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L-arginine-NO-cGMP signalling pathway in pancreatitis

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The role of nitric oxide (NO) in the human pancreas and in pancreatitis still remains controversial. Furthermore, conflicting conclusions have been reached by different laboratories about the localization of the NO-generating enzyme (NO synthase, NOS) in the pancreas. Here, we investigated the co-expression of NOS with enzymes involved in regulation of NO signalling in the normal human pancreas and in pancreatitis. We found that the whole NO signalling machinery was up-regulated in pancreatitis, especially within the exocrine compartment. Furthermore, the exocrine parenchymal cells revealed higher levels of oxidative stress markers, nitrotyrosine and 8-hydroxyguanosine, in pancreatitis, which reflects the exceptional susceptibility of the exocrine parenchyma to oxidative stress. This study provides a direct link between oxidative stress and the enzymatic control of the NO bioavailability at the cellular level and endows with further insight into fundamental mechanisms underlying pancreatic disorders associated with disruptions in the L-arginine-NO-cGMP signalling enzyme cascade.

Recognition of pathophysiological significance of nitric oxide (NO) as an important biological mediator has led to an intensive research and development of therapies based on the interception of the L-arginine-NO-cGMP signalling cascade. The boundary between biology and pathobiology of the L-arginine-NO-cGMP signalling is demarcated by the delicate balance between superoxide production rate and the ability of the body to maintain the homeostatic cellular level of the bioactive NO[1]. NO is generated by a family of enzymes termed NO synthases (NOS) that convert L-arginine to NO and citrulline. The major target of L-arginine-derived NO in many tissues is soluble guanylyl cyclase (sGC)[2]. NO activates sGC, and the latter converts GTP to cGMP. cGMP plays a central role in NO signalling and in regulation of physiologic responses[1]. Its intracellular level may be decreased by the complex superfamily of cyclic nucleotide phosphodiesterases[3] 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[4] that wrangles with NOS for the common substrate, L-arginine. Furthermore, NO can be withdrawn from its regular physiological course by reactive oxygen species (ROS) under inflammatory oxidative stress.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[5]. 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 and enzymes engaged in detoxification of ROS, such as catalase and superoxide dismutase[6]. 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[1,7]. However, the role of NO in the course of pancreatitis remains controversial. Both beneficial[8–12] and detrimental[13–16] consequences of induced NO synthesis in pancreatitis have been described. Moreover, the presence and localization of the NO-generating enzyme NOS in the exocrine pancreatic parenchyma are far from being elucidated. As yet, the three NOS isoforms (NOS1, NOS2 and NOS3) could be detected in pancreatic islets, but not in the exocrine compartment and not in the vasculature[17–20].

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. The aim of this study was the direct *in situ* visualization of enzymes involved in L-arginine-NO-cGMP signalling in the normal human pancreas and in pancreatitis. Here, we examined expression of the three NOS isoforms, arginase, sGC, PDE as well as of oxidative stress markers, nitrotyrosine and 8-hydroxyguanosine (8OHG).

Results

To start with, we had to re-examine the NOS expression in the normal pancreas in the light of new evidence. As earlier demonstrated by other groups employing conventional immunocytochemical techniques[17–20], all three NOS isoforms including the inducible isoform, NOS2, could be detected in pancreatic islets, but not in the exocrine compartment and not in the vasculature. In this study, we used a highly sensitive tyramide signal amplification (TSA) technique[21,22] and revealed all three NOS isoforms (NOS1, NOS2 and NOS3) not only in pancreatic islets but also in the exocrine compartments (**Figs. 1a–c**) with a dramatic up-regulation in pancreatitis (**Figs. 1d–e**). Along with NOS up-regulation in pancreatitis, we observed a transformation of acinar cells with their conversion to the morphology of ductal cells (**Figs. 1d, e**). Strong NOS immunostaining was also observed in infiltrated inflammatory cells in areas adjacent to the affected parenchyma zones (**Figs. 1f**).

Immunostaining of arginase, sGC and PDE showed striking parallels with that of NOS in exocrine glandular cells and in pancreatic islets. Figure 2 shows preferential expression of arginase in centroacinar and ductal cells in the normal human pancreas (**Fig. 2a**) with an up-regulation in pancreatitis (**Fig. 2b**). A similar expression pattern was registered also for sGC (**Fig. 2c**) and PDE (**Figs. 2d** and **3a**). PDE was additionally found, like NOS3 (**Fig. 2c**), also in capillaries (**Fig. 2d**).

In Langerhans islets, NOS1 (**Fig. 1a**), NOS2 (**Fig. 1b**), arginase and sGC (**Fig. 2a, c**) were immunolabelled generally equally in all islet cells, whereas NOS3 (**Fig. 1c**) and PDE (**Fig. 2d**) revealed a stronger preferential immunostaining in single scattered cells. In pancreatitis, we were unable to register any noteworthy increase in NOS expression in pancreatic islets possibly because of a rather high positive basal level of immunostaining in the islets of normal pancreas. A high expression of the constitutive NOS isoforms, NOS1 and NOS3, and the inducible NOS isoform, NOS2, was found in pancreatic islets also by other authors[17–20].

In the vasculature, all three NOS isoforms were found to be co-expressed with sGC, arginase and PDE in vascular smooth muscle cells (VSMC) both in the normal pancreas, like it was earlier shown by us for other tissues[23,24], and in pancreatitis. Constitutive expression of sGC, arginase and PDE in VSMC was also reported earlier by other groups[25,26]. In an exemplary way, we show here the localization of PDE in VSMC and intima of a blood vessel in the normal pancreas (**Fig. 3b**). In accord with our earlier report[23], endothelial cells in pancreas were found to express all three NOS isoforms in blood

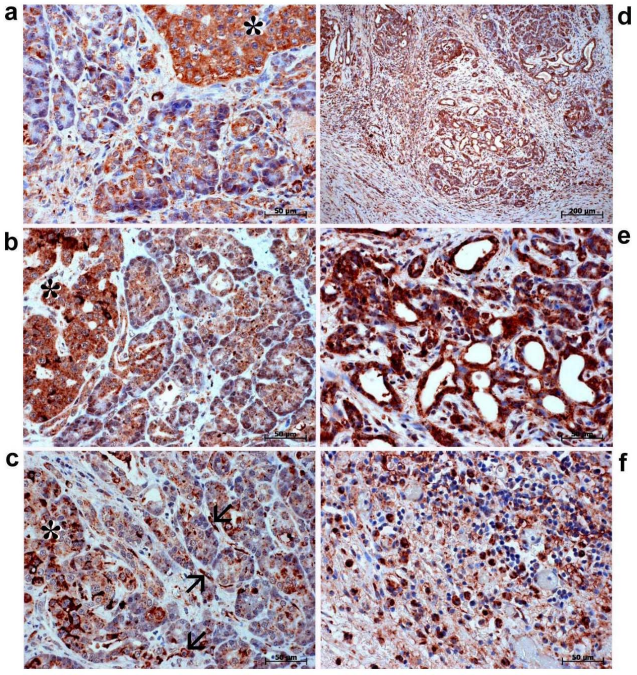

Figure 1 | Localization of NOS in the normal human pancreas and in pancreatitis. NOS1 (a), NOS2 (b) and NOS3 (c) in the normal human pancreas preferentially in the apical cytoplasm of acinar cells with a markedly stronger immunostaining of Langerhans islets (asterisks). NOS3 (c) was additionally observed in capillaries (arrows). (d–f) Enhanced expression of NOS1in the exocrine parenchyma in pancreatitis shown by lower (d) and higher (e) magnification. Similar up-regulation in pancreatitis was observed also for NOS2 and NOS3 (not shown). (f) Strong expression of NOS1 in infiltrated inflammatory cells in an area adjacent to pancreatitis zones. DAB-HRP staining, tyramide-biotin blast amplification, nuclei are counterstained with haematoxylin.

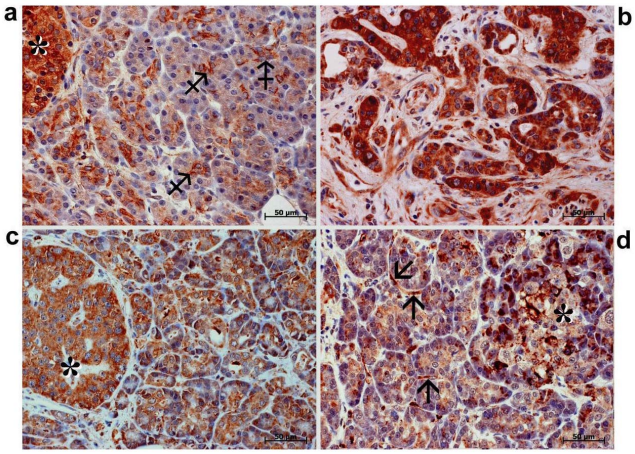

Figure 2 | Localization of arginase, sGC and cGMP-specific PDE V in the human pancreas and in pancreatitis. (a) Localization of arginase in the normal human pancreas preferentially in centroacinar cells and in duct radicles (tailed arrows) with a markedly stronger immunostaining of Langerhans islets (asterisks). (b) Enhanced expression of arginase in the exocrine parenchyma in pancreatitis. Similar up-regulation in pancreatitis was observed also for sGC and cGMP-specific PDE (not shown). Localization of sGC (c) and PDE (d) in the human pancreas preferentially in the apical cytoplasm of acinar cells with a markedly stronger immunostaining of Langerhans islets (asterisks). cGMP-specific PDE (d) was additionally observed in capillaries (arrows). DAB-HRP staining, tyramide-biotin blast amplification, nuclei are counterstained with haematoxylin.

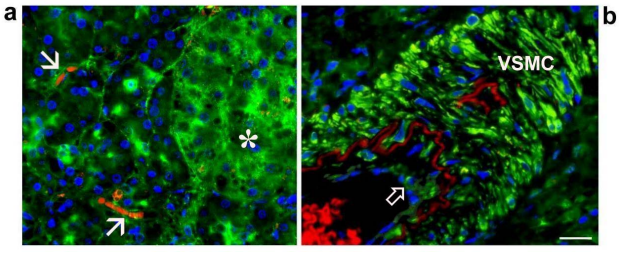

Figure 3 | Immunofluorescent (FITC-tyramide) demonstration of cGMP-specific PDE V in the human pancreas. (a) Preferential immunostaining of cGMP-specific PDE V in the apical cytoplasm of acini and capillaries (arrows) with a markedly stronger staining of Langerhans islets (asterisks). (b) cGMP-specific PDE V in media (vascular smooth muscle cells, VSMC) and intima (hollow arrow) in a diagonal section of a blood vessel of the normal pancreas. Red color in (a) and (b) accounts for autofluorescence of the lamina elastica interna and that of erythrocytes. Nuclei are counterstained with DAPI. 20 μm scale bar for entire layout.

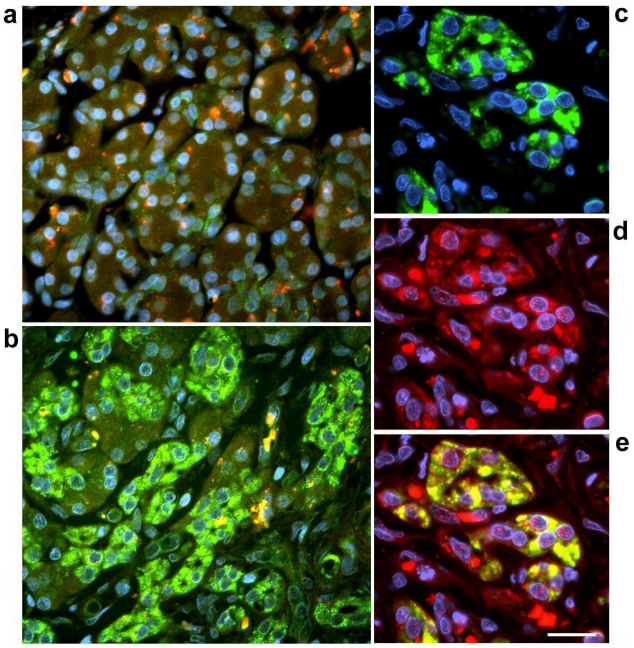

Figure 4 | Oxidative stress in pancreatitis. 8-OH-dG immunostaining with FITC-tyramide (green) is insignificant in the normal pancreas (a), but it markedly increases in the exocrine parenchyma in pancreatitis (b). Yellow and orange colour accounts for autoflurescent tissue components, especially erythrocytes. (c–e) Double immunolabelling of NOS2 and nitrotyrosine in pancreatitis. Composite image (e) exhibits a hybrid orange colour resulting from a coincidence of two labels – FITC-tyramide for NOS2 (c, green) and Cy3 for nitrotyrosine (d, red). Pure red in the composite image accounts for autofluorescence of erythrocytes. Nuclei are counterstained with DAPI. 20 μm scale bar for entire layout.

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 (**Fig. 1c**).

In view that oxidative stress has been asociated with the pathophysiology of chronic pancreatitis[7], we performed immunolabelling of oxidative stress footprints - nitrotyrosine and 8OHG (**Fig. 4**). Nitrotyrosine is considered a marker of NO-dependent, reactive nitrogen species-induced nitrative stress and has been identified as an indicator of cell damage and inflammation. 8OHG is produced by oxidative damage of RNA. Figure 4 demonstrates a dramatic increase in immunostaining with goat antiserum to 8OHG in pancreatitis vs. normal pancreas (**Figs. 4a, b**). According to the manufacturer (Serotec), this antiserum cross-reacts also with 8-hydroxydeoxyguanosine (8OHdG). Nuclear immunostaining due to 8-hydroxydeoxyguanosine was less prominent than that of the cytoplasm, which is indicative of a pronounced preferential oxidative damage of cytoplasmic nucleic acids. 8OHG-positive cells in pancreatitis could be easily identified as cells corresponding to ductal phenotype. In pancreatitis, the same cellular phenotype appeared also as a target for enhanced nitrotyrosine. A co-expression with the oxidative stress markers - 8OHG and nitrotyrosine - was observed for other runners of L-arginine-NO-cGMP signalling pathway. In an exemplary way (**Figs. 4c–e**), we show here a co-expression of nitrotyrosine with the inducible NOS isoform - NOS2. In the normal exocrine pancreas, we could not detect any discernible nitrotyrosine immunostaining, whereas pancreatic islets revealed a significant nitrotyrosine immunoreactivity (not shown) like it was earlier reported by Nakazawa et al.[27]. It is noteworthy, that despite the persistent presence of nitrotyrosine[27] and NOS2[17–20] in pancreatic islets, there is no evidence that this might have a deleterious effect on the islet viability or function[28].

Discussion

The state-of-the-art immunohistochemical technology (TSA) permitted us for the first time to demonstrate in the pancreas the principal runner of the L-arginine-NO-cGMP signalling pathway, NOS, not only in Langerhans islets as reported earlier[17–20], but also in the exocrine parenchyma. Immunostaining of arginase, sGC and PDE showed striking parallels with that of NOS expression in both exocrine glandular cells and in pancreatic islets. The co-expression of enzymes engaged in L-arginine-NO-cGMP signalling is indicative of an autocrine fashion of NO signalling in pancreas parenchymal cells, like it was earlier demonstrated by us for VSMC in the vasculature[24].

Along with the up-regulation of the enzymes engaged in NO signalling, the most significant feature of pancreatitis is a transformation of acinar cells. Acinar cells were earlier reported to lose progressively their zymogen granules and subsequently convert to the morphology of ductal cells[29]. Strobel et al.[30], however, noted that transdifferentiation from acinar to ductal cells has never been confirmed in the adult pancreas. These authors believe that the ductal component appears more resistant and could proliferate giving rise to a tubular network described as "ductal metaplasia" or "acinar-to-ductal transdifferentiation"[30]. A similar phenomenon in the thyroid was also explained by a higher vulnerability of the acinar thyroid cells[31]. The reduced population of acinar cells on account of a ductal cellular phenotype may be the reason for disorders in the rates of both protein synthesis and intracellular transport of secretory proteins observed in pancreatitis[29,32]. This might also explain a marked diminishing of water and bicarbonate secretion with pancreatic juice observed in chronic pancreatitis patients[33].

NOS expression in the exocrine parenchyma, especially in ductal cells and in duct radicles including centroacinar cells, is in line with reports about an active involvement of NO signalling in the regulation of water and secretion of bicarbonate and chloride ions in pancreatic ductal cells[33,34]. Taken together with reports from other groups about NOS localization in endocrine and exocrine secretory granules[19,35], our findings of NOS and other enzymes engaged in NO signalling in secretory cells may imply an involvement of NO signalling in maturation and/or concentration of zymogens in zymogen granules. 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[17–20]. This point definitely deserves further studies.

In the vasculature, all three NOS isoforms were found to be co-expressed with sGC, arginase and PDE in VSMC both in the normal pancreas, like it was earlier shown by us for other tissues[23,24], and in pancreatitis. Our findings of NOS expression in VSMC suggest an alternative mechanism by which NOS expression by medial cells may locally modulate vascular functions in an endothelium-independent manner[36]. PDE and arginase were reported to counteract NO-mediated vasodilatory and antiproliferative function[25,26] and therefore may be involved in haemodynamic and microcirculatory disturbances associated with acute pancreatitis.

And, last but not least, we have demonstrated the co-localization of the oxidative stress footprints - nitrotyrosine and 8OHG- with the enzymes engaged in NO signalling in the pancreas revealing a high vulnerability of the exocrine parenchyma, especially acini, to oxidative stress induced by pancreatitis. In pathophysiological states, NO formation and its bioavailability is impaired by oxidative stress[1], and patients with pancreatitis develop a systemic inflammatory response syndrome[37]. Superoxides, well known as NO scavengers, drastically reduce the NO bioavailability. As a result, the cell is getting short of bioactive NO and responses with NOS up-regulation. The ability of

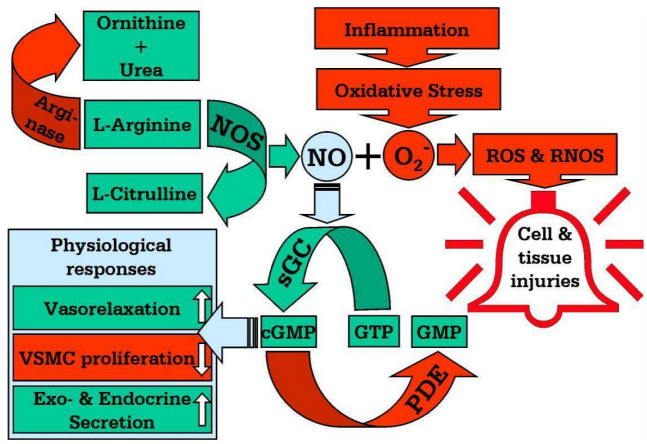

Figure 5 | Factors affecting NO bioactivity and bioavailability in pancreas. NOS and sGC together with their targets and physiological responses in pancreas (vasorelaxation and secretion) are represented in green colour. Negative events activated under pathological conditions (up-regulation of arginase and PDE, VSMC proliferation and oxidative stress-induced superoxides with resulting cell and tissue injuries) are represented in red colour.

the body to maintain the homeostatic cellular level of bioactive NO is determined by the interplay between the main runners of L-arginine-NO-cGMP signalling pathway in the pancreas (**Fig. 5**). Cytokines and other mediators including endotoxins can also activate NOS expression resulting in overproduction of NO[3,15].

It is thought that NO may play a destructive role in conjunction with superoxides[37]. On the other hand, exogenous NO donors have been reported to exert a beneficial effect on edema formation in acute pancreatitis conferring still more important protection against ectopic trypsinogen activation, which correlated with mortality, inflammation, and necrosis[12]. NO-releasing drugs such as glyceryl trinitrate have been used in 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[38]. Along with NO-releasing drugs, stimulators of sGC[1], radical scavengers like superoxide dismutase/catalase[37] and inhibitors of PDE[3] have also been successfully used in efforts to normalize the NO bioactivity and availability in pancreatitis.

To summarize, we have illustrated the co-localization of the main runners of L-arginine-NO-cGMP signalling pathway in the pancreas in both the endo- and exocrine secretory compartments, as well as in the vasculature. This might be indicative of an autocrine fashion of NO signalling in the regulation of both secretion and local vascular tone. Increased superoxides in the exocrine parenchyma indicate a high vulnerability of this cellular compartment to oxidative stress. The state-of-the-art immunohistochemical technology permitted us for the first time to demonstrate NOS in the pancreas not only in Langerhans islets but also in the exocrine parenchyma. This report endows with further insight into fundamental mechanisms underlying pancreatic disorders and might have implications in the interception of the L-arginine-NO-cGMP signalling enzyme cascade for designing new adjunctive therapies for pancreatitis.

Methods

Case selection. Routinely processed formalin-fixed paraffin-embedded tissue probes were retrieved from the files of the Institute of Pathology of the University of Muenster and the Institute for Hematopathology, Hamburg, Germany. Pancreatic tissue specimens from 9 Caucasian men (n = 6) and women (n = 3) (age from 55 to 76 years) suffering from chronic pancreatitis were obtained by surgical resection and classified on the basis of clinical features and histological findings at least by four pathologists independently (W.B., K.T., A.B. and C.P.). Informed consent was obtained from all subjects. Patients with malignant diseases or clinical signs of infection were excluded from the study. Pancreatic tissues from 3 organ donors served as controls. The procedure for the use of human tissue from surgical pathology was approved by the institutional review boards and done in accordance with the ethical standards of the regional committees on human studies.

Antibodies and immunohistochemical staining. For paraffin embedding, tissue probes were routinely fixed with 4% formaldehyde in PBS, pH 7.4. PBS was used for all washes and dilutions. Paraffin tissue sections (4 mμ thick) were deparaffinized with xylene and graded ethanol, and antigen retrieval was achieved by heating the sections in 10 mM sodium citrate buffer, pH 6.0, at 95°C for 30 min. After antigen retrieval, sections were immunoreacted with primary antibodies (Abs) over night at 4°C. All steps were preceded by rinsing with PBS (pH 7.4). Immunostaining was performed according to the standard protocol routinely used for immunohistopathology[21]. We recently reported that endogenous Fc receptors in routinely fixed cells and tissue probes do not retain their ability to bind Fc fragments of antibodies[39]; therefore, blocking the endogenous Fc receptors prior to incubation with primary antibodies was omitted. Characterisation of rabbit primary Abs recognising NOS1, NOS2 and NOS3 (Transduction Laboratories, Lexington, KY) including Western blotting were described elsewhere[23]. Primary anti-NOS Abs were diluted to a final concentration of 0.5 – 1 μg/ml. Polyclonal rabbit antisera raised against soluble guanylate cyclase and against phosphodiesterase V were from Calbiochem-Novabiochem GmbH, Bad Soden, Germany) and used at a dilution of 1 : 100 and 1 : 250, respectively. Rabbit Abs to arginase was purchased from Polyscience, Inc., Warrington, PA. They were applied at a final concentration of 1 μg/ml. Goat antiserum to 8-hydroxyguanosine (8OHG, a marker of oxidative damage to nucleic acids, purchased from Serotec Ltd, Oxford, UK) was applied at a dilution of 1 : 200. According to the manufacturer, this antiserum cross reacts also with 8-hydroxydeoxyguanosine (8OHdG), which was also proved by us with preabsorption control with a 10-fold molar excess of corresponding substrates (both from Calbiochem). Monoclonal mouse anti-nitrotyrosine Abs (Zymed Laboratories Inc., South San Francisco, USA) was applied at concentration of 5 μg/ml. After immunoreacting with primary Abs and following washing in PBS, the sections were treated for 10 min with methanol containing 0.6% H_2O_2 to quench endogenous peroxidase. Additionally, the sections were treated with avidin-biotin blocking kit (Vector Laboratories, USA) in the case of the following use of biotin-tyramide amplification for detection of NOS, sGC, PDE and arginase.

Bright-field microscopy. For bright-field microscopy, bound primary Abs to NOS, sGC and PDE and arginase were visualized with blast amplification[21] employing AmpliStain™ HRP conjugate (SDT GmbH, Baesweiler, Germany) and biotin-labeled tyramine in combination with HRP-avidin-biotin complex (Vectastain "Elite" ABC-kit, Vector Laboratories, USA). Biotin-labeled tyramine conjugate was synthesised by us[21] from tyramine-HCl (Sigma) and biotin-succinimidyl ester (Sulfo-NHS-LC-biotin, #127062-22-0 from Thermo Scientific, Pierce, USA) in DMF. Incubation with biotin-tyramine conjugate was carried out at a dilution of 1 : 1000 in PBS in the presence of 0.02% H_2O_2 for 3–5 min. To visualize the HRP label, the samples were incubated with DAB or NovaRed Kits (both from Vector Laboratories) and counterstained with Ehrlich haematoxylin for 30 sec.

Immunofluorescent microscopy. For fluorescent visualization, bound primary Abs were detected with an AmpliStain™ HRP conjugate (SDT GmbH, Baesweiler, Germany). The HRP label was amplified with FITC-conjugated tyramine. FITC-tyramine conjugate was synthesised by us[21] from tyramine-HCl (Sigma) and FITC-succinimidyl ester (FITC-NHS, #C1311 from Molecular Probes) in DMF. Incubation with FITC-tyramine conjugate was carried out at a dilution of 1 : 500 in PBS in the presence of 0.02% H_2O_2 for 3–5 min. Bound goat anti-8OHG Ab (marker of oxidative damage to proteins) was visualized with FITC-conjugated donkey-anti-goat AB (Dianova, Hamburg, Germany).

To visualize NOS simultaneously with nitrotyrosine (marker of oxidative damage to proteins), sections were immunoreacted with anti-NOS Abs in the presence of mouse monoclonal anti-nitrotyrosine Abs (Zytomed GmbH, Berlin, Germany, applied at a final concentration of 5 μg/ml). Bound anti-nitrotyrosine Abs were detected with Cy3-conjugated AffiniPure goat anti-mouse IgG (H + L) (minimal cross-reaction with human serum proteins, Dianova). Finally, samples were counterstained for 15 sec with DAPI (5 μg/ml PBS; Sigma) and mounted with Vectashield (Vector Laboratories, USA).

Controls. The exclusion of either the primary or the secondary antibody from the immunohistochemical reaction, substitution of primary antibodies with the corresponding IgG at the same final concentration, or preabsorption of primary antibodies with corresponding control peptides resulted in lack of immunostaining. Simultaneous or sequential application of immunosera in the case of double immunolabelling resulted in equally powerful detection of every antigen. The TSA step alone did not contribute to any specific immunostaining that might have influenced the analysis.

Images shown are representative of at least 3 independent experiments which gave similar results. At least 10 images from different fields of view were taken from all immunostatained probes. Three morphologists (I.B., V.S. and W.B.) evaluated the immunostaining intensity independently and stated an obvious increase in the immunolabelling of the oxidative stress markers and enzymes involved in L-arginine-NO-cGMP signalling by all patients under study regardless of the age and gender.

Image acquisition. Immunostained sections were examined on a Zeiss microscope "Axio Imager Z1". Microscopy images were captured using AxioCam digital microscope cameras and AxioVision image processing (Carl Zeiss Vision, Germany). The images were acquired at 96 DPI and submitted with the final revision of the manuscript at 300 DPI.

1. Nossaman, B., Pankey, E. & Kadowitz, P. Stimulators and Activators of Soluble Guanylate Cyclase: Review and Potential Therapeutic Indications. *Critical Care Research and Practice* **2012** (2012).
2. Martin, E., Berka, V., Tsai, A. L. & Murad, F. Soluble guanylyl cyclase: the nitric oxide receptor. *Methods Enzymol* **396**, 478–492 (2005).
3. Gomez-Cambronero, L. *et al*. Pentoxifylline ameliorates cerulein-induced pancreatitis in rats: role of glutathione and nitric oxide. *J Pharmacol. Exp. Ther.* **293**, 670–676 (2000).
4. Waddington, S. N. Arginase in glomerulonephritis. *Kidney Int.* **61**, 876–881 (2002).
5. Napoli, C. & Ignarro, L. J. Nitric oxide and atherosclerosis. *Nitric. Oxide.* **5**, 88–97 (2001).
6. McCord, J. M. Superoxide dismutase in aging and disease: an overview. *Methods Enzymol.* **349**, 331–341 (2002).
7. Tandon, R. K. & Garg, P. K. Oxidative stress in chronic pancreatitis: pathophysiological relevance and management. *Antioxid Redox Signal* **15**, 2757–2766 (2011).
8. Andrzejewska, A. & Jurkowska, G. Nitric oxide protects the ultrastructure of pancreatic acinar cells in the course of caerulein-induced acute pancreatitis. *Int. J. Exp. Pathol.* **80**, 317–324 (1999).
9. Jaworek, J. *et al*. Protective role of endogenous nitric oxide (NO) in lipopolysaccharide--induced pancreatic damage (a new experimental model of acute pancreatitis). *J. Physiol Pharmacol.* **51**, 85–102 (2000).
10. Qui, B., Mei, Q. B., Ma, J. J. & Korsten, M. A. Susceptibility to cerulein-induced pancreatitis in inducible nitric oxide synthase-deficient mice. *Pancreas* **23**, 89–93 (2001).
11. Vaquero, E., Molero, X., Puig-Divi, V. & Malagelada, J. R. Contrasting effects of circulating nitric oxide and nitrergic transmission on exocrine pancreatic secretion in rats. *Gut* **43**, 684–691 (1998).
12. Werner, J. *et al*. On the protective mechanisms of nitric oxide in acute pancreatitis. *Gut* **43**, 401–407 (1998).
13. Al Mufti, R. A., Williamson, R. C. & Mathie, R. T. Increased nitric oxide activity in a rat model of acute pancreatitis. *Gut* **43**, 564–570 (1998).
14. Alhan, E., Kucutulu, U. & Ercin, C. The effects of nitric oxide synthase inhibitors on acute necrotising pancreatitis in rats. *Eur. J. Surg.* **164**, 697–702 (1998).
15. Ayub, K., Serracino-Inglott, F., Williamson, R. C. & Mathie, R. T. Expression of inducible nitric oxide synthase contributes to the development of pancreatitis following pancreatic ischaemia and reperfusion. *Br. J. Surg.* **88**, 1189–1193 (2001).
16. Cuzzocrea, S. *et al*. Inducible nitric oxide synthase-deficient mice exhibit resistance to the acute pancreatitis induced by cerulein. *Shock* **17**, 416–422 (2002).
17. Alm, P., Ekstrom, P., Henningsson, R. & Lundquist, I. Morphological evidence for the existence of nitric oxide and carbon monoxide pathways in the rat islets of Langerhans: an immunocytochemical and confocal microscopical study. *Diabetologia* **42**, 978–986 (1999).
18. Burrell, M. A., Montuenga, L. M., Garcia, M. & Villaro, A. C. Detection of nitric oxide synthase (NOS) in somatostatin-producing cells of human and murine stomach and pancreas. *J. Histochem. Cytochem.* **44**, 339–346 (1996).
19. Lajoix, A. D. *et al*. A neuronal isoform of nitric oxide synthase expressed in pancreatic beta-cells controls insulin secretion. *Diabetes* **50**, 1311–1323 (2001).
20. Yu, W. *et al*. Calmodulin overexpression causes Ca(2+)-dependent apoptosis of pancreatic beta cells, which can be prevented by inhibition of nitric oxide synthase. *Lab Invest* **82**, 1229–1239 (2002).
21. Buchwalow, I. B. & Boecker, W. *Immunohistochemistry: Basics and Methods,* Edn. 1st Edition. (Springer, Heidelberg, Dordrecht, London, New York; 2010).
22. Buchwalow, I. B. Increasing the power of immunohistochemistry. *Proc. Roy. Microsc. Soc.* **36** 57–59 (2001).
23. Buchwalow, I. B. *et al*. Vascular smooth muscle and nitric oxide synthase. *FASEB J.* **16**, 500–508 (2002).
24. Buchwalow, I. B. *et al*. An in situ evidence for autocrine function of NO in the vasculature. *Nitric Oxide* **10**, 203–212 (2004).
25. Wei, L. H., Wu, G., Morris, S. M., Jr. & Ignarro, L. J. Elevated arginase I expression in rat aortic smooth muscle cells increases cell proliferation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 9260–9264 (2001).
26. Zhang, C., Hein, T. W., Wang, W., Chang, C. I. & Kuo, L. Constitutive expression of arginase in microvascular endothelial cells counteracts nitric oxide-mediated vasodilatory function. *FASEB J.* **15**, 1264–1266 (2001).
27. Nakazawa, H. *et al*. Nitrotyrosine Formation and its Role in Various Pathological Conditions. *Free Radic. Res.* **33**, 771–784 (2001).
28. Feng, X. *et al*. Inducible nitric oxide synthetase is expressed in adult but not fetal pig pancreatic islets. *Xenotransplantation.* **7**, 197–205 (2000).
29. Aghdassi, A. *et al*. Animal models for investigating chronic pancreatitis. *Fibrogenesis & Tissue Repair* **4**, 26 (2011).
30. Strobel, O. *et al*. In vivo lineage tracing defines the role of acinar-to-ductal transdifferentiation in inflammatory ductal metaplasia. *Gastroenterology* **133**, 1999–2009 (2007).
31. van Gijlswijk, R. P. *et al*. Fluorochrome-labeled tyramides: use in immunocytochemistry and fluorescence in situ hybridization. *J Histochem Cytochem* **45**, 375–382 (1997).
32. Antonin, W., Wagner, M., Riedel, D., Brose, N. & Jahn, R. Loss of the zymogen granule protein syncollin affects pancreatic protein synthesis and transport but not secretion. *Mol. Cell Biol.* **22**, 1545–1554 (2002).
33. Laugier, R. *et al*. Exocrine pancreatic secretion in normal controls and chronic calcifying pancreatitis patients from Burundi: possible dietary influences. *Digestion* **54**, 54–60 (1993).
34. Ishiguro, H., Steward, M. C., Wilson, R. W. & Case, R. M. Bicarbonate secretion in interlobular ducts from guinea-pig pancreas. *J. Physiol* **495 (Pt 1)**, 179–191 (1996).
35. Garcia-Vitoria, M., Garcia-Corchon, C., Rodriguez, J. A., Garcia-Amigot, F. & Burrell, M. A. Expression of neuronal nitric oxide synthase in several cell types of the rat gastric epithelium. *J. Histochem. Cytochem.* **48**, 1111–1120 (2000).
36. Buchwalow, I. B. *et al*. The role of arterial smooth muscle in vasorelaxation. *Biochem Biophys Res Commun* **377**, 504–507 (2008).
37. Rau, B. *et al*. Modulation of endogenous nitric oxide synthase in experimental acute pancreatitis: role of anti-ICAM-1 and oxygen free radical scavengers. *Ann.Surg.* **233**, 195–203 (2001).
38. Velosy, B. *et al*. The effects of somatostatin and octreotide on the human sphincter of Oddi. *Eur. J. Gastroenterol. Hepatol.* **11**, 897–901 (1999).
39. Buchwalow, I., Samoilova, V., Boecker, W. & Tiemann, M. Non-specific binding of antibodies in immunohistochemistry: fallacies and facts. *Scientific Reports* **1** (2011).

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Author contributions

I.B., J.S., K.T., J.N. and W.B. designed experiments, analyzed and interpreted results, and wrote the manuscript, whereas all the authors contributed to its revision; V.S. did immunohistochemistry; A.B., C.P., K.T., W.B. and C.S. selected the tissue samples for this study and contributed to the morphological assessment of tissue probes.

Additional information

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