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Abstract
Although the pancreatic heat shock response has already been reported to confer protective effects during experimental pancreatitis, the mechanism of action remains unknown. We investigated the effects of hyperthermia in cerulein-induced pancreatitis. Heat shock protein 70 (HSP70) expression in rats was induced by a 20-min period of water immersion (42 degrees C). The severity of pancreatitis as well as the pancreatic expression of cytokines, nuclear factor-kappaB (NF-kappaB), and inhibitory factor kappaB-alpha (IkappaB-alpha) were evaluated in the presence and absence of hyperthermia. We found that hyperthermia resulted in time-dependent expression of HSP70 within the pancreas associated with a reduction in the severity of acute pancreatitis. Tumor necrosis factor-alpha and intercellular adhesion molecule-1 expression was significantly reduced in the presence of hyperthermia. Moreover, NF-kappaB activity was delayed in the presence of hyperthermia whereas IkappaB-alpha was stabilized in the cytoplasm. These results suggest that hyperthermia decreases the severity of cerulein-induced pancreatitis by decreasing cytokine [...]

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Effect of hyperthermia on NF-κB binding activity in cerulein-induced acute pancreatitis

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Frossard, Jean Louis, Catherine M Pastor, and Antoine Hadengue. Effect of hyperthermia on NF-κB binding activity in cerulein-induced acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 280: G1157–G1162, 2001.—Although the pancreatic heat shock response has already been reported to confer protective effects during experimental pancreatitis, the mechanism of action remains unknown. We investigated the effects of hyperthermia in cerulein-induced pancreatitis. Heat shock protein 70 (HSP70) expression in rats was induced by a 20-min period of water immersion (42°C). The severity of pancreatitis as well as the pancreatic expression of cytokines, nuclear factor-κB (NF-κB), and inhibitory factor κB-α (IκB-α) were evaluated in the presence and absence of hyperthermia. We found that hyperthermia resulted in time-dependent expression of HSP70 within the pancreas associated with a reduction in the severity of acute pancreatitis. Tumor necrosis factor-κB and intercellular adhesion molecule-1 expression was significantly reduced in the presence of hyperthermia. Moreover, NF-κB activity was delayed in the presence of hyperthermia whereas IκB-α was stabilized in the cytoplasm. These results suggest that hyperthermia decreases the severity of cerulein-induced pancreatitis by decreasing cytokine expression in the pancreas through the modulation of NF-κB activity.

EXPOSURE OF PROKARYOTIC and eukaryotic cells to hyperthermia induces a rapid and constant modification of cell metabolism called the heat shock response (17, 18). This response is mainly the consequence of the modified expression of stress proteins that were first described by Ritossa (22) 39 years ago. Raising the temperature of Drosophila above its physiological norm decreased the expression of several genes that were active before heat exposure and increased the expression of other genes encoding for a group of proteins referred as the heat shock proteins (HSPs). The main role of HSPs is of thermotolerance; a brief sublethal heat exposure of isolated cells induces quick expression of HSPs, conferring total protection against a subsequent but lethal exposure to hyperthermia. Additionally, HSPs also protect cells from other sources of stress such as endotoxins (30) and oxygen radicals (1, 30). Recently, we (9) also showed that HSPs prolong the in vivo survival time of rats suffering from anoxia.

Among the HSPs, HSP70 protects against experimental pancreatitis. The protective role of hyperthermia during experimental pancreatitis has already been investigated (29). Wagner et al. (29) demonstrated that hyperthermia partially protected acute pancreatitis by reducing acinar cell necrosis. Other studies (11, 12) showed that HSP70 expression after hyperthermia decreased the severity of cerulein-induced acute pancreatitis by lowering intrapancreatic trypsin activity. However, the protective mechanism of HSP70 is not completely understood. For example, whether hyperthermia decreases the severity of cerulein-induced acute pancreatitis is still under discussion. Among the mechanisms involved in the protection conferred by hyperthermia, a modification of nuclear factor-κB (NF-κB) activity might be important. Indeed, the transcription NF-κB is a pleiotropic regulator of many genes involved in inflammatory responses. NF-κB is rapidly activated after the induction of acute pancreatitis by cerulein (14–16). NF-κB activates genes encoding for proinflammatory cytokines (13, 16), such as TNF-α and interleukin-6 (IL-6), which in turn aggravate the severity of pancreatitis (19). Additionally, Rossi et al. (23) demonstrated that HSPs inhibit NF-κB activation through activation of the heat shock transcription factor.

The aim of our study was to better define the cytoprotective action of hyperthermia in a well-defined model of cerulein-induced acute pancreatitis. This experimental model induces edematous pancreatitis, the severity of which is correlated with pancreatic TNF-α concentrations (19), endothelial ICAM-1 expression (10), and neutrophil infiltration (2, 10, 25). Because previous studies (27) showed that hyperthermia can inhibit the activation of NF-κB and NF-κB is known as an activator of genes encoding for proinflammatory cytokines such as IL-6 and TNF-α, which can in turn upregulate the expression of adhesion molecules (27), we hypothesized that hyperthermia might abolish NF-κB nuclear migration and decrease TNF-α and ICAM-1 expression with a concomitant reduction of pancreatic injury.

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METHODS

**Animals.** All experiments were performed according to protocols approved by institutional animal care and the veterinary office. Male Wistar rats (75–125 g), obtained from BRL, were bred and housed in standard cages in a thermostated (23 ± 2°C) environment with 12:12-h light-dark cycles. The rats ate standard laboratory chow, drank water ad libitum, and were then randomly assigned to control or treated groups.

**Drugs.** Cerulein, the decapeptide analog of the pancreatic secretagogue, was purchased from Research Plus (Bayonne, NJ). Superfrost Plus slides were provided by Fisher Scientific (Pittsburgh, PA). All other chemicals and reagents were purchased from Sigma Chemical (Basel, Switzerland).

**Induction of pancreatitis.** Rats were intraperitoneally injected twice at a 1-h interval with a supramaximally stimulating dose (10 μg/kg) of cerulein to elicit acute pancreatitis (see Fig. 1). Control rats received similar injections of saline solution. The animals were killed 2 or 4 h after the last cerulein or saline injection with pentobarbital sodium injection (50 mg/kg ip).

**Induction of hyperthermia.** To produce hyperthermia rats were immersed in hot water (42 ± 2°C), and rectal temperature was monitored. Whole body temperature slowly increased over 25 min to reach 42°C and remained at this level 12 h after hyperthermia at the peak of heat. The rats were then randomly assigned to control or treated groups.

**Blood and tissue preparation.** Blood and pancreatic tissue were processed as described previously (10). Briefly, at the time of death blood was withdrawn from the heart and centrifuged, and the serum was kept at 4°C and remained at this level 12 h after hyperthermia at the peak of heat shock protein 70 (HSP70) expression within the pancreas. Rats were injected twice at a 1-h interval with a supramaximally stimulating dose of cerulein and killed 2 and 4 h after the second injection of cerulein, unless otherwise cited.

**Serum amylase activity.** Serum amylase activity was measured as described by Pierre et al. (21) using 4,6-ethylidene (G1)–D-maltoheptoside (Sigma Chemical, St. Louis, MO) as the substrate. The extent of pancreatic edema was quantitated by measuring tissue wet and dry tissue weights was calculated and expressed as a percentage of the tissue wet weight.

**Electrophoretic mobility shift assay.** To detect HSP70 protein, Western blot analysis of IκB, and the nuclear pellet was resuspended in the high-salt buffer containing 20 mM HEPES (pH 7.6), 25% (vol/vol) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM glycercophosphate, 10 mM Na2MgSO4, 1 mM DTT, and 1 mM PMSF. After being rotated at 4°C overnight, the nuclear membranes were pelleted by microcentrifugation for 10 min, and the clear supernatant (nuclear extract) was aliquoted and stored at −80°C. The protein concentration in the nuclear extract was determined by the Bio-Rad protein assay.

**Nuclear protein extracts.** To allow NF-κB measurement, nuclear protein extracts were prepared essentially as described by Dignam et al. (7). A 150- to 200-mg pancreatic tissue sample was lysed on ice in a hypotonic buffer by 20 or 25 strokes in a glass Dounce homogenizer. The hypotonic buffer was supplemented with phenylmethylsulfonyl fluoride (PMSF, 1 mM), dithiothreitol (DTT, 1 mM), and protease inhibitors, including pepstatin (5 μg/ml), leupeptin (5 μg/ml), and aprotenin (5 μg/ml). The homogenate was left on ice for 15–20 min, and Nonidet P-40 was added to a final concentration of 0.3–0.4% (vol/vol). The samples were briefly vortexed and incubated on ice for an additional 1–2 min. Crushed nuclear pellet was collected by centrifugation of the lysed tissue or cell samples for 30 s in a microfuge. The supernatant (cytosolic protein) was saved for Western blot analysis of IκB, and the nuclear pellet was resuspended in the high-salt buffer containing 20 mM HEPES (pH 7.6), 25% (vol/vol) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM glycercophosphate, 10 mM Na2MgSO4, 1 mM DTT, and 1 mM PMSF. After being rotated at 4°C overnight, the nuclear membranes were pelleted by microcentrifugation for 10 min, and the nuclear protein extracts were prepared essentially as described by Dignam et al. (7). A 150- to 200-mg pancreatic tissue sample was lysed on ice in a hypotonic buffer by 20 or 25 strokes in a glass Dounce homogenizer. The hypotonic buffer was supplemented with phenylmethylsulfonyl fluoride (PMSF, 1 mM), dithiothreitol (DTT, 1 mM), and protease inhibitors, including pepstatin (5 μg/ml), leupeptin (5 μg/ml), and aprotenin (5 μg/ml). The homogenate was left on ice for 15–20 min, and Nonidet P-40 was added to a final concentration of 0.3–0.4% (vol/vol). The samples were briefly vortexed and incubated on ice for an additional 1–2 min. Crushed nuclear pellet was collected by centrifugation of the lysed tissue or cell samples for 30 s in a microfuge. The supernatant (cytosolic protein) was saved for Western blot analysis of IκB, and the nuclear pellet was resuspended in the high-salt buffer containing 20 mM HEPES (pH 7.6), 25% (vol/vol) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM glycercophosphate, 10 mM Na2MgSO4, 1 mM DTT, and 1 mM PMSF. After being rotated at 4°C overnight, the nuclear membranes were pelleted by microcentrifugation for 10 min, and the clear supernatant (nuclear extract) was aliquoted and stored at −80°C. The protein concentration in the nuclear extract was determined by the Bio-Rad protein assay.

Fig. 1. Study protocol is shown. Rats were immersed in hot water (42°C) for 20 min and studied 12 h after hyperthermia at the peak of heat shock protein 70 (HSP70) expression within the pancreas. Rats were injected twice at a 1-h interval with a supramaximally stimulating dose of cerulein and killed 2 and 4 h after the second injection of cerulein, unless otherwise cited.

**SDS-PAGE and Western blot transfer for HSP70 protein, ICAM-1, and IκB proteins.** To detect HSP70 protein, Western blot was performed according to the method of Laemmli (16a) using minigels (Bio-Rad, Zurich, Switzerland). Proteins (10 μg) were loaded in each lane. After gel electrophoresis, proteins were transferred to nitrocellulose membrane according to Towbin et al. (28). The membrane was then incubated for 2 h with primary antibody (mouse monoclonal anti-HSP70, Sigma Chemical). After rinsing in PBS-Tween, the membrane was incubated with secondary antibody (antimouse IgG, Bio-Rad) for 1 h at room temperature. The same procedure was used to detect ICAM-1 expression (30 μg of protein were loaded in each lane) within the pancreas using mouse anti-ICAM-1 monoclonal antibody (mouse CD54, R&D Systems). For IκB measurement, cellular protein extracts were diluted in SDS-PAGE loading buffer and then resolved by conventional electrophoresis. The blot was incubated with a primary antibody against IκBα for 2 h (Santa Cruz Biotechnology), and after washing the blot was incubated with a secondary antibody (goat anti-mouse IgG, Bio-Rad).

**Nuclear protein extracts.** To allow NF-κB measurement, nuclear protein extracts were prepared essentially as described by Dignam et al. (7). A 150- to 200-mg pancreatic tissue sample was lysed on ice in a hypotonic buffer by 20 or 25 strokes in a glass Dounce homogenizer. The hypotonic buffer was supplemented with phenylmethylsulfonyl fluoride (PMSF, 1 mM), dithiothreitol (DTT, 1 mM), and protease inhibitors, including pepstatin (5 μg/ml), leupeptin (5 μg/ml), and aprotenin (5 μg/ml). The homogenate was left on ice for 15–20 min, and Nonidet P-40 was added to a final concentration of 0.3–0.4% (vol/vol). The samples were briefly vortexed and incubated on ice for an additional 1–2 min. Crushed nuclear pellet was collected by centrifugation of the lysed tissue or cell samples for 30 s in a microfuge. The supernatant (cytosolic protein) was saved for Western blot analysis of IκB, and the nuclear pellet was resuspended in the high-salt buffer containing 20 mM HEPES (pH 7.6), 25% (vol/vol) glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM glycercophosphate, 10 mM Na2MgSO4, 1 mM DTT, and 1 mM PMSF. After being rotated at 4°C overnight, the nuclear membranes were pelleted by microcentrifugation for 10 min, and the clear supernatant (nuclear extract) was aliquoted and stored at −80°C. The protein concentration in the nuclear extract was determined by the Bio-Rad protein assay.
nondenaturing 4.5% polyacrylamide gel at 200 V. Gels were dried and directly exposed at −80°C to Kodak RX film with intensifying screens. The intensity of the bands on the gel was quantified using the image analysis system from Zeiss (Zurich, Switzerland).

**TNF-α assay.** TNF-α was quantitated using a commercially available ELISA kit (Endogen, Woburn, MA). Pancreas tissue cytokine levels were measured by homogenizing a sample of freshly obtained tissue in 2 ml of phosphate buffer (20 mM, pH 7.4), subjecting it to centrifugation (14,000 g for 5 min at 4°C), and quantitating TNF-α in the resulting supernatant. Results were expressed as picograms per microgram of DNA in the sample.

**Morphology.** Sections of pancreatic tissues were rapidly removed at the time of death, fixed, embedded in paraffin, and sectioned (5 μm). After staining with hematoxylin and eosin, the sections were examined by an experienced morphologist who was not aware of the sample identity. The extent of acinar cell necrosis was quantitated by computer-assisted morphometry as previously described (10) and expressed as a percentage of total acinar tissue.

**Analysis of data.** The results are expressed as means ± SE of values obtained from at least three different determinations. The significance of changes was evaluated using Student’s t-test when data included two groups or ANOVA when three or more groups were compared.

**RESULTS**

**Hyperthermia induced intrapancreatic HSP70 protein expression.** Whole body hyperthermia resulted in time-dependent expression of HSP70 within the pancreas with a peak occurring 12 h after the heating procedure (Fig. 2). For the following experiments, all animals were studied at the peak of HSP70 expression within the pancreas (12 h after hyperthermia) because the cytoprotective effects of hyperthermia were maximal at that time. Twelve hours after whole body hyperthermia, rats were killed and in vitro amylase secretion from acini was studied as a function of cerulein concentration. The pattern of secretion was identical to the pattern observed in control animals, indicating that the receptor remained functionally intact after hyperthermia (data not shown).

**Effects of hyperthermia on severity of cerulein-induced acute pancreatitis.** The severity of the cerulein-induced acute pancreatitis was assessed by serum amylase concentration, edema within the gland, the amount of neutrophils sequestered within the gland as reflected by MPO activity, and acinar cell necrosis. Supramaximal stimulation of rats with cerulein increased concentrations of serum amylase, edema within the gland, and acinar cell necrosis 3 and 5 h after the start of cerulein injection (Fig. 3). Cerulein injections, in the absence of hyperthermia, concomitantly increased intrapancreatic MPO activity. In contrast, when rats were exposed to hyperthermia 12 h before cerulein injections, the severity of acute pancreatitis decreased. Hyperthermia by itself had no effect on MPO activity in control rats.

**Intrapancreatic TNF-α concentrations in cerulein-treated rats in the presence or absence of hyperthermia.** Injection of cerulein increased pancreatic TNF-α concentrations over time (Fig. 4). Pancreatic TNF-α concentrations also increased in rats previously exposed to hyperthermia before cerulein injections. However, the peak of TNF-α was higher in the absence of hyperthermia (3.56 ± 0.3 pg/μg DNA at 3 h; 6.44 ± 0.48 pg/μg DNA at 5 h) than in the presence of hyperthermia (1.42 ± 0.2 pg/μg DNA at 3 h; 2.56 ± 0.26 pg/μg DNA at 5 h) (P < 0.01).

**Intrapancreatic ICAM-1 expression in cerulein-treated rats in presence or absence of hyperthermia.** Cerulein injections induced a small but significant rise of ICAM-1 expression in the absence of hyperthermia (Fig. 5) 3 h after the start of cerulein injection and a
more significant rise after 5 h. The ICAM-1 expression was mainly located on the endothelium surface (data not shown). When rats had been previously exposed to hyperthermia, pancreatic ICAM-1 content was lower. The optical density of the ICAM-1 band was 2.91 times higher in cerulein-treated animals at 3 h and 3.24 times higher at 5 h compared with pancreas isolated from animals injected with saline. ICAM-1 expression within the pancreas of cerulein-injected animals previously exposed to hyperthermia was slightly increased (optical density 1.15 times higher than in animals injected with saline at 3 h and 1.77 times higher at 5 h), but the increase was significantly lower than the increase observed in pancreas isolated from animals injecting with cerulein in the absence of hyperthermia ($P < 0.02$).

NF-$\kappa$B binding activity in normal and cerulein-treated rats in presence or absence of hyperthermia. In normal animals, there was no detection of NF-$\kappa$B activity and hyperthermia alone did not induce the activation of NF-$\kappa$B (Fig. 6). Cerulein injections increased NF-$\kappa$B binding activity over time in the absence of hyperthermia. NF-$\kappa$B binding activity rapidly increased 30 min after cerulein injections and peaked at 60 min. Cerulein injection in rats previously exposed to hyperthermia completely abolished the increase of NF-$\kappa$B binding activity within the first 45 min. However, the activity appeared at 45 min and reached a maximum by 90 min. Thus after cerulein treatment, NF-$\kappa$B activity was significantly modified by hyperthermia. Because previous studies showed that the heat shock response inhibits NF-$\kappa$B nuclear translocation by sta-
bilizing the NF-κB inhibitory protein IκB-α (23), we collected cytoplasmic extracts from rats treated with cerulein in the absence and presence of hyperthermia and analyzed them by Western blot using anti-inhibitory κB-α (IκB-α) antibody (Fig. 7). The cytoplasmic IκB-α signal decreased 30 min after cerulein administration in the absence of hyperthermia (−57% compared with pancreas isolated from animals injected with saline), suggesting the nuclear translocation of NF-κB, whereas IκB-α signal remained in the cytoplasm until 60 min in the presence of hyperthermia, suggesting the preservation of the NF-κB-IκB-α complex at this time (Fig. 7).

**DISCUSSION**

Our study clearly shows that hyperthermia is associated with a time-dependent increase of pancreatic HSP70 expression, which peaked 12 h after heat stress. Concomitantly, hyperthermia protects against cerulein-induced acute pancreatitis as evidenced by decreased serum amylase concentration, pancreatic edema, and acinar cell necrosis extent. Moreover, TNF-α and ICAM-1 expression, two parameters correlated with the severity of cerulein-induced pancreatitis (10, 20), were significantly reduced in the presence of hyperthermia. Finally, the nuclear migration of NF-κB was delayed by hyperthermia whereas the NF-κB-IκB-α complex was stabilized in the cytoplasm. These results suggest that hyperthermia decreases the severity of cerulein-induced pancreatitis by decreasing cytokine expression in the pancreas through the modulation of NF-κB activity.

Because in this model we clearly demonstrated that the CCK-receptor binding characteristics after hyperthermia are unaltered by in vitro amylase secretion study (12), the beneficial effects due to hyperthermia are not related to a decreased binding of cerulein to the receptor. In our study, TNF-α was upregulated in the pancreas during cerulein hyperstimulation as previously shown by Gukovskaya et al. (14). TNF-α levels rise in a time-dependent manner in animals injected with cerulein, whereas these levels were significantly lower when rats were previously exposed to hyperthermia. The deleterious role of TNF-α in inducing organ injury has already been investigated. TNF-α might promote organ injury by upregulating ICAM-1 (19), an important adhesion molecule involved in the adhesion of circulating activated inflammatory cells to the microvascular endothelial surfaces and in the leukocyte sequestration within areas of pancreatic injury and inflammation (10). In our study, ICAM-1 expression was markedly elevated during cerulein-induced pancreatitis, as was MPO content, which reflects the sequestration of inflammatory cells. We also found that both ICAM-1 expression and MPO content increases were significantly attenuated in the presence of hyperthermia. Thus rats exposed to hyperthermia are partially protected from cerulein-induced pancreatitis, because both TNF-α and ICAM-1 expression were blunted with a concomitant decrease of neutrophil recruitment into the pancreas, which plays an important role in the development of injury (2, 10, 25).

As shown by Gukovskaya et al. (15), NF-κB was rapidly activated by cerulein in the rat pancreas. The activity peaked by 45 min after the start of cerulein injections (15). Interestingly, the pattern of NF-κB activity was different in pancreatic tissue expressing a high amount of HSP70 protein. Indeed, in the presence of hyperthermia, NF-κB activity was first blunted within the first 45 min while its activity appeared only by 60 min. In the absence of hyperthermia, NF-κB activity peaked at 60 min in the nucleus while the IκB α signal disappeared in the cytoplasm. However, in the presence of hyperthermia, NF-κB nuclear translocation was delayed and was probably associated with stabilization of IκB α in the cytoplasm. Thus the delayed NF-κB activity was likely to decrease cytokine expression in the pancreas, which in turn reduced the severity of acute pancreatitis.

Indeed, HSPs might directly interfere with NF-κB, which is a key regulator of inducting cytokines such as TNF-α, IL-1, and IL-6 (13). Heat exposure is also known to inhibit cytokine-mediated inducible nitric oxide synthase gene expression through the activation of the same transcriptional regulator NF-κB (26). Several studies found (23, 24) that the heat shock response inhibits cytokine-mediated NF-κB nuclear translocation in human isolated cells. The heat shock response also inhibits cytokine-mediated IκB degradation by a chaperone cytoprotective mechanism in the rat liver (4, 5). Lastly, Curry et al. (6) demonstrated that HSPs transiently inhibited radiation-induced NF-κB DNA binding activity by preventing IκB kinase activation. Thus the increased expression of HSP in our model might be responsible for the delayed NF-κB activity observed in the presence of hyperthermia.

Our data suggest that the heat shock response protects against cerulein-induced pancreatitis by interfering with transcriptional mechanisms involving, at least in this study, the NF-κB-IκB pathway. However, it is possible that HSPs may act through other transcription factors to influence the severity of pancreatitis. Indeed, HSPs were shown (3) to modulate the cellular activator protein-1, a transcription factor in-
in multiple aspects of cell regulation (8). The molecular protective mechanism of hyperthermia is not fully understood; further study is needed to comprehend it.

In conclusion, our study shows that hyperthermia decreases cytokine expression in the pancreas with a concomitant reduction of the severity of acute pancreatitis. Because HSP70 is highly expressed within the pancreas 12 h after hyperthermia and HSP protein has been shown to decrease NF-κB activity, the delayed activity of NF-κB observed in our study is likely to stem from HSPs. However, the mechanisms involved between NF-κB and HSP70 and between NF-κB and cytokine expression need further study. Our study suggests that the heat shock pathway is able to simultaneously switch on cytoprotective genes and downregulate genes encoding for proinflammatory cytokines such as TNF-α.

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