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Increased iNOS activity in vascular smooth muscle cells from diabetic rats: Potential role of Ca^{2+}/calmodulin-dependent protein kinase II delta 2 ($CaMKII\delta_2$)

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ABSTRACT

Objective: Inducible nitric oxide synthase (iNOS) expression may be increased by cytokine plasma levels contributing to vascular damage in diabetes. Besides transcriptional regulation, Ca^{2+}/CaMKII may play a role in post-translationally controlled iNOS activity. We accordingly investigated the involvement of the Ca^{2+}/$CaMKII\delta_2$ signaling pathway in regulating lipopolysaccharide (LPS)-induced iNOS activity in cultured aortic vascular smooth muscle cells (VSMCs) from diabetic rats.

Methods and results: VSMCs obtained from 10 diabetic rats (DR) and 10 control rats (CR) were stimulated with 20 μg/ml LPS. After 24 h, iNOS protein levels were 1.37 fold increased in DR- *vs* CR-VSMCs ($p < 0.05$; Western Blot), while iNOS activity (conversion L-(^{3}H)-arginine into L-(^{3}H)-citrulline) and intracellular nitrotyrosine levels (immunofluorescence) were about 2.7 fold greater in DR- than in CR-VSMCs. Interestingly, LPS increased intracellular Ca^{2+} levels (Fluorescence video imaging) more markedly in DR- than in CR-VSMCs. This was associated with CaMKII activation by phosphorylation, a decreased amount of co-immunoprecipitating iNOS/$CaMKII\delta_2$ (Western Blot) and increased iNOS activity. The CaMKII inhibitor KN-93 abolished all the LPS-effects.

Conclusion: These results indicate that the Ca^{2+}/$CaMKII\delta_2$ signaling pathway may be an important regulator of iNOS activity in diabetes, and hence contribute to the potential development of innovative therapeutic strategies for vascular complications in diabetes.

1. Introduction

Cardiovascular disease is one of the main causes of high morbidity and mortality in people with diabetes mellitus [1]. Although it has been shown to be associated with endothelial dysfunction and it is known that endothelial-dependent vasodilation is significantly impaired in diabetic patients [2], whether and how elevated glucose levels might affect nitric oxide (NO) synthesis and bioavailability is still a matter of controversy [3,4]. NO plays a key role in preserving physiological endothelial function, it is mainly produced by endothelial nitric oxide synthase (eNOS) and it plays a pleiotropic role in preserving and mediating vascular wall functions and proliferative homeostasis [5–10].

Although endothelial cells are by far the main site of vascular NO synthesis, VSMCs do express all NOS isoforms and NO synthesis in VSMCs might be important in vessel function [11].

Interestingly, our previous work showed that in rat aortic VSMCs chronic hyperglycemia could induce a cell proliferative/secretory phenotype associated with increased intracellular oxidative stress and reduced NO bioavailability [12]. Since these cells are directly involved in atherosclerotic plaque formation and development, our data supported the idea that chronic hyperglycemia may increase nitro-oxidative stress, leading to the loss of vascular proliferative and functional homeostasis.

It is known that inducible NOS (iNOS) is the NOS isoform that is mainly expressed in VSMCs, its expression being induced by inflammation and septic shock [13]. In particular, iNOS is expressed in atherosclerotic plaque while local release of large amounts of NO and O^{2-} has been linked to the production of harmful oxidative

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products such as peroxynitrite [14]. Of note, nitrotyrosine levels (NT, a permanent marker of protein nitrosylation) are significantly increased in the vascular wall of diabetic mice, confirming the idea that in diabetes chronic hyperglycemia can increase both oxidative stress and peroxynitrite formation and thus decrease NO availability [15].

However, although diabetes mellitus represents a pro-atherogenic condition and is associated with increased levels of inflammatory markers [16], the *in vivo* or *in vitro* evidence supporting increased VSMC iNOS expression and/or activity is so far very scanty [17].

Recently, discoveries concerning post-translational modifications of iNOS have raised the possibility of complex iNOS regulation separate from transcriptional regulation [18]. In particular, Jones et al. [19] provide evidence for iNOS-specific trafficking and activity regulation by multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), following cytokine induction. CaMKII is a ubiquitous, multifunctional serine/threonine protein kinase with complex structural and auto-regulatory properties [20]. Moreover, supporting the potential involvement of CaMKII in the pathways leading to the loss of vascular functions in diabetes, Yousif et al. [21] have recently demonstrated that inhibition of CaMKII-mediated signaling could be an effective way to antagonize the surge of injury-promoting factor activity in diabetic rats with hypertension. However, how chronic hyperglicemia and/or inflammation may directly or indirectly influence CaMKII activity in diabetes has not been investigated.

Several results show that activation of CaMKII is required for VSMC migration and proliferation [22] and, in particular, CaMKIIδ_2-isoform (the primary CaMKII isoform expressed in undifferentiated cultured rat aortic VSMCs) is co-immunoprecipitated and co-localized with iNOS in an aggresome-like structure that potentially functions as a reservoir for latent iNOS [18,19].

Thus, it is conceivable that activation of CaMKII by phosphorylation may lead in turn to decreased CaMKIIδ_2-iNOS interaction and thus iNOS activation. Supporting this idea, it has recently been demonstrated that cell culture treatment with KN-93, a known inhibitor of CaMKII, significantly inhibits iNOS activity [19].

Although these data suggest the CaMKIIδ_2-iNOS pathway is a contributor to vascular dysfunction in diabetes, no-one so far has investigated the potential post-translational regulation of iNOS activity by CaMKIIδ_2 in a model of VSMCs from diabetic rats.

In the present study we show a greater increase in LPS-induced iNOS activity in VSMCs from diabetic than control rats. Building on this, we investigate whether CaMKII activation may be involved in the abnormal modulation of iNOS activity in a model of cultured VSMCs from diabetic rats.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Use and Care Committee and the investigation conforms to the Directive 2010/63/EU of the European Parliament. Ten male Sprague–Dawley rats (Charles River Laboratories, Lecco, Italy) aged 3–4 weeks (80–100 g) underwent 90% pancreatectomy (diabetic rats, DR) and ten additional animals underwent sham surgery as a control (CR) according to the partially modified technique described by Foglia [23]. Briefly, the animals were anesthetized with 2% inhaled isoflurane and a pre-emptive analgesia was performed by intraperitoneal injection of buprenorphine (0.02 mg/kg body wt.). Since the first week after surgery, the animals that underwent 90% pancreatectomy, showed markedly increased blood glucose values and were therefore considered diabetic.

To obtain thoracic aortas, five–six weeks following surgery (9–10 weeks of age), CR and DR (respective average weights were 235 ± 30 g and 255 ± 45 g) were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body wt.), preceded by subcutaneous injection of buprenorphine (0.02 mg/kg body wt). Briefly, thoracic aortas were rapidly excised sterilely as described by Pandolfi et al. [12].

2.2. Cell cultures and experimental protocols

VSMCs were isolated by explant technique [12] from two pools of aortas respectively obtained from 5 control and 5 diabetic rats. Both CR- and DR-VSMCs were used between passages 4 and 8. When confluent, cells were starved in a quiescent medium (0.1% FBS) for 72 h and then stimulated with 20 μg/ml of LPS (Sigma–Aldrich St. Louis, MO, USA) for 24 h. In some experiments cells were pre-incubated for 40 min before LPS stimulation with 30 μM KN-93 or 10 μM KN-62 (both CaMKII inhibitors), or with 30 μM KN-92 (negative control of KN-93) (all compounds from Calbiochem; San Diego, CA, USA). After stimulation, cells were collected and used for analysis.

2.3. Semi-quantitative RT-PCR

Total RNA was extracted from VSMCs (Nucleospin RNA II kit; M-Medical, Florence, Italy) and quantified. Semi-quantitative RT-PCR for iNOS was performed as described by Pandolfi et al. [12].

2.4. Immunoblotting

For CR- and DR-VSMC protein analysis, 25 μg of proteins were processed to determine the iNOS expression and 15 μg to determine the CaMKII and P-CAMKII expression. Primary iNOS antibodies (1:2500; BD Bioscence, San Joe CA, USA), β-actin (1:10,000; Sigma–Aldrich, St. Louis, MO, USA), CaMKII and P-CaMKII (both1:200; Santa Cruz Biotechnology, S. Cruz, CA, USA) were used. Immune complexes were visualized by ECL Plus detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, England) and data were quantified and processed by Bio-Rad Image Processing and Analysis System [12].

2.5. Immunoprecipitation

For iNOS/CaMKIIδ_2 co-immunoprecipitation 400 μg of proteins were used. Briefly, proteins were incubated overnight with 2 μg of CamKIIδ_2 antibody (kindly provided by professor Harold A. Singer) at 4 °C gently rocking; the day after, 40 μl of 50% slurry protein A/G beads (Santa Cruz Biotechnology, S. Cruz, CA, USA) were added and incubated for 4 h at 4 °C gently rocking. Immunoprecipitates were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Thereafter, membranes were blocked and then incubated with primary antibodies (iNOS 1:2500) overnight at 4 °C. The co-immune complexes were quantified and processed as described above.

2.6. NOS activity

NOS activity was evaluated by measuring the conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline as described by Pandolfi et al. [12]. NOS activity was expressed as pmoles citrulline/min/mg protein.

2.7. Immunofluorescence analysis

Semi-confluent VSMCs were stimulated as described above and then fixed and permeabilized. For nitrotyrosine (NT)