Expression of stress proteins heme oxygenase-1 and -2 in acute pancreatitis and pancreatic islet βTC3 and acinar AR42J cells

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Abstract Oxygen-derived free radicals have been implicated in the pathogenesis of acute pancreatitis, yet adaptive responses in the pancreas in vivo to oxidative stress remain poorly defined. We have investigated expression of the stress protein heme oxygenase in the intact pancreas of rats with caerulein-induced pancreatitis and in cultured pancreatic acinar and islet cell lines. Expression of inducible heme oxygenase-1 (HO-1) in the pancreas in vivo was enhanced 12–24 h after induction of pancreatitis. In murine islet (βTC3) and rat acinar (AR42J) pancreatic cells, H_2O_2, methyl viologen, cadmium chloride and diethylmaleate enhanced HO-1 expression in a dose- and time-dependent manner, without altering expression of constitutive HO-2. Enhanced expression of HO-1 in the pancreas in vivo and pancreatic islet and acinar cells may contribute to cellular defences against oxidative stress associated with acute pancreatitis.

Key words: Heme oxygenase; Pancreatitis; Oxidative stress; Islets of Langerhans; Exocrine pancreas

1. Introduction

Microsomal heme oxygenase (HO) has been identified as a stress protein induced in many cell types by various stimulants such as oxidative stress and sulfhydryl-reactive agents [1]. HO is the most efficient heme degrading system and produces almost exclusively biliverdin and carbon monoxide (CO) as heme degradation products [1,2]. The cytosolic enzyme biliverdin reductase subsequently catalyzes the two-electron reduction of biliverdin to bilirubin, a powerful chain-breaking antioxidant [3–5]. Two isoforms of HO have been identified, an inducible form HO-1 (~32 kDa) and a constitutive form HO-2 (~34 kDa). Accumulating evidence suggests that heme oxygenase plays an important role in regulating vascular tone through carbon monoxide mediated increases in intracellular cGMP levels in smooth muscle cells [6,7] and neuronal transmission [8]. Constitutive HO-2 has recently been identified immunocytochemically in endothelial cells, adventitial nerves of blood vessels and ganglia of the myenteric plexus in the intestine [9], suggesting that HO and nitric oxide synthase may act in concert to modulate vascular tone and the autonomic nervous system.

Pancreatitis is a disease of increasing incidence and covers a spectrum of pathophysiological alterations in the pancreas and other organs [10,11]. Oxidative stress has been implicated in the pathophysiology of pancreatitis [12], yet the cellular mechanisms underlying secretory dysfunction remain to be elucidated. Glutathione (GSH) metabolism and lipid peroxidation have been measured as indicators of free radical mediated damage in acute pancreatitis, and there is general agreement that GSH and other sulfhydryl compounds are depleted while lipid peroxidation is increased in pancreatic tissue (see reviews [13,14]). Moreover, other indices, such as acinar cell injury and oedema show a marked improvement following treatment with enzymic antioxidants, vitamin C analogues, GSH monoethyl ester and GSH prodrugs [14]. Thus, endogenous antioxidant defence mechanisms in both pancreatic acinar and islet cells may be critically important in preventing the onset or diminishing the severity of acute pancreatitis.

In the present study we have used the caerulein-induced model of acute pancreatitis to investigate expression of HO during the progression of pancreatitis in the rat in vivo. To resolve whether pancreatic endocrine and exocrine cells exhibit a differential sensitivity to oxidative stress, we also investigated the effects of H_2O_2 and methyl viologen and sulfhydryl-reactive agents diethylmaleate and cadmium chloride on HO expression in pancreatic islet (βTC3, [15]) and acinar (AR42J, [16]) cell lines.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), cadmium chloride, diethylmaleate and hydrogen peroxide were purchased from Sigma Chemical Co., Poole, UK. Horseradish peroxidase conjugated goat anti-rabbit immunoglobulin and BCA protein assay reagents were purchased from Pierce, Chester, UK. Polyvinylidene difluoride membrane (Immobilon-P) was purchased from Millipore, Watford, UK. Enhanced chemiluminescence Western blotting detection reagents and Hyperfilm-MP autoradiography film were purchased from Amersham International, Amersham, UK.

2.2. Induction of caerulein pancreatitis in rats in vivo

Male Sprague-Dawley rats were kept on a 12-h light-dark cycle with free access to water and a standard laboratory diet (No. 491, Grain Harvesters Ltd., Kent, UK). As described previously [17], rats weighing 190–250 g received four intraperitoneal injections of either saline (0.9%) or 20 μg/kg body weight of caerulein (a pancreatic secretagogue) at hourly intervals. Animals were killed by cervical dislocation 1, 12, 24 h after the last intraperitoneal injection of caerulein or saline, and pancreatic, heart and liver tissues were the removed surgically. Tissue specimens were rinsed with ice-cold phosphate-buffered saline containing 0.1 mM phenylmethylsulfonyl fluoride and homogenized in SDS sample buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl (pH 6.8) and 0.1 mM phenylmethylsulfonyl fluoride).

2.3. Culture of pancreatic βTC3 islet and AR42J acinar cell lines

The pancreatic islet βTC3 cell line (see [15]) was kindly provided by Dr. Elaine Baileys from Addenbrooks Hospital, University of Cam-

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bridge. The pancreatic AR42J acinar cell line (see [16]) was obtained from the American Type Culture Collection, Rockville, MD, USA. Both cell lines were cultured routinely in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin in an incubator at 37°C in a 5% CO_2 atmosphere. Cells were subcultured every 4 or 5 days with a split ratio of 1:2 or 1:3 for βTC3 cells, or every 2 or 3 days with a split ratio from 1:5 to 1:10 for AR42J cells.

βTC3 and AR42J cells were seeded into 24-well microtitre plates at a density of 5×10^5 and 2×10^5 cells/ml, respectively. Cells were cultured for 24 h and then incubated for specified time intervals with or without stress agents. The medium was then removed, and the cells were rinsed three times with Dulbecco's phosphate-buffered saline and lysed with 0.1 ml of SDS sample buffer.

2.4. Determination of heme oxygenase protein expression

Sample total protein content was determined using the BCA protein assay reagents and 3% 2-mercaptoethanol was added. An equal protein concentration from each sample was then boiled for 3 min and separated by 13% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene difluoride membrane and probed with polyclonal rabbit anti-rat HO-1 and HO-2 antibodies [18,19]. A horseradish peroxidase conjugated goat anti-rabbit secondary antibody was used in conjugation with enhanced chemiluminescence to visualise the HO bands on autoradiography film.

3. Results

3.1. Expression of HO-1 in pancreatic tissue in caerulein-induced pancreatitis

Caerulein, a decapeptide cholecystokinin analogue, causes acute pancreatitis in rats when administered in supraphysiological doses [20]. To investigate the effects of acute pancreatitis on expression of HO in vivo, pancreatitis was induced in rats by administering caerulein intraperitoneally (see Section 2). Protein extracts of pancreatic, heart and liver were then analyzed by Western blotting (Fig. 1A). HO-1 expression was not detected in the pancreas 1 h after treatment of rats with either caerulein or 0.9% NaCl, although interstitial oedema was observed in caerulein-treated animals. HO-1 was expressed selectively in pancreatic tissue 12–24 h after treatment of rats with caerulein, whereas expression of HO-1 in the liver remained unchanged and HO-1 was hardly detected in the heart. Constitutively expressed HO-2 was barely detectable in the pancreas or heart of rats treated with caerulein or NaCl. Although HO-2 was clearly expressed in the liver of rats treated with either NaCl or caerulein (Fig. 1B), we were unable to detect changes in HO-2 expression in the liver, pancreas or heart over the 1–24 experimental period (data not shown).

3.2. Expression of HO-1 in pancreatic islet βTC3 and acinar AR42J cell lines

Basal expression of HO-1 was low in untreated pancreatic βTC3 islet cells (Fig. 2). Cadmium chloride and diethylmaleate enhanced HO-1 expression in a dose- and time-dependent manner (Fig. 2A,B). Moreover, H_2O_2 and methyl viologen, which generates O_2^- in cells [21], also enhanced HO-1 expression. With the exception of methyl viologen, maximal expression of HO-1 was observed after 12–24 h exposure to the stress agents. Methyl viologen induced maximal HO-1 expression only after 48 h.

Basal expression of HO-1 was also very low in untreated pancreatic AR42J acinar cells (Fig. 3), and as in βTC3 islet cells, cadmium chloride, diethymaleate, H_2O_2 and methyl viologen increased HO-1 expression in a dose-dependent manner (Fig. 3A). When the time-dependent effects of cadmium chloride and the electrophilic agent diethylmaleate were examined, both agents increased HO-1 expression maximally over 6–12 h (Fig. 3B), and levels of protein expression then decreased by 48 h. A similar pattern of HO-1 expression was observed following exposure of AR42J cells to H_2O_2 or methyl viologen for up to 48 h (data not shown).

Constitutive HO-2 was detected under basal conditions in pancreatic βTC3 islet cells, and expression of HO-2 was unaffected by oxidative stress and sulfhydryl-reactive agents

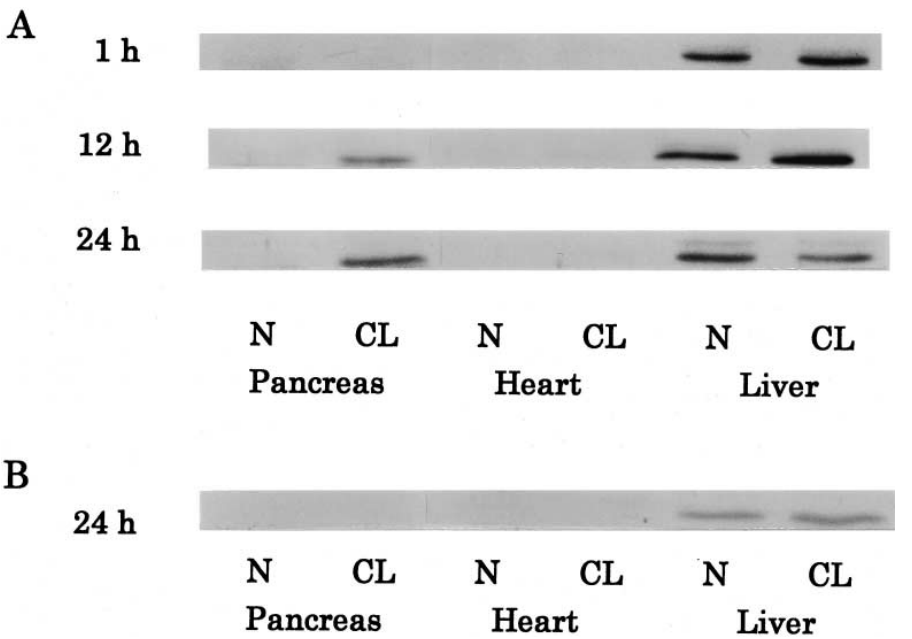

Fig. 1. Expression of heme oxygenase in the pancreas, heart and liver in vivo. Pancreatic, heart and liver tissues were removed at 1, 12, 24 h after the last of four intraperitoneal injections of either 0.9% saline (N) or caerulein (CL, 20 μg/kg body weight). Expression of HO-1 (A) and HO-2 (B) was determined by Western blot analysis. Data are representative of measurements in 3 animals.

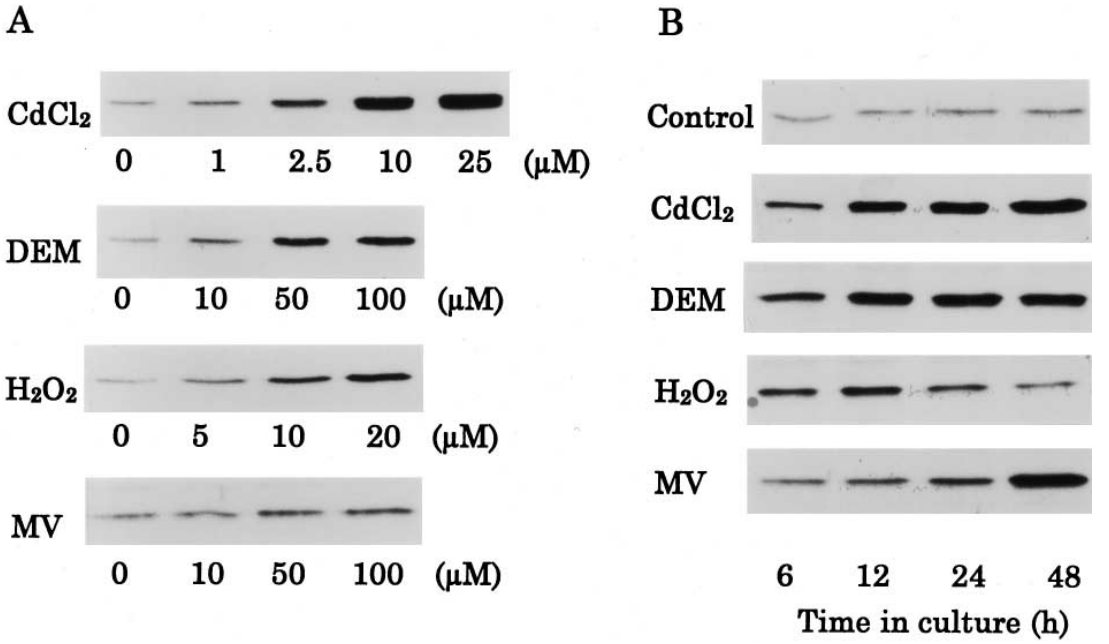

Fig. 2. Dose- and time-dependent enhancement of HO-1 expression in pancreatic βTC3 islet cells. (A) Cells were incubated in the presence of increasing concentrations of either cadmium chloride ($CdCl_2$), diethylmaleate (DEM) or H_2O_2 for 12 h, or with methyl viologen (MV) for 48 h. (B) Cells were incubated for 6–48 h in the absence (control) or presence of 10 μM $CdCl_2$, 100 μM DEM, 20 μM H_2O_2 or 100 μM methyl viologen. Equal amounts of proteins were electrophoresed and analyzed by Western blotting. Data are representative of experiments in 3–4 different cell cultures.

(data not shown). By comparison, expression of HO-2 in pancreatic AR42J acinar cells was very low and not enhanced by stress agents. Interestingly, caerulein (10^{-12}–10^{-6} M) had no effect on the expression of either HO-1 or HO-2 in AR42J acinar cells when added directly to the culture medium (24 h, data not shown). Treatment of either βTC3 or AR42J cells with oxidative stress or sulfhydryl-reactive agents at concentrations and time periods used in this study did not affect cell viability, as determined by the exclusion of trypan blue dye.

4. Discussion

Evidence for the involvement of oxygen-derived free radicals in the pathogenesis of pancreatitis is increasing (see [12–14]), yet the molecular events mediating secretory dysfunction in both the exocrine and endocrine pancreas remain to be investigated. Although some studies have shown that heat shock proteins (HSP70, HSP90) and HO-1 are expressed in pancreatic cells in response to pro-inflammatory cytokines

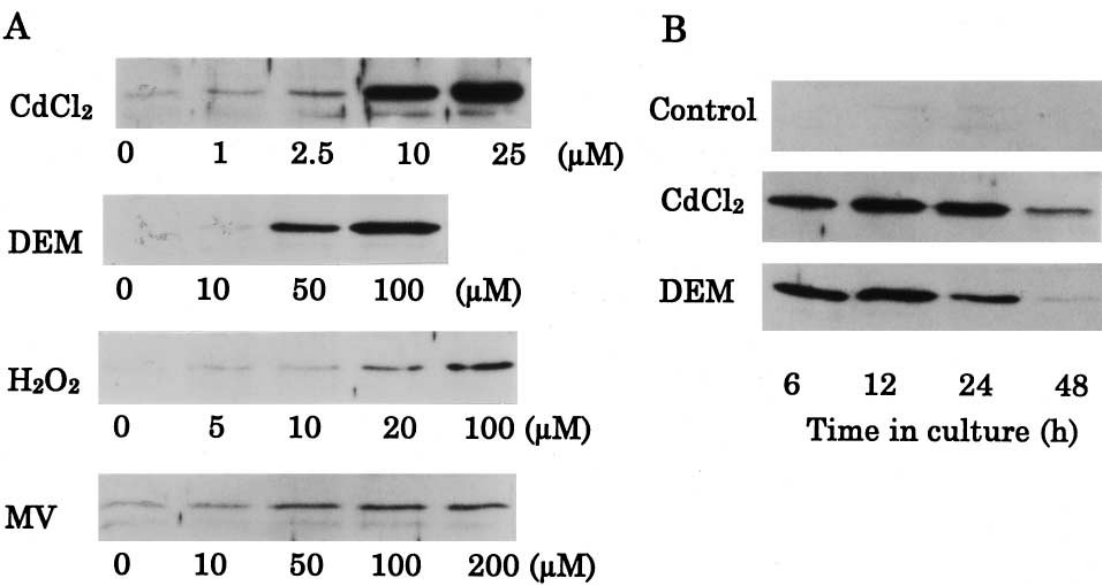

Fig. 3. Dose- and time-dependent enhancement of HO-1 expression in pancreatic AR42J acinar cells. (A) Cells were incubated in the presence of increasing concentrations of either $CdCl_2$, DEM, H_2O_2 or methyl viologen for 12 h. (B) Cells were incubated for 6–48 h in the absence (control) or presence of 10 μM $CdCl_2$ and 100 μM DEM. Equal amounts of proteins were electrophoresed and analyzed by Western blotting. Data are representative of experiments in 3 different cell cultures.

[22–24], there is no consensus as to whether these proteins are involved in the protection against oxygen radical cytotoxicity.

We have established that caerulein-induced acute pancreatitis in rats in vivo is associated with a significant increase in the expression of HO-1 in pancreatic tissue, occurring 12–24 h after the first signs of interstitial oedema in the pancreas. Although we cannot exclude the possibility that infiltrating phagocytes in pancreatic tissue may have contributed to the enhanced expression of HO-1, it seems more likely that enhanced expression of HO-1 in vivo is attributable to pancreatic cells. The time course of this selective induction of HO-1 in the pancreas in vivo may reflect an adaptive response to the generation of reactive oxygen radicals in the early stages of acute pancreatitis [13,14]. Interestingly, induction of the heat shock protein HSP60 in the rat pancreas in response to stress induced by water immersion has been reported to prevent the pancreatic interstitial oedema and serum amylasaemia associated with pancreatitis induced subsequently with caerulein [25]. As constitutive HO-2 was hardly detectable in the pancreas in vivo in the present study, enhanced expression of HO-1 and heat shock proteins may constitute a key cellular defence against both oxidative and environmental stress.

Adaptive responses in HO-1 expression were also detected in pancreatic islet (βTC3) and acinar (AR42J) cell lines exposed to oxidative or sulfhydryl stress agents, with βTC3 cells exhibiting a greater sensitivity to oxidative stress. The enhanced expression of HO-1 detected in βTC3 cells in response to H_2O_2 confirms a previous report in rat islets [23]. As islet cells express only low levels of MnSOD and glutathione peroxidase [26], synthesis of HO-1 and heat shock proteins may offer an alternative defence against oxidative stress in β-cells. Unlike HO-1, only very low levels of HO-2 expression were detected in cultured pancreatic islet and acinar cells.

The mechanisms by which oxidative stress and sulfhydryl-reactive agents enhance expression of HO-1 still remain unclear. One possibility is that alterations in the levels of the key intracellular antioxidant glutathione (GSH) may influence HO-1 expression. The stress agents employed in the present study are known to decrease GSH levels and/or form GSH conjugates. In Chinese hamster ovary cells, a decrease in intracellular GSH induces expression of HO-1 [27], while lowering GSH levels in murine peritoneal macrophages is not sufficient for HO-1 induction [28]. In animals treated with high doses of caerulein, the total pancreatic glutathione content falls during the early stages acute oedematous pancreatitis [14,29]. Thus, it seems reasonable to conclude that the decrease in intracellular GSH caused by reactive oxygen radicals represents one pathway for enhanced expression of HO-1 in acute caerulein-induced pancreatitis. However, addition of caerulein directly to the culture medium in our experiments had no effect on HO-1 expression in AR42J acinar cells (data not shown). Caerulein is known to bind to cholecystokinin receptors (CCKA) in AR42J acinar cells [30]. The lack of an effect of caerulein on HO-1 expression in AR42J cells in vitro may reflect a differential sensitivity to caerulein in the cultured cell line.

Carbon monoxide (CO) generated from heme metabolism by HO elevates cGMP levels in smooth muscle cells [6,7] and has been implicated as a regulator of cGMP production in the brain, where expression of HO-1 compensates for a depression in nitric oxide (NO) synthase activity [31]. Generation of NO mediates carbachol-stimulated increases in cGMP and calcium influx in pancreatic acinar cells, events involved in exocrine pancreatic secretion [32,33]. Furthermore, Seo et al. [34] have recently shown that treatment of rat pancreatic tissue with cholecystokinin results in rapid NO mediated increases in the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, cGMP levels and amylase release. These findings are, however, at variance with the study by Gilon et al. [35], who concluded that the increase in intracellular cGMP level depends on, rather than mediates, increases in intracellular calcium.

The present study has shown that only very low basal levels of heme oxygenase are expressed in the pancreas in vivo and acinar cells in vitro. It thus seems likely that in oxidative stress associated with acute pancreatitis enhanced expression of HO-1 would lead to an increased generation of CO which in concert with NO may contribute to the regulation of cGMP levels in acinar cells and exocrine pancreatic secretion. As regards the antioxidant potential of HO-1, recent evidence clearly demonstrates that overexpression of heme oxygenase moderates cell damage induced in rabbit coronary microvessel endothelial cells by recombinant haemoglobin and heme [36] and enhances the resistance of human pulmonary epithelial cells to non-heme mediated oxidant injury [37]. Thus, increased expression of HO-1 detected in pancreatic endocrine and exocrine cells may serve as a key defence mechanism against oxidative stress associated with acute inflammatory pancreatitis.

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