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Review

New Insight into the Role of Nitric Oxide Pathways in Pancreas

Igor Buchwalow[1], Jürgen Schnekenburger[2], Vera Samoilova[1], Werner Boecker[1], Joachim Neumann[3] and Katharina Tiemann[1]

[1]Institute for Hematopathology Hamburg, Fangdieckstr. 75a, 22547 Hamburg, Germany, [2]Biomedical Technology Center, Westfälische Wilhelms-University Münster, 48149 Münster, Germany and [3]Institute for Pharmacology and Toxicology, Medical Faculty, Martin-Luther-University Halle-Wittenberg, 06112 Halle (Saale), Germany

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Nitric oxide (NO) is generated by a family of enzymes termed NO synthases (NOS) that convert L-arginine to NO and citrulline. The role of NO as an important biological mediator and recognition of the pathophysiological significance of superoxides/NO interaction has led to an intensive research and development of therapies based on the interception of the NO signaling cascade in the pancreatitis course. However, the presence and localization of the NO-generating enzymes in various organs including pancreas are subject to controversy. We assumed that this controversy might reflect rather the diversity of experimental approaches and an insufficient sensitivity of the methods used. Applying tyramide signal amplification (TSA) immunohistochemical technology, we were able detect all three NOS isoforms both in exocrine and endocrine compartments and in the vasculature in the normal pancreas and in pancreatitis. This also allowed us to demonstrate that oxidative stress runs ahead of NOS up-regulation, which implies that the NO enhancement in the course of pancreatitis is likely to be an adaptive mechanism aimed at maintaining the homeostatic cellular level of the bioactive NO. The aims of this minireview are to describe normal intrapancreatic NO pathways and the role of NO in the pancreatitis course.

Key words: nitric oxide, nitric oxide synthase, pancreas, pancreatitis

I. NO-sGC-cGMP Pathway in Health and Disease

Nitric oxide (NO) is generated by a family of enzymes termed NO synthases (NOS) that convert L-arginine to NO and citrulline. The major target of NO in many tissues is soluble guanylyl cyclase (sGC) [27]. NO activates sGC, and the latter converts GTP to cGMP. cGMP plays a central role in NO signalling and in regulation of physiologic responses [34]. Its intracellular level may be decreased by the complex superfamily of cyclic nucleotide phosphodiesterases [20] that degrade cGMP to its relative inactive isomer, GMP. At this point, the NO message may be abridged or even interrupted. The NO message may also be deteriorated by arginase [42] that wrangles with NOS for the common substrate, L-arginine.

Further, NO can be withdrawn from its regular physiological course by reactive oxygen species (ROS) under inflammatory oxidative stress. ROS, or superoxides, are known as NO scavengers [33]. Consequently, the NO bioavailability gets drastically reduced. As a result, the cell is getting short of bioactive NO and responses with up-regulation of NOS [29]. Combined with ROS and degraded further to reactive nitrogen oxide species (RNOS), NO can damage the cell of its origin and thereby cause a variety of diseases [35, 38]. The scheme in Figure 1 is intended to facilitate an overview of the cross talk between enzymes engaged in the L-arginine-NO-cGMP signaling—NOS isoforms, arginase, sGC and PDE in pancreas and pancreatitis. Mechanisms of developing chronic pancreatitis may differ from those of acute pancreatitis, but finally they induce

Correspondence to: Igor Buchwalow, Institute for Hematopathology Hamburg, Fangdieckstr. 75a, 22547 Hamburg, Germany.
E-mail: buchwalow@pathologie-hh.de

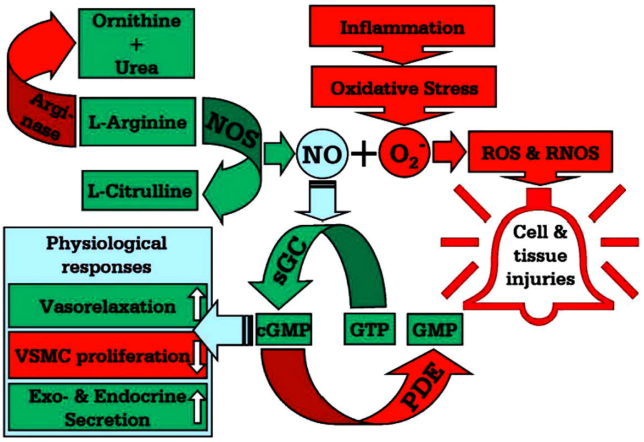

Fig. 1. Factors affecting NO bioactivity and bioavailability in pancreas. NOS and sGC together with their targets and physiological responses in pancreas (vasorelaxation and secretion) are represented in green colour. Negative events activated under pathological conditions (upregulation of arginase and PDE, VSMC proliferation and oxidative stress induced superoxides with resulting cell and tissue injuries) are represented in red colour. White arrows by physiological responses indicate acceleration or deceleration of the process, respectively [11].

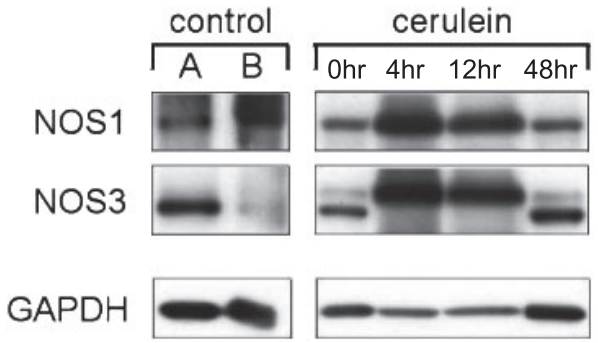

Fig. 2. Western blotting of NOS isoforms in the normal rat pancreas and in cerulein-induced pancreatitis. **A** and **B**: positive controls. 0 hr, 4 hr, 12 hr and 48 hr: time-course experiments. Comparability of protein content in the samples used was confirmed by determination of GAPDH expression (house-keeping protein). These gels are representative of 3 experiments with similar results [39].

modulations of NO pathways leading to similar pancreatic tissue damage.

We have shown earlier that oxidative stress runs ahead of NO-synthase (NOS) up-regulation in pancreatic inflammation [10, 11, 39], which implies that the increased NO generation in the course of acute pancreatitis is likely to be an adaptive mechanism aimed at maintaining the homeostatic cellular level of the bioactive NO.

II. NO-generating Enzymes in Pancreas

Three different NOS isoforms (EC 1.14.13.39) have been described and represent the products of three distinct genes [3, 32]. Neuronal and endothelial NOS (also designated NOS1 and NOS3) were identified in and cloned from neuronal and endothelial cells, respectively, whereas inducible NOS was isolated originally from activated macrophages and was therefore called macrophage NOS (also designated NOS2).

The presence and localization of the NO-generating enzymes NOS in various organs including pancreas are subject to controversy. By many authors a high expression of the constitutive NOS isoforms (NOS1 and NOS3) and the inducible NOS isoform (NOS2) was detected exclusively in pancreatic islets but not in the exocrine compartment or in the vasculature [5, 15, 25, 35, 45].

Subsequently it has been, however, reported that NOS3 and NOS1 are constitutively expressed in pancreas in the vasculature and in neurons, respectively, and that NOS3, also in acinar cells, whereas inducible NOS (NOS2) was not detected in either control or during the acute pancreatitis time-course experiment [17]. These authors ascribed to NOS3 the role of the main isoform in the pancreas and assumed that NOS3 is protective in the initiation of caerulein-induced acute pancreatitis, though this NOS isoform expression in their experiment did not change during early and inflammatory phases of acute pancreatitis. Contrariwise in our study on the same pancreatitis model, Western blotting of pancreatic NOS protein expression during 48 hr pancreatitis time-course experiments revealed marked NOS1 and NOS3 up-regulation at the time points of 4 and 12 hr [39], which is in agreement with data on NO overproduction by acinar cells found in response to cerulein hyperstimulation at these time points [7]. Remarkably, we detected double bands for NOS3 with a pronounced molecular weight shift to a higher molecular weight at the time points of 4 and 12 hr (Fig. 2). A double protein band for NOS3 was also found earlier in the Western blots of blood vessels [14]. The shift in molecular weight of NOS3 during an acute pancreatitis may account for a strong phosphorylation of the protein. Phosphorylation of NOS3 at several sites is involved in the activation of nitric oxide synthesis [18].

III. State-of-the-art Immunohistochemical Technology for NOS Detection *In Situ*

Conflicting conclusions reached earlier by different laboratories about the NOS localization in pancreas rather reflect the diversity of experimental approaches and an insufficient sensitivity of conventional indirect immunohistochemical methods. In order to verify the current knowledge about NOS expression in pancreas, we resorted to a highly sensitive tyramide-signal-amplification (TSA) technology increasing antigen detectability up to 100-fold compared with customary immunohistochemical methods (Fig. 3).

TSA technology was developed by Litt's group [9] at DuPont NEN (now a part of PerkinElmer Corporation) and licensed to Molecular Probes for in-cell and in-tissue applications. TSA permits to reduce the working concentration

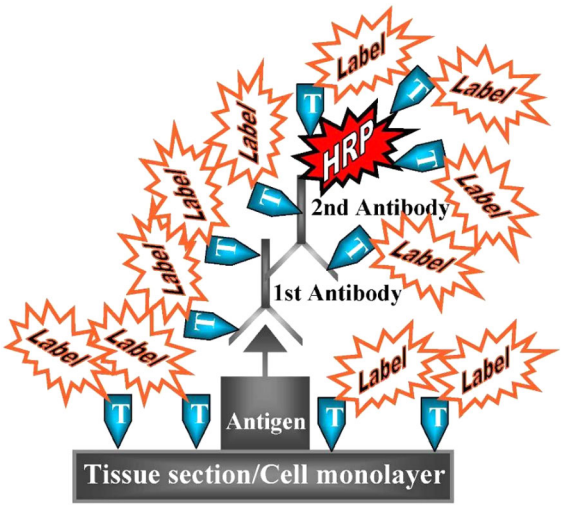

Fig. 3. Tyramide signal amplification. "T" is the labeled tyramine and "HRP", horse radish peroxidase. The "Label" can be a fluorochrome or biotin. The fluorochrome can be visualized directly in a fluorescence microscope. Biotin can be visualized via labeled streptavidin [12].

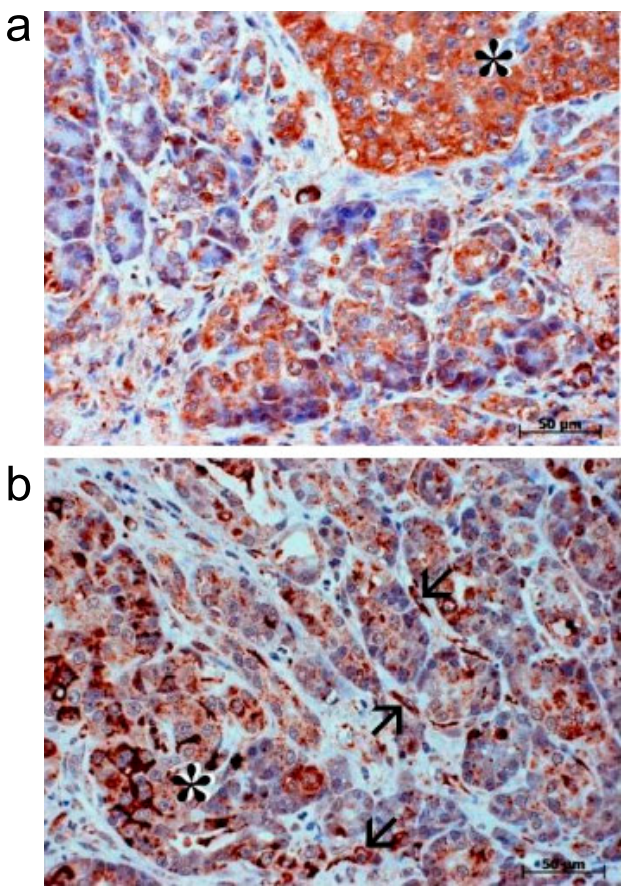

Fig. 4. Localization of NOS in the normal human pancreas. NOS1 (**a**) and NOS3 (**b**) in the normal human pancreas preferentially in the apical cytoplasm of acinar cells with a markedly stronger immunostaining of Langerhans islets (asterisks). NOS3 was additionally observed in capillaries (arrows). DAB-HRP staining, tyramide-biotin blast amplification, nuclei are counterstained with haematoxylin [11].

of primary antibodies bringing the background staining to a negligible level and thereby increasing the specificity of immunolabeling [44]. The use of TSA technique is nowadays documented for immunohistochemical staining as well as for *in situ* hybridization and immunoelectron microscopy [1, 12]

This platform allowed us to detect NOS (constitutive isoforms, both NOS1 and NOS3, and an inducible isoform, NOS2) in the human and rat pancreas not only in pancreatic islets, but also in the exocrine compartments and in the vasculature [10, 11, 39]. As an example, we show here the localization of NOS1 and NOS3 with the use of TSA technology in the normal human pancreas in the cytoplasm of acinar cells and in capillaries with a markedly stronger immunostaining of Langerhans islets (Fig. 4 and Fig. 5a).

Application of TSA technology permitted to detect all three NOS isoforms in vascular smooth muscle cells both in the normal pancreas and in pancreatitis [10, 11, 39], like

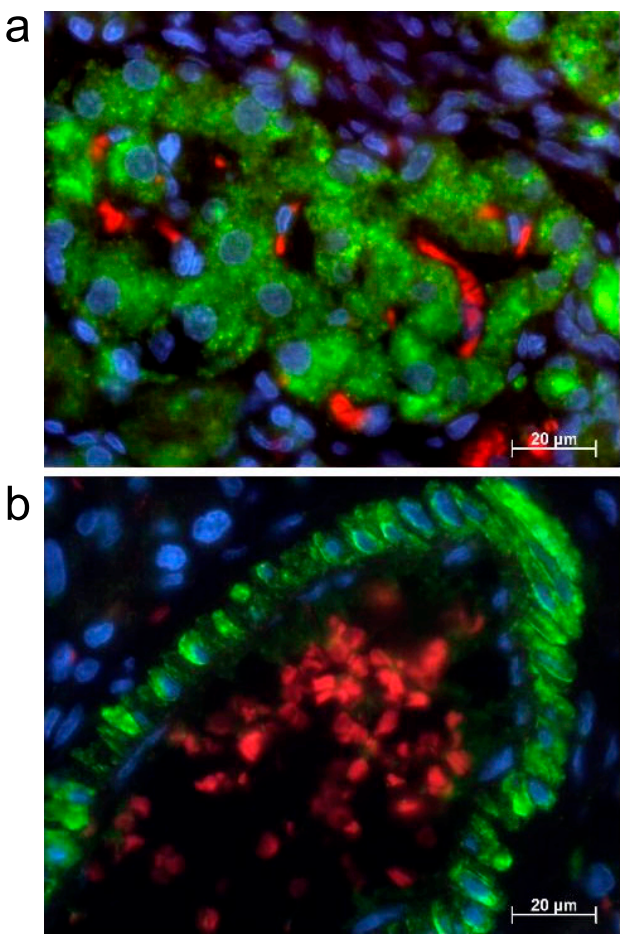

Fig. 5. Immunofluorescent demonstration of NOS1 (TSA-FITC, green channel) in acini and in an arteriole in the normal rat pancreas. (**a**) In acini, a stronger immunostaining is visible in secretory granules. (**b**) In an arteriole, NOS1 is localized in the media in smooth muscle cells. Red autofluorescence of erythrocytes was captured with a filter exciting the autofluorescence in the red spectrum under an exposure longer than with the filter exciting specific fluorescence in the green spectrum. Nuclei are counterstained with DAPI (blue channel).

it was earlier shown by us for other tissues [14]. Our findings of NOS expression in vascular smooth muscle cells (Fig. 5b) suggest an alternative mechanism by which NOS expression in medial cells may locally modulate vascular functions independently of the so called endothelial derived relaxing factor (EDRF) [13] being directly involved in haemodynamic and microcirculatory disturbances associated with acute pancreatitis. This might be indicative of an autocrine fashion of NO signaling in the regulation of the local vascular tone. In accord with our earlier report [14], endothelial cells in pancreas were found to express all three NOS isoforms in blood vessels of smaller diameter, whereas the intima of larger blood vessels revealed as a rule a positive immunoreaction for NOS1 and NOS3. Endothelial cells of capillaries were immunostained only for NOS3.

Our data on NOS expression in the exocrine parenchyma, especially in ductal cells and in duct radicles including centroacinar cells [11], is in line with reports about an active involvement of NO signaling in the regulation of water and secretion of bicarbonate and chloride ions in pancreatic ductal cells [23, 26]. Taken together with reports from other groups about NOS localization in endocrine and exocrine secretory granules [19, 25], our findings of NOS and other enzymes engaged in NO signaling in secretory cells [11] may imply an involvement of NO signaling in maturation and/or concentration of zymogens in zymogen granules.

We have also shown that in Langerhans islets NOS1 and NOS2 were immunolabeled generally equally in all islet cells, whereas NOS3 revealed a stronger preferential immunostaining in single scattered cells that apparently might correspond to the cells that were earlier reported by other authors as somatostatin-, glucagon- or insulin-immunoreactive cells [5, 15, 25, 45]. This might be indicative of an autocrine fashion of NO signaling in the regulation of endocrine as well as of exocrine secretion.

IV. NO in Pancreatitis: Functions or Malfunctions?

Both beneficial [6, 17, 24, 31, 36, 40, 43] and detrimental [2, 4, 8, 16] consequences of induced NO synthesis in pancreatitis have been described. The obvious controversy and confusion in this area necessitates a more holistic interpretation of NO pathways at the cellular level with a reassessment of the role of NO in pathology.

NO indeed plays a destructive role in conjunction with superoxides [37]. Therefore, up-regulation of NOS has been implicated in the initiation of pancreatic tissue damage and impairment of the pancreatic microcirculation in pancreatitis. However, exogenous NO donors revealed a beneficial effect on edema formation in acute pancreatitis and conferred still more important protection against ectopic trypsinogen activation, which correlated with mortality, inflammation, and necrosis [43]. The susceptibility to cerulein toxicity in inducible NOS-deficient mice was abolished by NO donor treatment, which supports the view that NO plays a protective role in acute pancreatitis [36]. NO-releasing drugs such as glyceryl trinitrate have been used in the treatment of ischemic heart disease for more than a century and proved presently to be beneficial also in the treatment of acute pancreatitis or pancreatitis following endoscopic retrograde cholangiopancreatography [41]. The ability of NO to limit endothelial activation and inhibit leukocyte adhesion contributed to its anti-inflammatory properties in acute pancreatitis [22, 28]. NO synthase inhibitors were reported to inhibit pancreatic secretion *in vivo* and to aggravate cerulein induced pancreatitis [40]. They also enhanced the ultrastructural degenerative alterations in the pancreatic acinar cells in the course of caerulein-induced acute pancreatitis thus confirming the protective role of endogenous NO in this disease [6].

Taken together, these data imply that an enhanced NOS expression in pancreatitis is an adaptive mechanism, which needs to be supported by corresponding therapies. However, aggravating effects of NO have also been described [2, 4, 8, 16]. This predicament necessitates the reassessment of the NO role in pathology with a more holistic interpretation of NO pathways and the associated metabolism (summarized in Fig. 1). Pancreatitis is associated with oxidative stress and concomitantly with overproduction of superoxides, ROS and RNOS. Known as NO scavenger, superoxides remove NO from its regular physiological course thereby reducing NO bioactivity and bioavailability. In inflammatory diseases like pancreatitis, superoxides up-regulate the enzymes engaged in the L-arginine-NO-cGMP signaling, including PDE and arginase [21, 42]. This results in further reduction of the NO bioactivity. PDE deteriorates NO signaling by degrading cGMP to its inactive isomer, GMP, whereas arginase restricts NO generation on a competitive basis. As a result, the cell is getting short of bioactive NO and responses with up-regulation of NOS and sGC.

Thus, the boundary between biology and pathobiology of the L-arginine-NO-cGMP signaling is demarcated by the delicate balance between superoxides production rate and the ability of the body to maintain the homeostatic cellular level of the bioactive NO. Therapeutic interventions that attenuate the superoxide attack and inflammatory responses within the pancreas in acute pancreatitis or restore the bioactive NO level with exogenous NO donors [20, 30, 43] might be useful adjuvants in the treatment of acute pancreatitis. Our findings on the co-localization and cooperative expressional fluctuation of the enzymes engaged in the L-arginine-NO-cGMP signaling in parenchyma and vasculature in both normal pancreas and in pancreatitis are indicative of an autocrine fashion of NO signaling in pancreas [11]. Previously, we have shown that oxidative stress runs ahead of NOS up-regulation, which implies that the NO enhancement in the course of pancreatitis is likely to be an adaptive mechanism aimed at maintaining the homeo-

static cellular level of the bioactive NO. This experimental paradigm provides a direct and causal link between oxidative stress and the enzymatic control of NO bioavailability at the cellular level, which endows with further insight into fundamental mechanisms underlying the processes of secretion and might have implications in the interception of the L-arginine-NO-cGMP signaling enzyme cascade for designing new adjunctive therapies for pancreatic disorders. Restoration of the bioactive NO level with exogenous NO donors or supplementation with antioxidants that are deficient in patients with pancreatitis might be a feasible option for designing new adjunctive therapies for pancreatic disorders.

V. Competing Financial Interests

The authors declare no competing financial interests.

VI. References

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