Nitric oxide signals are interlinked with calcium signals in normal pancreatic stellate cells upon oxidative stress and inflammation

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The mammalian diffuse stellate cell system comprises retinoid-storing cells capable of remarkable transformations from a quiescent to an activated myofibroblast-like phenotype. Activated pancreatic stellate cells (PSCs) attract attention owing to the pivotal role they play in development of tissue fibrosis in chronic pancreatitis and pancreatic cancer. However, little is known about the actual role of PSCs in the normal pancreas. These enigmatic cells have recently been shown to respond to physiological stimuli in a manner that is markedly different from their neighbouring pancreatic acinar cells (PACs). Here, we demonstrate the capacity of PSCs to generate nitric oxide (NO), a free radical messenger mediating, for example, inflammation and vasodilation. We show that production of cytosolic NO in PSCs is unambiguously related to cytosolic Ca2+ signals. Only stimuli that evoke Ca2+ signals in the PSCs elicit consequent NO generation. We provide fresh evidence for the striking difference between signalling pathways in PSCs and adjacent PACs, because PSCs, in contrast to PACs, generate substantial Ca2+-mediated and NOS-dependent NO signals. We also show that inhibition of NO generation protects both PSCs and PACs from necrosis. Our results highlight the interplay between Ca2+ and NO signalling pathways in cell–cell communication, and also identify a potential therapeutic target for anti-inflammatory therapies.

1. Background

Mammalian stellate cells (Latin stella—star) are retinoid-storing cells woven into the tissue of various organs [1] including the liver, pancreas, kidney, spleen, lung and vocal folds. Stellate cells are capable of transformations from a quiescent to an activated myofibroblast-like phenotype [2]. Activated stellate cells have attracted attention owing to the pivotal role they play in pathological fibrosis: they overproduce extracellular matrix proteins to repair the chronic stress-induced injuries in the tissue [1–3]. Nevertheless, the initial pathophysiological role of stellate cells—prior to activation—remains enigmatic. Here, we studied the primary signalling events, evoked by either oxidative stress or proinflammatory mediators, in stellate cells (PSCs) and neighbouring acinar cells (PACs) in the normal mouse pancreas, and identified a link between calcium and nitric oxide signalling pathways in PSCs.

In the normal pancreas acetylcholine (ACh) or cholecystokinin (CCK) evoke Ca2+ signals regulating the processes of enzyme release from zymogen granules deposited in the apical parts of PACs [4–10]. However, under pathological conditions (e.g. bile reflux into the pancreatic duct, high-fat diet together with...
excessive alcohol intake), Ca\(^{2+}\) signals become abnormally large and this elicits premature activation of enzymes within PACs and subsequent necrosis [8], followed by sterile (non-microbial) inflammation leading to acute pancreatitis (AP) [11,12].

Reactive oxygen/nitrogen species (ROS/RNS), such as NO, are highly chemically active radical and non-radical molecules that initiate and propagate reactions of oxidative stress, and thus act as second messengers in various inflammatory processes [13–16]. Both endogenous and exogenous ROS can modulate store-operated Ca\(^{2+}\) entry (SOCE) [17–20] and release [21]. Excessive Ca\(^{2+}\) influx into PACs, together with the sustained elevation of the cytosolic calcium ion concentration ([Ca\(^{2+}\)\(_{c}\)] ) [22–24], underlies the mechanism of AP [25], and store-operated Ca\(^{2+}\) entry channels are therefore potential therapeutic targets [24,26–28]. Nevertheless, the roles of ROS/RNS (including NO) in the (patho)physiology of PSCs remain unexplored. Generation of NO has not yet been reported in PSCs, although a plausible link between PSC activation and NO has been established: in cultured rat PSCs, expression of nitric oxide synthase 2 (NOS2) is increased after stimulation with pathogen-associated molecular patterns (PAMP) that activate Toll-like receptors (TLR) of innate immunity [29]. TLRs also mediate responses to damage-associated molecular patterns (DAMP) released from injured tissues (e.g. the necrotizing pancreas) [12].

The proinflammatory mediator bradykinin (BK) induces NO production in vascular endothelial cells [30,31]. BK was found to elicit Ca\(^{2+}\) signals in PSCs [32], at physiologically relevant concentrations [33], and this was linked to AP via specific action on PSCs through bradykinin receptor B2 [33]. So far, it is unknown whether BK elicits NO generation in PSCs and, if so, what the role of this process might be. Bile acids (BA), employed extensively in cellular studies, expression of nitric oxide synthase 2 (NOS2) is increased after stimulation with pathogen-associated molecular patterns (PAMP) that activate Toll-like receptors (TLR) of innate immunity [29]. TLRs also mediate responses to damage-associated molecular patterns (DAMP) released from injured tissues (e.g. the necrotizing pancreas) [12].

In order to explore such interactions, we inhibited either Ca\(^{2+}\) or NO signal generation in PSCs. Caffeine is known to reduce Ca\(^{2+}\) signals via inhibition of inositol 1,4,5-triphosphate receptors in PACs [39,40]; what is more, a caffeine-dependent decline in severity of pancreatic injury was recently demonstrated in three animal models of AP [41]. Thus, caffeine was used here to test whether the blockade of CA-elicited Ca\(^{2+}\) responses might attenuate NO signals. The latter were also blocked pharmacologically by inhibitors of enzymatic NO synthesis (NOS inhibitors), widely used in therapy of various inflammatory diseases [42], including AP [43,44]. Nevertheless, so far, the actual outcome of NOS inhibition in AP remains unclear. The aim of this study was to investigate the role of normal PSCs in the initial signalling events upon proinflammatory stimulation. We report here that PSCs, in contrast to PACs, generate substantial Ca\(^{2+}\)-mediated and NOS-dependent NO signals, and that inhibition of NO generation protects both PSCs and PACs from necrosis.

2. Methods

**Animals:** C57BL/6/J mice (Charles Rivers). **Antibodies:** Alexa Fluor 488 goat anti-mouse, Alexa Fluor 635 goat anti-rabbit (Thermo Fisher Scientific); mouse anti-BDKRB2 (Santa Cruz); rabbit anti-NOS2 (Merck). **Cell culture:** human pancreatic stellate cell line, SteCM complete stellate cell medium (ScienCell). **Chemicals:** aminoguanidine (AG), bradykinin, i-NAM (Tocris); PBS, ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific); other chemicals were obtained from Sigma. **Fluorescent dyes:** DAF-2 (Santa Cruz); DAF-FM, Fluo-4, Fura-2, Hoechst 33342, propidium iodide (Thermo Fisher Scientific).

2.1. Isolation of pancreatic lobules

Six- to eight-week-old male mice were sacrificed by cervical dislocation, the pancreases were dissected and the lobules were immediately isolated by collagenase digestion. Briefly, the pancreas was injected intraductally with NaHEPES-based collagenase solution and incubated (5–6 min, 37°C) to allow partial digestion of the tissue.

2.2. Primary human pancreatic stellate cell line

hPSCs were cultured (up to the fifth passage) at 37°C, 5% CO\(_{2}\) in complete stellate cell medium and split once a week.

2.3. Cytosolic calcium or nitric oxide measurements

Unless otherwise indicated, NaHEPES-based media, containing (mM): NaCl, 140; KCl, 4.7; HEPES, 10; MgCl\(_{2}\), 1; glucose, 10; and pyruvate, 1, were supplemented with 1 mM Ca\(^{2+}\) for calcium measurements and with 1 mM Ca\(^{2+}\) together with 0.5 mM L-Arg for nitric oxide recordings. For Ca\(^{2+}\) measurements, the lobules were loaded with 10 \(\mu\)M Fluo-4 (1 h, 30°C), and hPSC with 1 \(\mu\)M Fluo-4 (30 min, 37°C). For NO measurements, the lobules were loaded with 20 \(\mu\)M DAF-2 or DAF-FM (1 h, 30°C), and hPSC with 0.1 \(\mu\)M DAF-2 or DAF-FM (1 h, 37°C). The lobules were transferrered to a flow chamber and allowed to adhere to the glass surface; and for hPSC imaging, the coverslips with growing cells were used for flow chamber assembly. Experiments were performed in continuous perfusion with extracellular buffer-based solution; and the cells were visualized using a TCS SP5 II two-photon confocal microscope (Leica) with a 63 x 1.2 NA water objective. Fluo-4 or DAF dyes were excited with a 488 nm Ar laser, at 1–4% power, and emitted light was collected in the three-dimensional recording mode at 495–580 nm. The speed of recordings was approximately one image per 10 s, and varied dependent on thickness of the samples (up to 15 \(\mu\)m, z-axis resolution 1 \(\mu\)m). Images were captured at 512 x 512, and series of images were recorded at 256 x 256 pixel resolution, respectively, and analysed using Leica software. In order to reconstruct the three-dimensional signal, the individual signals from z-stacks were cropped, and the maximal projection was applied. Fluorescence signals were plotted as \(F/F_0\), where \(F_0\) was an averaged signal from first ten baseline images, and normalized as previously described [45].

2.4. Simultaneous cytosolic calcium and nitric oxide measurements

For simultaneous Ca\(^{2+}\) and NO measurements, the lobules were loaded with 10 \(\mu\)M Fura-2 and 10 \(\mu\)M DAF-2 (1 h,
30°C). After the loading, the lobules were transferred to the chamber, perfused and visualized as described above. Fura-2 fluorescence was excited with 355 nm and 405 nm lasers, at 8% and 16% power, respectively; and emitted light was collected in the three-dimensional recording mode at 500–600 nm; DAF-2 fluorescence was excited and collected as described above.

2.5. Measurements of necrosis level in the lobules

The lobules were treated with 5 mM cholate, 5 mM taurocholate or 0.2 mM TLC-S challenge (2 h, room temperature), and in some experiments, 0.6 mM L-NAME was present. The lobular PSCs were visualized using Fluo-4 (10 μM, 2 h); the lobules were co-stained with Hoechst 33342 (32 μM, 30 min), and dead cells were identified by PI staining (1.5 μM, 15 min) as described [33]. The cells were visualized, using the confocal microscope with a 63 × 1.2 NA water objective. Fluo-4, Hoechst 33342 and PI were excited with 488 nm Ar (1%), 355 nm diode (10%) and 543 nm HeNe laser (10%), respectively; and corresponding emissions were collected at 505–535, 415–485 and 615–720 nm. The fluorescence signal was collected sequentially between frames in the three-dimensional mode from 20 μm thick lobules and 512 × 512 pixel resolution. Five pictures of independent lobules were taken in each of four experimental replicates (n = 20), and live (PI-negative) and dead (PI-positive) cells were counted.

2.6. Immunohistochemistry

Unless otherwise indicated, the procedure was performed at room temperature, and double distilled water (ddH2O) was used for preparation of all solutions. 0.1% Tween 20 was used as a washing buffer and 1% BSA in PBS with 0.1% Tween 20 was a blocking buffer. Mouse pancreatic tissue samples were fixed in formalin, embedded in paraffin and cut into 4 μm sections. The sections were heated in a dry oven (30 min, 65°C), then deparaffinized in xylene (2 × 10 min) and graded ethanol, and then incubated in 50 mM NH4Cl (20 min). Antigen retrieval was achieved by autoclaving (20 min, 120°C) the samples in TAE buffer (pH 8.1), followed by slow cooling to room temperature (30 min). Permeabilization was performed in 0.4% Triton X-100 (10 min). In order to quench autofluorescence, the sections were incubated in 0.2% Sudan black B [46]. The sections were then transferred to a humid chamber, and blocking of non-specific binding sites was performed (1 h), followed by incubation with primary anti-BDKRB2 and anti-NOS2 Abs (0.5 μg ml−1) for 1 h at room temperature, and then overnight at 4°C. The negative controls were incubated in blocking solution with no primary Abs. The following day, the sections were incubated (1 h) with goat anti-rabbit secondary Ab (4 μg ml−1), washed, and then incubated (1 h) with goat anti-mouse secondary Ab (4 μg ml−1). The sections were embedded in antifade mounting medium with DAPI, and imaged immediately using the confocal microscope (excitation wavelengths: 355, 488 and 633 nm). The slides were stored at 4°C.

2.7. Statistics

The quantitative results were expressed as means ± s.d. or s.e.m. (see the text for details). Statistical analysis was performed using the Student’s t-test or ANOVA, and the significance threshold was set at 0.05.

3. Results

3.1. Oxidative stress elicits (patho)physiological calcium and nitric oxide signals

Hydrogen peroxide (H2O2) was used as an initiator of oxidative stress in human pancreatic stellate cells (hPSCs; figure 1a–b) and mouse pancreatic tissue lobules (figure 1c–h). In hPSCs, a sustained increase in [Ca2+]c was evoked by 0.5 mM H2O2 (blue), which was markedly (p < 0.001) attenuated by removal of external Ca2+ (orange; figure 1a–b). In the lobules, oxidative stress elicited rises in [Ca2+]c in both PSCs (red) and PACs (blue; figure 1c), although cytosolic NO signals (figure 1d; see also electronic supplementary material, figure S1 and video S1) were limited spatially to PSCs (red), manifest as a sharp increase and sustained plateau phase. Three-dimensional reconstruction of the DAF-FM fluorescence signal collected from the 1 mM H2O2-stimulated lobules confirmed the increase in [NO]c in situ in PSCs (cells of well-defined projections, red arrows), but not in adjacent PACs (cells that form the acini, blue arrows; figure 1c). The amplitudes of the H2O2-elicited increases in cytosolic NO in the PSCs varied, depending on the peroxide concentration (figure 1f). The development of NO responses was modulated by the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; figure 1g), so that, at a concentration of 0.6 mM (pink trace and bar), it attenuated the 0.25 mM H2O2-evoked (black trace and bar) NO signals (figure 1g), reducing significantly (p < 0.001) the area under the response curve (figure 1h).

3.2. The proinflammatory mediator bradykinin evokes simultaneous calcium and nitric oxide signals in stellate cells

Double loading of the lobules—with Ca2+- and NO-sensing fluorescent indicators—revealed simultaneous development of both Ca2+ and NO signals: upon stimulation of the lobules with 20 nM BK (figure 2a), a rapid increase in [Ca2+]c, with an initial peak and subsequent plateau phase (navy blue), and a sustained increase in cellular NO [NO]c (green) were observed solely in PSCs, with no Ca2+/NO signals detected in PACs (figure 2a). In hPSCs, a sustained increase in [NO]c was evoked by 1 μM BK (figure 2b), and additional stimulation of the cells with 0.5 mM H2O2 brought a further rise in [NO]c.

3.3. Bile-acid-elicited calcium and nitric oxide signals have different profiles in acinar and stellate cells

Robust [Ca2+]c elevations in the lobules (figure 3a) were evoked by the natural bile sodium salts: 5 mM cholate (left), 5 mM taurocholate (middle) and 0.2 mM tauro lithocholic acid 3-sulfate (TLC-S; right). The pattern of the responses was markedly different in adjacent PACs and PSCs: cholate and taurocholate-elicited increases in [Ca2+]c almost exclusively in PSCs (red), with robust Ca2+-signals in cholate- and
Figure 1. Oxidative stress evokes cytosolic calcium and nitric oxide signals in the pancreatic cells. (a) Average traces (mean ± s.e.m.) of cytosolic Ca\(^{2+}\) responses to 0.5 mM H\(_2\)O\(_2\) in hPSCs, in the presence of 1.0 mM Ca\(^{2+}\) (blue, \(n = 38\)) or the absence of Ca\(^{2+}\) (orange, \(n = 35\)). (b) Bar chart (mean ± s.e.m.) comparing the areas under the response curves to 0.5 mM H\(_2\)O\(_2\) in the presence of 1.0 mM Ca\(^{2+}\) (blue, \(n = 38\)) or the absence of Ca\(^{2+}\) (orange, \(n = 35\))—summary of (a). (c) Typical cytosolic Ca\(^{2+}\) responses elicited by 0.5 mM H\(_2\)O\(_2\) in PSCs (red, \(n = 6\)) and PACs (blue, \(n = 6\)) in pancreatic lobules. (d) Typical cytosolic NO responses elicited by 0.5 mM H\(_2\)O\(_2\) in PSCs (red, \(n = 5\)) and PACs (blue, \(n = 8\)) in pancreatic lobules. See also electronic supplementary material, video S1 and figure S1. (e) Photomicrograph of the lobules loaded with fluorescent NO probe DAF-FM, upon stimulation with 1 mM H\(_2\)O\(_2\). Arrows: PSCs, red; PACs, blue. Scale bar, 50 μm. (f) Bar chart (mean ± s.e.m.) comparing amplitudes of cytosolic NO responses in PSCs, elicited by 0.05 – 0.5 mM H\(_2\)O\(_2\) in the lobules (\(n ≥ 5\)). (g) Average traces (mean ± s.e.m.) of cytosolic NO responses to 0.25 mM H\(_2\)O\(_2\) (arrow) in the absence (black, \(n = 13\)) or the presence of 0.6 mM L-NAME (pink, \(n = 10\)), in PSCs in the lobules. (h) Bar chart (mean ± s.e.m.) comparing the areas under the response curves of PSCs to 0.25 mM H\(_2\)O\(_2\) in the absence (black, \(n = 13\)) or the presence of 0.6 mM L-NAME (pink, \(n = 10\))—the summary of (g).
were significantly reduced in the presence of 0.6 mM L-NAME and the irreversible inhibitor of NOS2 aminoguanidine (AG), reduced BA-evoked NO responses in the lobules (figure 4c–d).

The role of pharmacological blockade of NO production was analysed after 2 h incubation of the lobules with 5 mM cholate, 5 mM taurocholate or 0.2 mM TLC-S (figure 4e; see also electronic supplementary material, figure S3), and L-NAME was used as NOS inhibitor. Because the levels of necrosis in the lobules that received 0.6 mM L-NAME (and no BA) treatment did not change in comparison with NaHEPES controls (less than 5%), L-NAME at this concentration was tested as a protective agent against the BA challenge. Lobular PSCs (dashed red) were substantially more susceptible to cholate or taurocholate than PACs (dashed blue): the PSCs/PACs necrosis ratios were approximately 83/47 (1.8) in cholate- and approximately 31/8 (3.8) in taurocholate-treated lobules. The presence of L-NAME, however, reduced necrosis by almost 50%, irrespective of cell type, reaching approximately 48/22 (2.2) for the cholate- and approximately 13/6 (1.3) for taurocholate-challenged lobules, respectively. TLC-S induced necrosis at similar levels in both pancreatic cell types: the PSCs/PACs necrosis was approximately 43/38 (1.1), and in L-NAME-protected lobules approximately 16/16 (1.0), similarly the cholate- or taurocholate-treated lobule necrosis decreased by almost 50%.

3.5. Blockade of nitric oxide production reduces necrosis in the pancreatic lobules

3.6. Source of nitric oxide signals in pancreatic stellate cells

Co-immunolocalization of NOS2 and BK receptor B2 (BDKRB2) in the pancreas was assessed in formalin-fixed paraffin-embedded mouse tissue sections (figure 4f–i; see also electronic supplementary material, figure S4), and DAPI was used to stain the nuclei (PSCs: elongated, indicated with arrows; PACs: large round, indicated with arrows; figure 4f). The PSCs were localized in the interacinar spaces, encircling the base of adjacent acini with fine cytoplasmic processes. The processes, as well as the areas that surround PSCs nuclei, were visualized using anti-NOS2 (figure 4g, red) and anti-BDKRB2 (figure 4h, green) antibodies (Ab). The areas of co-immunolocalization (figure 4i, hybrid yellow to orange) were identified as PSCs. The transmitted light image along with the high-resolution NOS2 staining images are shown in electronic supplementary material, figure S4.

4. Discussion and conclusion

It has previously been shown that cytosolic calcium signals can be elicited in cultured [32] and in normal (lobular) [33] pancreatic stellate cells. This study now reveals that these signals are interlinked with cytosolic nitric oxide signals.

We show that NO signals are evoked in PSCs upon induction of oxidative stress (figure 1) or application of inflammatory mediators (figures 2–4). Oxidative stress originates from the imbalance between the production and neutralization of ROS/RNS [13,47], and is implicated in the oscillatory Ca$^{2+}$ signals in taurocholate-stimulated PSCs, whereas only very modest responses—single spikes—were detected in PACs (blue). In contrast, stimulation with TLC-S evoked Ca$^{2+}$ signals with an oscillatory pattern in PACs (blue), and almost no detectable responses in PSCs (red). NO levels were shown to rise in PSCs (red) upon stimulation (blue, and almost no detectable responses in PSCs (red). NO levels were shown to rise in PSCs (red) upon stimulation with cholate (figure 3b, left; see also electronic supplementary material, figure S2) and taurocholate (figure 3b, middle), but not with TLC-S (figure 3b, right); no detectable NO signals accompanied the Ca$^{2+}$ signals in PACs (blue). 20 mM caffeine blocked the development of cholate-evoked NO signals in both lobular PSCs and hPSCs (electronic supplementary material, figure S2).

3.4. Nitric oxide synthase inhibitors modulate bile-acid-evoked nitric oxide signals

Pharmacological NOS inhibitors, the non-specific L-NAME and the irreversible inhibitor of NOS2 aminoguanidine (AG), reduced BA-evoked NO responses in the lobules (figure 4a–d). The developments of 5 mM cholate- (figure 4a) and 5 mM taurocholate-elicited (figure 4b) responses (black traces and bars) were significantly reduced in the presence of 0.6 mM L-NAME (pink traces and bars; figure 4a–b). The aforementioned reductions in taurocholate-elicited NO generation were dependent on L-NAME concentration (figure 4c; $p < 0.05^{*}$, $p < 0.01^{**}$ and $p < 0.001^{***}$), as well as AG concentration (figure 4d; $p < 0.01^{**}$ and $p < 0.0001^{****}$), as shown in the bar charts (0.04–0.6 mM inhibitor; figure 4c–d) and the representative traces in the insets (0.04, 0.15 and 0.3 mM inhibitor; figure 4c–d).

Figure 2. Bradykinin elicits simultaneous cytosolic calcium and nitric oxide responses in stellate cells. (a) Average traces (mean ± s.e.m.) of cytosolic Ca$^{2+}$ and NO responses to 20 nM BK (arrow), recorded in pancreatic lobules. The lobules were loaded with Fura-2 and DAF-2 fluorescent probes, and Ca$^{2+}$ (navy blue) and NO responses (green) were registered simultaneously in PSCs (n = 12; top curves); no responses were detected in PACs (n = 6; bottom curves). (b) Average traces (mean ± s.e.m.) of cytosolic NO responses to 1 μM BK, and then to 0.5 mM H$_2$O$_2$ recorded in human PSCs (purple, n = 24); the control cells received placebo and H$_2$O$_2$ treatment (orange, n = 24).
mechanisms of numerous inflammatory diseases [15,16,20], including AP [11,16,19]. However, for pancreatitis, there remains the ‘chicken or the egg’ question regarding the roles of ROS/RNS in the development of inflammation. Here, we show that H2O2 at concentrations that are (patho)-physiologically relevant [47,48] evoke large Ca2+ signals with an oscillatory pattern in both PACs and PSCs (figure 1a–c). Importantly, we demonstrate that it is only in the PSCs that these Ca2+ signals are accompanied by detectable NO signals (figure 1d–h).

Spatial separations of BK- and ACh- or CCK-elicited Ca2+ signals in pancreatic lobules have previously been reported. Whereas ACh and CCK evoke Ca2+ signals in PACs, which control normal acinar secretion [49], BK-evoked Ca2+ signals are entirely confined to PSCs [33]. BK-elicited Ca2+ signalling events in PSCs are mediated via BDKRB2 [33] and pharmacological blockade of this receptor with the B2 antagonist WIN64338 protected lobular PACs from the necrosis evoked by alcohol/fatty acid or bile acids [33], indicating a possible paracrine interaction between PSCs and PACs. Here, we show that BK elicits simultaneous Ca2+ and NO signals in PSCs (figure 2a, upper curves), but fails to evoke responses in adjacent PACs (figure 2a, lower curves). Because large Ca2+ signals in PSCs are not accompanied by Ca2+ signals in PACs, and vice versa, it may be NO that mediates paracrine communication between stellate cells and other cell types in the pancreas.

Figure 3. Bile acids evoke toxic calcium overload and nitric oxide signals in the lobules. (a) Typical cytosolic Ca2+ responses elicited by 5 mM cholate (left), 5 mM taurocholate (centre) and 0.2 mM TLC-S (right) in PSCs (red; n = 5, 6, n = 4) and PACs (blue; n = 12, n = 7, n = 19). (b) Typical cytosolic NO responses elicited by 5 mM cholate (left), 5 mM taurocholate (central) and 0.2 mM TLC-S (right) in PSCs (red; n = 8, shown also in figure 4a and electronic supplementary material, figure S2a; n = 16, shown also in figure 4b; n = 5) and PACs (blue; n = 8, n = 6, n = 8). See also electronic supplementary material, figure S2.

Figure 3. Bile acids evoke toxic calcium overload and nitric oxide signals in the lobules. (a) Typical cytosolic Ca2+ responses elicited by 5 mM cholate (left), 5 mM taurocholate (centre) and 0.2 mM TLC-S (right) in PSCs (red; n = 5, 6, n = 4) and PACs (blue; n = 12, n = 7, n = 19). (b) Typical cytosolic NO responses elicited by 5 mM cholate (left), 5 mM taurocholate (central) and 0.2 mM TLC-S (right) in PSCs (red; n = 8, shown also in figure 4a and electronic supplementary material, figure S2a; n = 16, shown also in figure 4b; n = 5) and PACs (blue; n = 8, n = 6, n = 8). See also electronic supplementary material, figure S2.

Bile acids are natural compounds of the bile that facilitate enzymatic hydrolysis of lipids in the process of digestion [50]. In the case of gallstone-induced bile reflux into the pancreatic duct, bile acids in high concentrations will get in direct contact with pancreatic cells. Bile acids evoke large abnormal Ca2+ signals in PACs [22,34,35], followed by intracellular enzyme activation, PAC necrosis [25], autodigestion of the pancreas and finally pancreatitis [11,12,25]. Cytoplasmic and mitochondrial Ca2+ signals, elicited in isolated PACs by TLC-S, impair ATP synthesis and induce ROS production [11]. Nevertheless, TLC-S has little effect on [Ca2+]C in PSCs (figure 3a), and no detectable role in NO signalling in these cells (figure 3b). In contrast, robust Ca2+ signals elicited in PSCs by cholate and taurocholate (figure 3a) are accompanied by NO signals (figures 3b and 4a–d). A plausible explanation for the diverse sensitivity of pancreatic cells to bile acids might be the different pattern of bile-acid-transporting proteins in PSCs and PACs.

Our results demonstrating that even substantial Ca2+ signals generated in PACs fail to elicit detectable NO signals are in agreement with a previous study in which the non-enzymatic NO signal generation was explored in isolated PACs [45]. In that study, even supramaximal ACh concentrations failed to evoke detectable NO signals in more than 70% of intact PACs.

The potential benefit of blocking NO generation in the therapy of various diseases, including AP, remains controversial [15]: several studies show beneficial effects of NOS inhibitors in the therapy of cancer [51], arthritis [52] and pancreatitis [43,44], whereas others demonstrate that blockade of NO production aggravates liver injury [53], and exacerbates inflammation in the kidney [54]. Here, we show that L-NAME (blocker of NOS 1–3; figure 4a) and AG (irreversible inhibitor of NOS2; figure 4f) significantly reduce bile-acid-evoked NO signals in PSCs. Furthermore, we demonstrate that there is substantially more necrosis in PSCs than in PACs in cholate- or taurocholate-challenged lobules (figure 4e), which correlates with the presence of NO signals together with large Ca2+ signals in PSCs upon stimulation with these BAs (figure 3). Interestingly, the levels of necrosis in TLC-S-stressed lobules are comparable for both type of pancreatic cells (figure 4e)—possibly owing to the lack of detectable NO signals in PSCs (figure 3) that could exacerbate necrosis. The pharmacological inhibitor L-NAME significantly reduces necrosis in BA-challenged
Figure 4. Nitric oxide synthase inhibitors diminish cytosolic nitric oxide signals and protect from necrosis. (a) Typical cytosolic NO responses evoked by 5 mM cholate in the absence (black, n = 8) or the presence of 0.6 mM L-NAME (pink, n = 9). Inset: bar chart (mean ± s.e.m.) comparing the areas under the response curves. (b) Typical cytosolic NO responses evoked by 5 mM taurocholate in the absence (black, n = 16) or the presence of 0.6 mM L-NAME (pink, n = 8). Inset: bar chart (mean ± s.e.m.) comparing the areas under the response curves (showed also in figure 4c). (c) Bar chart (mean ± s.e.m.) comparing the areas under the response curves of PSCs to 5 mM taurocholate, in the presence of 0.04–0.6 mM L-NAME (n ≥ 3). Inset: typical cytosolic NO responses of PSCs to 5 mM taurocholate in the presence of 0.04 (dark grey), 0.15 (grey) and 0.3 mM L-NAME (light grey); the responses are presented from the moment of taurocholate and NOS inhibitor administration. (d) Bar chart (mean ± s.e.m.) comparing the areas under the response curves of PSCs to 5 mM taurocholate, in the presence of 0.04–0.6 mM AG (n ≥ 3). Inset: typical cytosolic NO responses of PSCs to 5 mM taurocholate in the presence of 0.04 (dark grey), 0.15 (grey) and 0.3 mM AG (light grey); the responses are presented as in figure 4c. (e) Bar chart (mean ± s.d.) comparing levels of necrosis in the lobules under different experimental conditions. The lobules were treated with 5 mM cholate, 5 mM taurocholate or 0.2 mM TLC-S, the presence of 0.6 mM L-NAME is indicated with +; NaHEPES-treated lobules served as control. PSCs (dashed red) and PACs (dashed blue) were counted after triple staining of the lobules with fluorescent probes: Fluo-4, Hoechst 33342 and PI (four replicates, n = 20). See also electronic supplementary material, figure S3. (f–i) Co-immunolocalization of the BDKRB2 and the NOS2 in mouse pancreas. (f) DAPI, (g) Ab anti-NOS2, (h) Ab anti-BDKRB2, (i) overlaid (f–h). Scale bar, 25 μm. Arrowheads, PSCs; arrows, PACs; V, blood vessel; dash line, pancreatic duct. See also electronic supplementary material, figure S5.
lobules, irrespective of bile type and cell type (figure 4e). In cholate- or taurocholate-stressed lobules the protective effect of l-NAME (blockade of NO signals ameliorates necrosis) is more prominent in the PSCs than in the PACs, which do not have detectable cytosolic NO signals. The protection of PACs might be the result of, for example, paracrine communication, PSCs→PACs, via intercellular messengers (plausibly NO) released by PSCs.

Expression of the NO-forming enzymes, NOS 1–3, in the exocrine pancreas has recently been confirmed using state-of-the-art immunohistochemical methods [55]. The authors of the study, however, did not refer directly to PSCs, although they report the presence of ‘cells of the morphology of ductal cells’ in chronic pancreatitis (CP) tissue specimens [55]. These cells could well have been PSCs, which are known to induce fibrosis in CP [2,56]. TLR-mediated expression of NOS2 has been detected in isolated PSCs upon stimulation with PAMP [29]. Importantly, TLRs are also activated upon stimulation with DAMP—endogenous molecules released from injured cells [12] (e.g. autodigested PACs). Thus, DAMP-exposed and then NOS2-expressing PSCs might be present in the pancreas (e.g. autodigested PACs). Thus, DAMP-exposed and then NOS2-expressing PSCs might be present in the pancrea-

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