pathogenesis has not been elicited yet.

Abbreviations: F, forward primer; R, reversed primer.

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In vivo studies in rats showed the presence and localization of NOS isoforms in callus samples after creation of a femoral fracture. mRNA and protein activity of inducible NOS (iNOS or NOS2) was present during the first phase of fracture repair and was mainly localized within the intramembranous region along the edge of the periosteal callus. The constitutive and calcium dependent endothelial (eNOS or NOS3) and neuronal NOS (nNOS or NOS1) were found in later stages of fracture healing and mainly in cells lining blood vessels and in the fibrochondral region between fibrous tissue and cartilage respectively [12–14].

We hypothesized that low amounts of NO and disturbances in arginine substrate metabolism due to an absence of either the *Nos*2 or *Nos*3 gene will inhibit callus formation and increase the risk of nonunion. Therefore, we studied the formation of callus and the arginine metabolism after a femur osteotomy with periosteal cauterization in a mouse model of delayed union.

2. Materials and methods

2.1. Animals and surgical procedure

In this study, skeletally mature, 20 to 24 week old specific pathogen free (SPF), female C57Bl6/J (RCC Switzerland) and *Nos2*$^{-/-}$ and *Nos3*$^{-/-}$, both backcrossed more than 10 generation into the C57Bl6/J background, with constructs previously described by Laubach et al. [15] and Shesely et al. [16] respectively (kindly provided by Dr. Theo Hakvoort, University of Amsterdam, The Netherlands), mice were used. All mice were housed in groups of five in individually ventilated cages (IVC) with a 12-h day-night cycle. Mice were fed standard diet (3436, Prowimi, Switzerland) and water *ad libitum*. All animals were allowed to acclimatize for 2 weeks prior to surgical intervention. After these 2 weeks, mice were randomly assigned to the micro-computed tomography (N = 9/group), the amino acid and RNA analysis (N = 6/group) or the histology (N = 9/group) groups for analysis. See Table 1 for a complete overview of animals per mouse strain, group of analysis and days of follow-up.

Anesthesia was induced by placing the mice in an induction box flooded with isoflurane (Isoflurane, Baxter AG, Switzerland). For intra-operative analgesia, 0.1 mg/kg s.c. buprenorphine (Temgesic, Essex Chemie AG, Switzerland) was administered. During surgery, animals were kept under 1.5–2% isoflurane inhalation anesthesia and on a heating pad to prevent hypothermia. After aseptic preparation of the surgical field, animals were placed in prone position and a lateral skin incision starting at the base of the tail towards the left knee was made. By blunt dissection between the quadriceps and biceps femoris muscles, the femur was exposed and a 1 mm segment of periosteum was cauterized circumferentially during 0.5 s. The soft tissue was protected by a Teflon foil during cauterization. Thereafter, an internal plate [17] (7 × 1.5 × 0.7 mm, MouseFix, RISystems Davos, Switzerland) was placed on the femur and after predrilling with a 0.33 mm drill bit the plate was fixed with four angular stable MouseFix screws (2.0 mm in length). Following fixation, a 0.45 mm mid-diaphyseal femoral gap osteotomy was performed with a Gigli wire saw in the center of the cauterized segment. Each screw was untightened by half a turn to induce secondary fracture healing [18]. Fascia and skin were closed in routine fashion (5–0 Vicryl Rapide, Ethicon and Proline, Ethicon, Belgium). At the end of surgery, plate placement and fixation was confirmed radiographically. In the following 48 h, mice received 0.1 mg/kg s.c. buprenorphine every 10–14 h and for the first 5 days postoperatively 8 mg paracetamol per os/mouse/day was given through the drinking water (Dafalgan, Upsamedica, Switzerland).

Mice were sacrificed using CO_2 following different periods of fracture healing (7, 28 and 42 days after osteotomy). The veterinary welfare and ethics committee of the Canton of Graubünden (Switzerland) approved the experimental set-up and procedures of this study (permit number GR 23/2006).

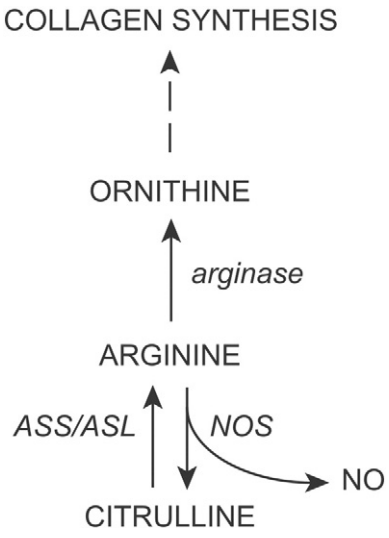

Fig. 1. Schematic representation of arginine metabolism. Arginine can be converted into ornithine by the enzyme arginase. Ornithine is a precursor of collagen. Arginine can also be converted into citrulline by one of the nitric oxide synthases (NOS); neuronal NOS1, inducible NOS2 or endothelial NOS3. During this process the free radical nitric oxide (NO) is produced. Citrulline can be converted back into arginine by the enzymes ASS (argininosuccinate synthase) and ASL (argininosuccinate lyase).

2.2. Amino acid measurements

To determine arginine, citrulline and ornithine concentrations, blood was collected post mortem in heparinized tubes on ice for amino acid measurements and centrifuged immediately (4 °C, 15 min at 8500 g) to obtain plasma. For amino acid analysis, plasma was deproteinized using acetonitrile (ratio plasma:acetonitrile 1:2), vortexed and stored until further analysis at −80 °C. Tissue samples for amino acid measurements were snap frozen in liquid nitrogen directly after harvesting. Before analysis, frozen homogenized tissue samples were added to 0.1 g of glass beads (1.0 mm diameter) in 250 μl of 5% sulfosalicylic acid for deproteinization, beaten for 30 s at maximum speed with mini-beadbeater (BioSpec Products, Bartlesville, Oklahoma, USA) and stored at −80 °C until further analysis. The contralateral right femurs of the mice were used as unfractured control bones, to determine possible local differences per mouse strain. Plasma and tissue amino acid concentrations were measured by HPLC as previously described [19].

The arginine availability in plasma and callus tissue was calculated as [arginine]/([ornithine] + [lysine]). This is based on the uptake of arginine, ornithine and lysine in cells via the y^+ transport system [20,21].

2.3. Immuno histochemistry

Following euthanasia, internal fixators were removed from the femurs and samples were fixed in 4% buffered paraformaldehyde solution and decalcified using EDTA. Samples were embedded in paraffin and 4 μm sections were prepared. For immuno histochemical analysis,

Table 1
Number of animals per mouse strain, days of follow-up and type of analysis.

Analysis	Days	Wild type (n)	Nos2$^{-/-}$ (n)	Nos3$^{-/-}$ (n)
Micro-CT	28	8	9	9
	42	9	9	9
RNA and amino acids	7	6	6	6
	28	6	5	6
Histology	28	8	9	9
	42	8	9	9
	Total	45	47	48