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.
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, 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 and revealed all three NOS isoforms (NOS1, NOS2 and NOS3) not only in pancreatic islets but also in the exocrine compartments with a dramatic up-regulation in pancreatitis. Along with NOS up-regulation in pancreatitis, we observed a transformation of acinar cells with their conversion to the morphology of ductal cells. Strong NOS immunostaining was also observed in infiltrated inflammatory cells in areas adjacent to the affected parenchyma zones.

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 with an up-regulation in pancreatitis. A similar expression pattern was registered also for sGC and PDE in the normal pancreas, like it was earlier shown by us for other tissues and in pancreatitis. Constitutive expression of arginase in the apical cytoplasm of acinar cells with a markedly stronger immunostaining of Langerhans islets (asterisks). 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.

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 pancrea. 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.

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 and in pancreatitis. Constitutive expression of sGC, arginase and PDE in VSMC was also reported earlier by other groups. In an exemplary way, we show here the localization of PDE in VSMC and intima of a blood vessel in the normal pancreas. In accord with our earlier report, endothelial cells in pancreatitis were found to express all three NOS isoforms in blood vessels.

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 NOS1 in 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 apical cytoplasm of acinar cells with a markedly stronger immunostaining of Langerhans islets (asterisks). (c) 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.
In view that oxidative stress has been associated with the pathophysiology of chronic pancreatitis, 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.22. It is noteworthy, that despite the persistent presence of nitrotyrosine27 and NOS217–20 in pancreatic islets, there is no evidence that this might have a deleterious effect on the islet viability or function23.

**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 earlier17–20, 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 vasculature24.

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 cells29. 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”29. A similar phenomenon in the thyroid was also explained by a higher vulnerability of the acinar thyroid cells31. 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 pancreatitis32–34. This might also explain a marked diminishing of water and bicarbonate secretion with pancreatic juice observed in chronic pancreatitis patients15.

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 cells35,36. Taken together with reports from other groups about NOS localization in endocrine and exocrine secretory granules37–39, 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 ofzymogens in zymogen granules. In Langerhans islets, NOS1 and NOS2 were immunolabelled 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 cells17–20. 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 tissues32–34, 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 manner34. PDE and arginase were reported to counteract NO-mediated vasodilatory and antiproliferative function35,36 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 stress15, and patients with pancreatitis develop a systemic inflammatory response syndrome37. 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...
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 (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.

It is thought that NO may play a destructive role in conjunction with superoxides. 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. 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. Along with NO-releasing drugs, stimulators of sGC, radical scavengers like superoxide dismutase/catalase and inhibitors of PDE 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 NO 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 histopathological 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 washing and dilutions. Paraffin tissue sections (4 μm 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 immunohistochemistry. 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; therefore, blocking the endogenous Fc receptors prior to incubation with primary antibodies was omitted. Characterisation of rabbit primary Abs reference in immunohistochemistry. 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% H2O2 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-tetramine amplification for detection of NOS, sGC, PDE and arginase.

Bright-field microscopy. For bright-field microscopy, bound primary Abs to NOS, sGC, PDE, and arginase were visualized with blast amplification employing AmpliStain™ HRP conjugate (SDT GmbH, Baesweiler, Germany). The HRP label was amplified with FITC-conjugated tyramine. FITC-tyramine conjugate was synthesised by us 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% H2O2 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 fluorescence 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 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% H2O2 for 3–5 min. Bound anti-HRP antibody (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 (Zymed Laboratories 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 immunohistochemistry 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 immunostained probes. Three morphologists (L.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 the 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.

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