The role of nitric oxide synthase emzymes in the pathogenesis of hypoxia-induced diseases

Thesis booklet

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1. Introduction

The ischemia induced pathophysiological processes play a central role in several clinical diseases so they contribute significantly to the mortality and morbidity statistics.

Nitric oxide (NO) is a multifunctional signaling molecule in mammals playing a key role in the physiological and pathological reactions given to hypoxia/ischemia.

NO is synthesized from L-arginine by three different isoforms of nitric oxide synthases (NOSs): neuronal (nNOS), inducible (iNOS) and endothelial nitric oxid synthase (eNOS).

As NO has no specific receptors, its function and activity in the different pathophysiological conditions mainly depend on the site and concentration of its production and the surrounding mediators.

It has a central function in neurotransmission, inflammatory processes and in the regulation of angiogenesis and vasodilatation.

Ischemia/reperfusion (I/R) injury of the kidney is the leading cause of organ dysfunction seen after restoration of blood flow after diverse events including shock and organ transplantation. Genes up-regulated in ischemia induced acute renal failure (ARF) are mainly involved in immunity and defense, ligand-mediated signaling, cell proliferation and differentiation, as well as in apoptotic processes, in which NO plays a major role.

The pathophysiology of ischemia induced ARF is multifactorial and there has been evidence accumulating for the role of NO and NOS isoforms in its pathogenesis, however, reports are often controversial.

Retinopathy of prematurity (ROP) is a major cause of blindness in infancy.

After preterm birth, the developing retina is exposed to a sudden increase in tissue oxygen tension resulting in the generation of reactive free radicals which may lead to impairment of retinal vascular development and even to loss of already developed retinal capillaries (ROP phase I).
This insufficient vascularization results in retinal hypoxia, which, in turn, induces a release of various growth factors, stimulating new and abnormal blood vessel growth (ROP phase II).

Several studies have investigated the role of eNOS in connection with ophthalmologic diseases. NO triggers the gene expression and activation of several angiogenic, cell-migration, and proliferation-inducing factors including fibroblast growth factor 2, vascular endothelial growth factor (VEGF), urokinase-type plasminogen activator, and matrix metalloproteinase. Peroxynitrite, the reaction product of superoxide and NO, is also an important mediator of hyperoxia-induced vaso-obliteration.

Summarizing, in the pathogenesis of both renal I/R injury and ROP is associated with hypoxia/ischemia derived signaling cascades in which NO play an important role.

2. Aims

The aim of our study was to investigate the effect of the NO substrate L-arginine and the selective nNOS inhibitor 7-nitroindazole (7-NI) on the pathogenesis of I/R injury of the kidney in a rat model.

Furthermore, we examined the association between two eNOS genetic polymorphisms and ROP.

Our main questions were as follows:

1. Is there any effect of 7-NI on the rat renal I/R injury?
2. Is there any effect of L-arginine on the rat renal I/R injury?
3. Are there any changes in the mRNA and protein expressions of the different NOS izoforms after ischemic kidney injury, L-arginine and 7-NI treatment?
4. Is there any association between the eNOS $T^{-786}C$ és 27-bp repeat genetic polymorphisms and the pathogenesis of ROP?

3. Methods and patients

3.1. In vivo renal I/R injury model

Experiments were performed on male (weighing 370-400 g) Sprague-Dawley rats. All animals were given standard food for laboratory rats and water *ad libitum*. Temperature was controlled to 20±1 °C.

Rats were randomized and divided into three groups (N=7/group): the first group of animals received only vehicle treatment (I/R+veh group). The second group was supplemented with L-arg (I/R+L-arg) for 7 days (2 g/kg/day) intraperitoneally, the third group received 7-NI treatment (I/R+7-NI) for 7 days (50 mg/kg/day) intraperitoneally before induction of renal ischemia.

After isoflurane general anesthesia and the abdomen was opened through a midline incision and the left kidney prepared together with the renal artery and vein. Left renal artery and vein were clipped by an atraumatic vascular clamp for 50 min and right nephrectomy was performed, after which the abdomen was temporarily closed. After removing the microvascular clamp the abdomen was closed again, and the animals remained on thermocontrolled tables until complete recovery from anaesthesia. Sham operated animals underwent identical surgical procedure without occlusion of the left renal pedicle.

At 24 h after reperfusion rats were re-anaesthetized and the remnant kidney was removed and blood samples were collected for separation of serum. The animals were then exanguinated.

3.2. Renal function studies

Serum creatinine and blood urea nitrogen (BUN) levels were analyzed by an automatic laboratory analyzer.

3.3. Renal histopathology

Paraffin sections of the kidneys fixed in 4% neutral buffered formalin were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) reagent. Samples were coded and examined in a blinded fashion. Tubular damage and leukocyte infiltration were semi-quantitatively evaluated on a scale [0-3]. The score of tubular necrosis and leukocyte infiltration were then added and shown as sum.

TUNEL studies were performed to assess the rate of apoptosis. Frozen sections fixed in paraformaldehyde 4% and incubated with TUNEL solution containing terminal deoxynucleotidyltransferase. Sections were counterstained with hematoxylin. All positive tubular epithelial cells in each section were counted at a magnification of 40x and related to the number of view fields per section.

3.4. RT-PCR

Total RNA was isolated from kidney samples using a commercially available kit and total RNA was reverse transcribed into complementer (c)DNA.

Samples were amplified with primers specific for nNOS, iNOS, eNOS and GAPDH. Signals of PCR reactions were quantified by densitometry and corrected for the GAPDH signal using an image analysis software program.

3.5. Western Blot

Renal samples were homogenized with a cell disrupter in a lysis buffer. Protein concentrations in the supernatant were determined by the Bradford method using bovine serum albumine as a standard. Samples (50 μg) were separated on 10% SDS-polycrylamide gel and transferred to nitrocellulose membranes. The membranes were then probed with mouse monoclonal anti-iNOS and -eNOS and then incubated with anti-mouse

immunoglobuline antibody, a horseradish peroxidase conjugated from sheep. Proteins were detected by enhanced chemiluminescence on X-ray film. Prestained standards were used as molecular weight markers. Signals were quantified by densitometry.

3.6. Allele-specific PCR reactions

3.6.1. Patients

We monitored 232 patients born with LBW (less than or equal to 2000 g). All infants enrolled in the study were of Caucasian race. All infants underwent ophthalmologic examination. Maximun ROP stage was assessed and therapy was decided after consultation with two out of the three available neonatal ophthalmologists. The patients were divided into two groups based on requirement for ROP treatment.

The first group consisted of 105 infants who had been treated with laser or cryotherapy due to ROP stage 2+ or 5. The mean gestational age was 28±2.5 weeks and mean birth weight was 1150±360 g (treated group).

The second group enrolled 127 preterm LBW infants with ROP stage 1 or 2 who did not require cryotherapy/photocoagulation. The mean gestational age was 30.5±3.5 weeks and birth weight was 1300±400 g (untreated group).

	ROP requiring treatment	ROP not requiring treatment	*P* value
Number of patients	127	105	
Boy/girl	60/67	67/38	0.017
Gestational age (week)	30.5±3.5	28.4±2.5	0.0001
Gestational weight (g)	1300±400	1150±360	0.003
Lenght of oxygen therapy (day)	7 [0-47]	15 [0-92]	0.0001

Table 1. Clinical characteristics of the patients enrolled into the study..

3.6.2. Detection of eNOS T^{-786}C és 27-bp repeat polymorphisms

DNA for genotyping was extracted from filter papers. Both *eNOS* T^{-786}C and 27-bp repeat polymorphisms were detected using allele-specific PCR with specific primer primers.

3.7. Statistical analysis

Results of the animal experiments are expressed as mean ± SD. Histological changes were analyzed using the Kruskal-Wallis-test followed by Dunns post-hoc test.

Parametric data were compared using one-way analysis of variance followed by the Newman-Keul test. $P<0.05$ was considered to be statistically significant.

Continuous clinical data were compared with Student's *t*-test. Logistic regression analysis was used to assess the association between the need for cryotherapy/photocoagulation and *eNOS* genotypes.

The association was adjusted for proven risk factors of ROP: gestational age, days on supplemental oxygen therapy, and their interaction. The Harlequin software was used to assess Hardy–Weinberg equilibrium of *eNOS* $T^{-786}C$ and *eNOS* 27-bp repeat polymorphisms.

We performed our statistical calculations with the STATISTICA.6 software. $P<0.05$ value was considered as statistically different.

4. Results

4.1. Effect of Larginine and 7-NI on renal I/R injury

4.1.1. Renal function parameters

In all three groups the level of serum creatinine and BUN was increased indicating severe ARF, however we could not detect differences between the 7-NI, L-arg or vehicle treated animals (all $P>0.05$).

4.1.2. Renal histopathology

In all three groups severe signs of ischemia-induced ARF developed, but there was no significant difference between the experimental groups in the grade of tissue injury (all $P>0.05$).

4.1.3. TUNEL staining

TUNEL assay revealed that there was no difference in the number of TUNEL-positive tubular cells between the experimental groups (all $P>0.05$).

4.1.4. The expression of different NOS isoforms in I/R injury

4.1.4.1. Effect of I/R on the expression of nNOS, iNOS and eNOS

nNOS mRNA expression decreased significantly ($P<0.05$), iNOS mRNA expression increased significantly, whereas eNOS mRNA expression remained unchanged in I/R animals 24 h after reperfusion compared to sham operated animals ($P<0.05$).

iNOS protein level increased according to the mRNA expression ($P<0.05$), while the protein expression of eNOS decreased significantly ($P<0.05$) versus I/R+veh group.

4.1.4.2. Effect of L-arg on the expression of nNOS, iNOS and eNOS

L-arg increased the mRNA expression of all three NOS isoforms compared to the vehicle treated group (all $P<0.05$).

However, only iNOS protein level was increased following L-arg treatment ($P<0.05$), while the eNOS protein expression was not changed as compared to the vehicle treated rats ($P>0.05$).

4.1.4.3. Effect of 7-NI on the expression of nNOS, iNOS and eNOS

7-NI decreased the nNOS mRNA expression compared to the vehicle treated animals ($P<0.01$).

7-NI had no effect on the iNOS mRNA expression ($P>0.05$), whereas it decreased the iNOS protein level ($P<0.05$). 7-NI did not change either the eNOS mRNA or the protein expression compared to vehicle treated rats ($P>0.05$).

4.2. Association of eNOS 27-bp repeat és $T^{-786}C$ polymorphisms with retinopathy of prematurity

Both assessed genotypes were in linkage disequilibrium and in Hardy–Weinberg equilibrium, irrespective of ROP treatment.

Analysis of genotype distributions revealed that the genotype distribution of *eNOS* 27-bp repeat polymorphism was significantly different in the treated group (p=0.015).

There was no difference in the genotype distribution of *eNOS* $T^{-786}C$ polymorphism compared to the untreated group (p=0.984).

A comparison of the allele frequencies revealed no significant difference in the allele distributions of *eNOS* 27-bp repeat "a" and *eNOS* $^{-786}$C between the two groups (p=0.153 and p=0.867, respectively).

Multiple logistic regression was performed to analyze the relevance of selected parameters (gender, gestational age, time on oxygen therapy and the interaction of gestational age and length of oxygen therapy, and genotypes of *eNOS* 27-bp repeat polymorphism).

We found that *eNOS* aa genotype and male gender were significant predictors of the onset of ROP requiring treatment among preterm infants (p=0.047 versus ab genotype and p=0.022 versus bb genotype and p=0.046 versus females). Based on the genotype distributions, we estimated and compared four haplotypes between the treated and untreated groups. We found that *eNOS* aT and bT haplotypes were significantly increased in the infants treated for ROP compared to the untreated group (p=0.0001 and p=0.0036, respectively;).

5. Summary and conclusions

To determine the role of nNOS in renal I/R, animals were treated with selective nNOS inhibitor 7-NI before induction of ischemia.

We did not detect any differences in the renal function parameters such as serum creatinine and BUN; in the histopathological changes and in the number of TUNEL-positive tubular cells either in comparison with vehicle treated rats. 7-NI decreased the nNOS mRNA expression suggesting that inhibition of the enzyme activity suppresses its transcription. Furthermore, we revealed that 7-NI decreased iNOS protein level, wehereas we could not detect any differences in either the mRNA or in the protein expression of eNOS after 7-NI administration.

Besides examining the effects of nNOS inhibition, the aim of our study was also to assess the impact of L-arg on renal I/R injury and in particular, on the expression of NOS isoforms in order to better define the dynamics and effect of the NO system under I/R circumstances.

Our present study demonstrates no effects on renal I/R injury due to L-arg. We showed that L-arg changed neither the levels of renal function parameters nor the grade of tissue injury nor the number of TUNEL-positive tubular cells. In this study we could also demonstrate that L-arg leads to enhanced mRNA expression of all NOS isoforms after renal I/R. We measured the highest rate of increase in the iNOS mRNA expression by which isoform we could detect a marked elevation in the level of protein expression as well.

In the second part of the study we investigated the relevance of functional *eNOS* $T^{-786}C$ and 27-bp repeat polymorphisms in the pathogenesis of ROP.

We found that the genotype distribution of *eNOS* 27-bp repeat was significantly different between the study groups; however there was no difference in the frequency of the “a” allele.

Using multiple logistic regression analysis *eNOS* aa genotype was proved to be associated with the onset of ROP requiring treatment. The association was adjusted for ROP risk factors such as gender, gestational age and time on oxygen therapy and for the interaction of gestational age and time on oxygen therapy. We found a significant difference in the birth weight between the two study groups as well, but because there was a strong correlation between the gestational age and birth weight (0.9), we made no adjustment for birth weight.

There was no significant difference in the genotype distribution of *eNOS* $T^{-786}C$ polymorphism nor in the frequency of ^{-786}C allele between the treated and untreated groups. Haplotype estimations revealed that prevalence of aT and bT haplotypes was significantly higher in the treated group.

In conclusion, our present study demonstrated that selective nNOS inhibitor 7-NI exerted no effect on renal I/R injury suggesting that nNOS does not play a central role in the pathogenesis of postischemic renal injury. Furthermore, we revealed that 7-NI changed the expression of iNOS. L-arg administration did not influence severe renal I/R injury in Sprague-Dawley rats supporting previous results showing diverse and time- and dose dependent effects of L-arg supplementation on I/R injury of the kidney.

We observed that the genotype distribution of *eNOS* 27-bp repeat polymorphism was significantly different in preterm infants treated for severe proliferating ROP compared to preterm infants infants with stage 1 or 2 ROP that did not require treatment. *eNOS* 27-bp aa genotype presented an independent risk factor for ROP requiring treatment. These findings suggest that *eNOS* 27-bp repeat polymorphism might be associated with the development of proliferative ROP and indicate the importance of determining the patient's genetic background when planning individual therapy.

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