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Effect of Hyperthermia on Premature Intracellular Trypsinogen Activation in the Exocrine Pancreas

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Hyperthermia, raising the body temperature from normal to above 40°C, has been shown to prevent pancreatitis in an experimental animal model of the disease, but the underlying cellular mechanisms of this protection remain unknown. We induced controlled hyperthermia in either laboratory rats and isolated pancreatic acini or, alternatively, raised the temperature of pancreatic homogenates *in vitro* from 37 to 41°C. *In vitro* controlled hyperthermia of up to 41°C increased the autoactivation-induced and enterokinase-induced trypsinogen activation as well as free trypsin activity. Conversely, in whole animal studies and in living acinar cells hyperthermia reduced or abolished premature intracellular trypsinogen activation in a time- and temperature-dependent manner and this protective effect was independent of either *de novo* protein synthesis, interference with acinar cell signal transduction, or confirmational changes in pancreatic trypsinogen. We conclude that hyperthermia, in a manner that is independent of the synthesis of pancreatic chaperone or heat shock proteins, can directly abolish the earliest initiating event involved in the onset of pancreatitis, namely the premature and intracellular activation of digestive zymogens.

Key Words: **autoactivation; hyperthermia; pancreatitis; serine proteases; trypsinogen.**

Acute pancreatitis is thought to represent an autodigestive disorder in which the exocrine pancreas is destroyed by its own proteolytic enzymes (1). Under physiological conditions digestive proteases are synthesised as inactive precursor zymogens, stored in the pancreas in membrane confined vesicles, and activated only in the small intestine after contact with the brush-border enzyme enterokinase [enteropeptidase] (2). Human (3) as well as experimental pancreatitis (4, 5, 6) have been found to be associated with a premature and intrapancreatic activation of digestive proteases, most notably trypsinogen, and this premature activation appears to begin in cytoplasmic vacuoles (7, 8).

One method of preventing pancreatitis, at least in an animal model of the disease, consists in raising the body temperature to above 40°C (9). This experimental hyperthermia has been found to not only protect against experimental pancreatitis but also to upregulate a variety of stress response proteins (i.e., heat shock proteins) in the pancreas (9, 10). The mechanisms, however, through which the pancreatic stress defence system either prevents the onset of pancreatitis or improves the outcome of pancreatitis are unknown. We have therefore investigated whether *in vivo* or *in vitro* hyperthermia affects the earliest known pathophysiological event in pancreatitis; the premature intracellular activation of trypsinogen. Our experiments using whole laboratory animals, isolated acini, or pancreatic homogenates indicate that controlled hyperthermia directly reduces or abolishes secretagogue-induced premature trypsinogen activation in the pancreas. This effect of hyperthermia, however, is independent of a direct effect on the stability and activity of trypsin and is also independent of the *de novo* synthesis or upregulation of heat shock proteins.

METHODS

All materials were obtained from Sigma (Deisenhofen, Germany) or Molecular Probes (Eugene, OR) unless stated otherwise in the text. Animals (male Wistar rats throughout) were obtained from Charles River (Sulzfeld, Germany), held in Nalgene shoebox cages for a minimum adjustment period of 5 days and used in experiments after an overnight fast with access to water *ad libitum*. All experiments involving animals were carried out in accordance to the guidelines of, and after prior protocol approval by, the institutional Animal Welfare Committee. To induce hyperthermia *in viv*o animals were kept anaesthetised using Ketamin (250 mg/kg; CuraMed, Karlsruhe, Germany), together with Rompun (40 mg/kg, BayerVital,

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Leverkusen, Germany), placed on a heated pad with an infrared overhead lamp until the core temperature, as measured by rectal probe, was elevated to 41°C over 30 min. Animals were then sacrificed and the pancreas was rapidly removed. Acini from animals after controlled hyperthermia and from control animals (kept at 37°C body temperature) were freshly prepared by collagenase digestion as previously reported (11). Acini were suspended in Hepes (24.5 mM) buffered medium (pH 7.5) containing NaCl (96 mM), KCl (6 mM), $MgCl_2$ (1 mM), NaH_2PO_4 (2.5 mM), $CaCl_2$ (0.5 mM), glucose (11.5 mM), Na-pyruvat (5 mM), Na-glutamat (5 mM), Na-fumarat (5 mM), minimum essential medium (1% v/v), and bovine serum albumin, fraction V (1% w/v).

To study intracellular protease activation in living acini they were adjusted to a biovolume concentration of 2 mm^3/ml and left to equilibrate for 30 to 60 min at temperatures ranging from 37 to 41°C. Thereafter the cholecystokinin analogue caerulein (Bachem, Heidelberg, Germany) was added at either supramaximal (10 nM) or maximal (0.1 nM) concentrations for up to 60 min and acini were then resuspended in medium without secretagogue but in the presence of the synthetic trypsin substrate $(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-rhodamine 110 (10 μM). For localisation experiments a high-resolution residual-light fluorescence imaging system (Till-Photonics, Martinsried, Germany; Ex 485 nm, Em 530 nm) was used. In order to quantify substrate cleavage acini were transferred to 96-well microtiter plates and the $\Delta F/\Delta t$ ratio was determined by cytofluorometry (CytoFluor 2350, Millipore, Bedford, MA) over 60 min as previously reported (8).

In order to study the effect of hyperthermia on the activation of trypsinogen *in vitro* the pancreas of control animals was quickly removed and homogenised (3 strokes of a Dounce homogenizer) in iced 0.2M TEA buffer at pH 7.5. Subsequently homogenates were incubated at either 37, 39, or 41°C in the presence of purified enterokinase (enteropeptidase; 0.1 μM, Boehringer, Mannheim, Germany) for 10 min.

For experiments addressing the autoactivation of trypsinogen Percoll-gradient isolates of secretory vesicles were prepared as previously described (8). Trypsinogen activation was measured spectrometrically using the specific substrate Bz-L-Arg-4-NA (L-BAPNA, Merck, Darmstadt, Germany).

For experiments in which we studied whether the effect of hyperthermia on zymogen activation in living acini depended on the synthesis or upregulation of heat shock proteins we completely blocked protein synthesis using the inhibitor cycloheximide (300 μM) for 30 min prior to the experiments, as well as throughout the incubation period, and as previously described (12). After exposure to temperatures ranging from 37 to 41°C and to either Caerulein (10 nM) or NaCl (controls) as indicated above acini were lysed in 250 μl Ripa-buffer at pH 8.0 containing Tris–HCl (100 mM), NaCl (300 mM), 0.2% SDS, 2% Triton-X-100, 2% Na-deoxycholate, and a protease inhibitor cocktail (1 ml/mg tissue) composed of Aprotinin (10 μg/ml), Leupeptin (10 μg/ml), sodiumpyrophosphate (0.01 M), sodiumfluoride (0.1 M), dihydrogenperoxide (1 mM), L-phenyl-methyl-sulfonyl-fluoride (PMSF, 1 mM), and 0.02% soybean-trypsin-inhibitor. Total lysates were centrifuged at 14,000 rpm (4°C) for 10 min, the protein concentrations were determined according to Bradford (BioRad-assay) and equal amounts of protein were used for SDS–polyacrylamide gel electrophoresis and Western blotting as previously reported (13). To study the synthesis and upregulation of heat shock proteins from different classes we used antibodies directed against the constitutive heat shock protein HSP-70/72 (14) and against the inducible form HSP-70 (15). Both antibodies were obtained from StressGen (Victoria, BC, Canada).

In order to study the structural and functional integrity during controlled hyperthermia amylase secretion and lactate dehydrogenase release were measured in the supernatant of acinar cells incubated with either physiological or supramaximal concentrations of secretoagogue over 60 min.

The data reported represent means ± SEM obtained from multiple determinations in 3 or more separate experiments for each group.

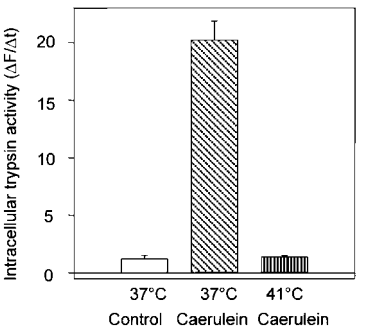

FIG. 1. Effect of *in vivo* hyperthermia on secretagogue-induced trypsinogen activation in the pancreas. The core temperature of anaesthetised rats was raised to 41°C for 30 min as described under Methods. The core body temperature of control rats was left at physiological levels. The pancreas was then removed and isolated acini incubated at 37°C with 10 nM caerulein for 10 min. Intracellular trypsinogen activation was measured cytofluorometrically in living acini over 40 min using the cell permeant trypsin substrate $(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-rhodamine 110. Bars represent mean intracellular trypsin activity ($\Delta F/\Delta t$) ± SEM of triplicates, representative for three experiments.

Data points in the graphs indicate means ± error bars (SEM). The absence of error bars indicates the deviation from the mean was to small to illustrate. The significance of changes was evaluated using Student's *t*-test when the data consisted of two groups and by analysis of variance (ANOVA) when comparing three or more groups. Differences were considered significant when the p value was <0.05.

RESULTS

Exposure of isolated pancreatic acini from control animals to supramaximal concentrations of caerulein (10 nM) resulted in a rapid intracellular activation of trypsinogen as indicated by the release of rhodamine 110 fluorescence from the specific trypsin substrate and as previously reported (8, 16). When acini were prepared from the pancreas of animals whose core body temperature had been raised to 41°C for 30 min before sacrifice this pretreatment by *in vivo* hyperthermia completely prevented the subsequent caerulein-induced intracellular activation of trypsinogen (Fig. 1). Because *in vivo* hyperthermia could have interfered with a number of biological processes that are not directly related to intracellular zymogen activation such as pancreatic blood supply or tissue oxygenation we prepared acini from untreated animals and studied them after raising the temperature of the incubation medium from 37 to 41°C.

When acini that were preincubated at 37°C were then exposed to supramaximal concentrations of caerulein evidence of a premature and intracellular trypsinogen activation on imaging studies first appeared as bright fluorescent foci at the apical pole of acinar cells (Fig. 2A) and spread from there to the entire cytosole of the affected cells. Prior (30 min) exposure of acini to incubation medium whose tempera-

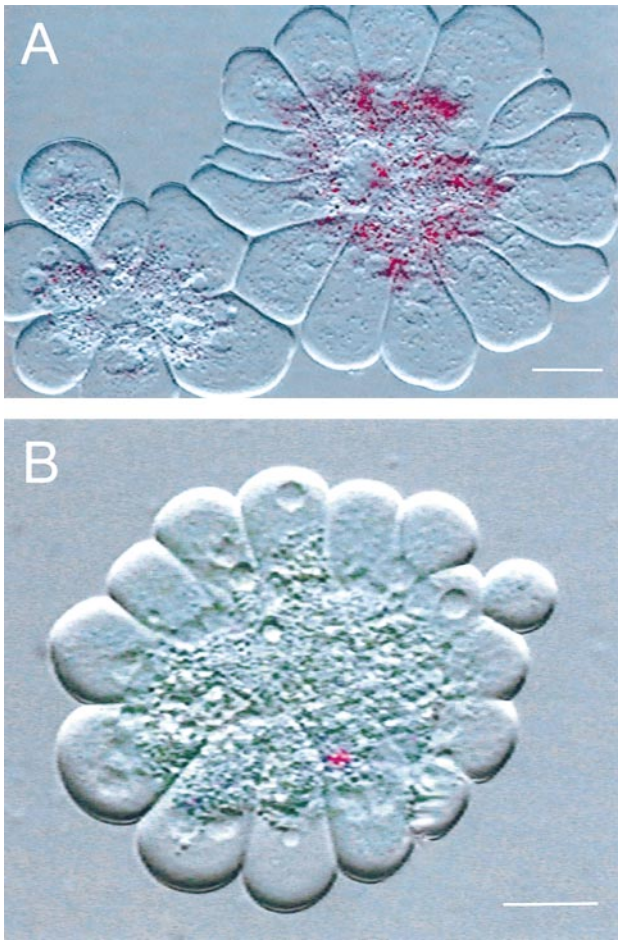

FIG. 2. Effect of *in vitro* hyperthermia on secretagogue-induced trypsinogen activation in pancreatic acini. Isolated acini were prepared by collagenase digestion, stimulated with supramaximal (10 nM) caerulein for 10 min, and placed under a coverslip for imaging of intracellular fluorescence after exposure to the cell permeant fluorogenic substrate $(CBZ\text{-}Ile\text{-}Pro\text{-}Arg)_2$-rhodamine 110 for 90 min. (A) The bright fluorescence (pseudocolour magenta for better contrast) at the apical pole of individual acinar cells indicates the location of the substrate cleavage by active intracellular trypsin. (B) Acinus in which hardly any trypsin activity developed over the same time interval had been preincubated at 41°C rather than at 37°C for 30 min before exposure to caerulein. Bars indicate 10 μm and images are representative for at least four separate experiments.

ture had been raised to 41°C completely abolished not only the cascade-like activity in the acinar cell cytoplasm but already prevented the initial activation in the membrane confined vesicles at the apical pole of acinar cells (Fig. 2B). When the same process was studied and quantitated by cytofluorometry of living acini an increase in the preincubation temperature resulted in a complete prevention of the caerulein-induced intracellular activation of trypsinogen. This effect was found to be time dependent (Fig. 3A) as well as temperature dependent (Fig. 3B).

In order to exclude that controlled hyperthermia had somehow interfered in the coupling of caerulein to the cholecystokinin receptor or with subsequent steps in acinar cell signal transduction we performed a series of experiments in which we tested the effect of preincubation at 41°C for 30 min on caerulein-evoked amylase secretion from isolated acini. These experiments indicated that stimulus-secretion-coupling in pancreatic acini remained unaffected by controlled hyperthermia (Fig. 4A) because amylase secretion was not impaired. To further exclude that controlled hyperthermia had damaged the acinar cells in a manner that precluded a subsequent activation of zymogens we measured the leakage of lactate-dehydrogenase (LDH) from pancreatic acinar cells in suspension. Over the course of 1 h the maximum LDH leakage of control acini amounted to 7.5% of the total LDH content. Prior hyperthermia of 41°C for 30 min increased the subsequent LDH leakage to a maximum of only 9.9% which indicates that the hyperthermia-induced acinar cell damage was minimal and did not account for the prevention of trypsinogen activation (Fig. 4B).

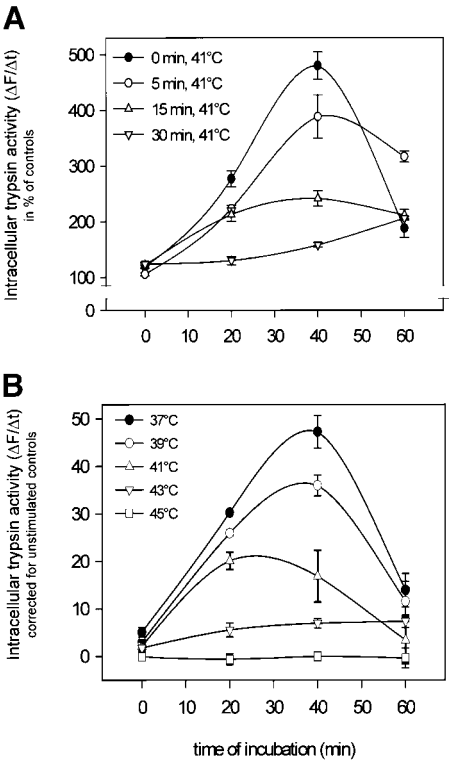

FIG. 3. Time dependence and temperature dependence of the effect of *in vitro* hyperthermia on secretagogue-induced trypsinogen activation in pancreatic acini. (A) Experiments isolated acini were preincubated in 41°C buffer for various time intervals from 0 to 30 min before exposure to caerulein (10 nM). (B) Experiments acini were always preincubated for 30 min but the buffer temperature was raised to different temperatures ranging form 37 to 45°C. Note that the effect of hyperthermia on intracellular trypsinogen activation depends on the time interval of preincubation as well as on the temperature of the preincubation buffer. Data points represent cytofluorometrically determined intracellular trypsin activity ($\Delta F/\Delta t$) ± SEM of triplicates, representative for five experiments.

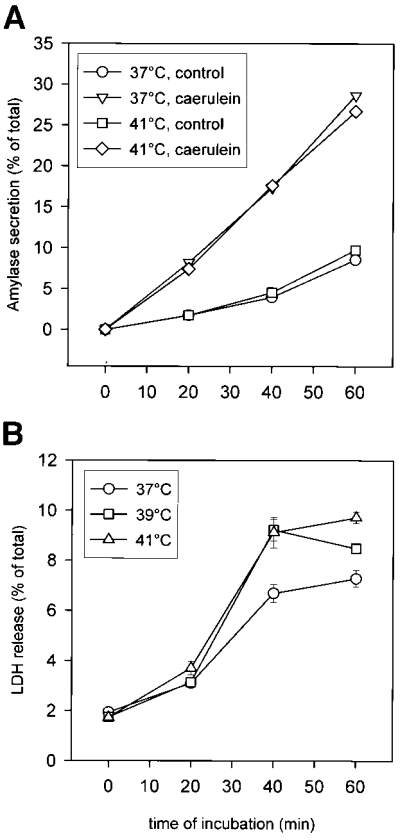

FIG. 4. Effect of *in vitro* hyperthermia on secretagogue-induced amylase secretion and LDH-release from pancreatic acini. (A) Acini were prepared as described under Methods and, after 30 min preincubation at either 37 or 41°C, exposed to a maximal concentration of caerulein (0.1 nM) over 60 min. Control acini were not exposed to caerulein. Amylase secretion into the medium in response to this stimulus was expressed as percentage of total amylase content of the acini. (B) Experiments acini were incubated for up to 60 min at temperatures ranging from 37 to 41°C and LDH leakage into the medium was expressed as percentage of total LDH content in acini. Data points in A and B are representative for three separate experiments.

When active trypsin and inactive trypsinogen are both present in the same solution two competing processes are known to occur. One is the activation of the zymogen trypsinogen by active trypsin and the other is an autodegradation of either trypsinogen or trypsin by active trypsin (17). In order to exclude that controlled hyperthermia of living pancreatic acini had either shifted the balance from trypsin-induced trypsinogen activation to trypsin-induced trypsin degradation or that it had led to a conformational change in the trypsin molecule which would have precluded trypsin activity to develop we performed another series of experiments. First, we incubated pancreatic acini in medium of 41°C for 30 min and measured their trypsinogen content as enterokinase-activatable trypsin activity in acinar homogenates. The results of these experiments indicate that total cellular trypsinogen content remained unchanged in response to controlled hyperthermia and that trypsinogen degradation had not occurred (Fig. 5A). Moreover, when we homogenised whole pancreas and incubated the homogenate in the presence of enterokinase at various temperatures an increase rather than a decrease in trypsin activity resulted (Fig. 5B). This indicates that the catalytic activity of trypsin is resistant to temperature increases of up to 41°C and that hyperthermia does not cause a conformational alteration of the trypsin molecule that would interfere with substrate cleavage.

To exclude that hyperthermia would affect the capacity of pancreatic zymogens to autoactivate we isolated a secretory vesicles fraction from pancreatic acini by Percoll density centrifugation. When this fraction was lysed with Triton X-100 and left at various temperatures for 60 min a spontaneous trypsin activity developed at 41°C, but not at 37°C (Fig. 5C). This observation indicates, again, that neither the enzymatic activity of trypsin nor its capacity to autoactivate were negatively influenced by controlled hyperthermia. Taken together these experiments show conclusively that, *in vitro*, a 4°C increase of the ambient temperature increases rather than decreases trypsin activity. The preventive effect of hyperthermia on premature and intracellular zymogen activation in living pancreatic acinar cells can therefore not be accounted for by a negative effect on either the acinar cell stimulus secretion coupling, nor on the cellular integrity of acini, nor on the stability and expression levels of their protease zymogens.

Because it has previously been shown that hyperthermia can induce an upregulation of stress response or chaperone proteins in various cell lines including pancreatic cells (18) and because an upregulation of heat shock proteins has previously been suggested to be responsible for the protective effect of hyperthermia on experimental pancreatitis (19) we inhibited protein synthesis in pancreatic acinar cells. The expression of the constitutive form of HSP-70/72 was neither affected by a temperature increase from 25°C (controls) to 37 or 41°C, nor by exposure to supramaximal concentrations of caerulein (10 nM), nor by preincubation with the protein synthesis inhibitor cycloheximide (Fig. 6A). Inducible HSP-70, on the other hand, was found to increase slightly in acini whose temperature was raised to 37°C and increased to a greater extend when the temperature was raised to 41°C. Additional exposure to supramaximal caerulein concentrations lead to an, at most, marginal increase of HSP-70 expression over the upregulation already obtained by raising the temperature. Preincubation with 300 μM cycloheximide completely prevented the expression of inducible HSP-70 under all of the above conditions. The same blockage of protein synthesis with cycloheximide before and during the induction of hyperthermia and during subsequent supramaximal caerulein stim-

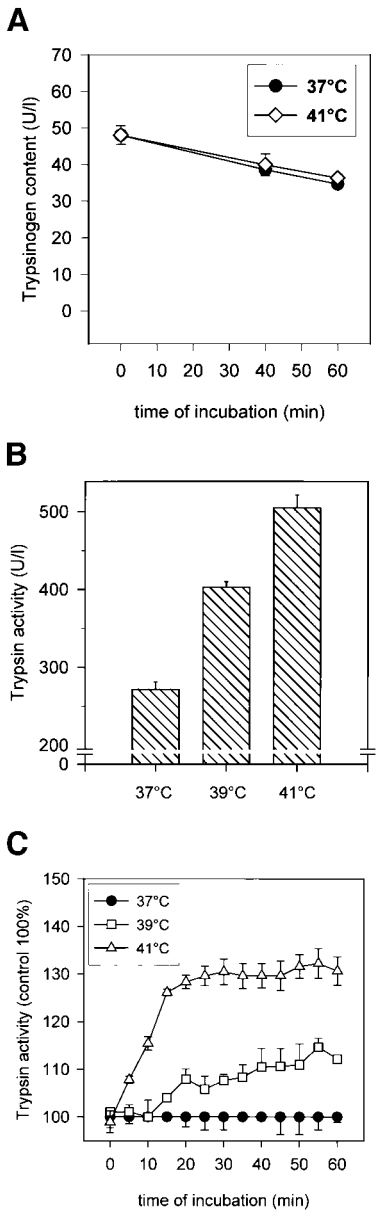

FIG. 5. Effect of hyperthermia on trypsinogen activation and trypsin activity *in vitro*. (A) Experiments acini were incubated for up to 60 min at either 37 or 41°C. Acini were then homogenised at different time intervals and their trypsinogen content determined as enterokinase activatable trypsin activity. (B) Experiments rat pancreas was homogenised in 200 mM TEA buffer at pH 7.5, centrifuged, brought to temperatures ranging from 37 to 41°C, and trypsinogen in the supernatant activated with enterokinase. Trypsin activity at the various temperatures was determined over 10 min as cleavage of the trypsin substrate Bz-L-Arg-4-NA (L-BAPNA, 0.8 mM). (C) To determine the effect of hyperthermia on trypsinogen autoactivation experiments rat pancreas was homogenised and a secretory vesicles-containing fraction was isolated by density gradient centrifugation with Percoll. Vesicles were transferred to TEA buffer (pH 7.5, no Ca^{2+}) and membranes were lysed with Triton X-100 (0.5%). At time intervals up to 60 min trypsinogen autoactivation was determined as spontaneous trypsin activity in the solution (L-BAPNA 0.8 mM, Ca^{2+} 20 mM). Note that under these conditions no autoactivation occurred in control samples (37°C, corrected for L-BAPNA alone). Data in A–C represent means ± SEM of triplicates, representative for at least three separate experiments.

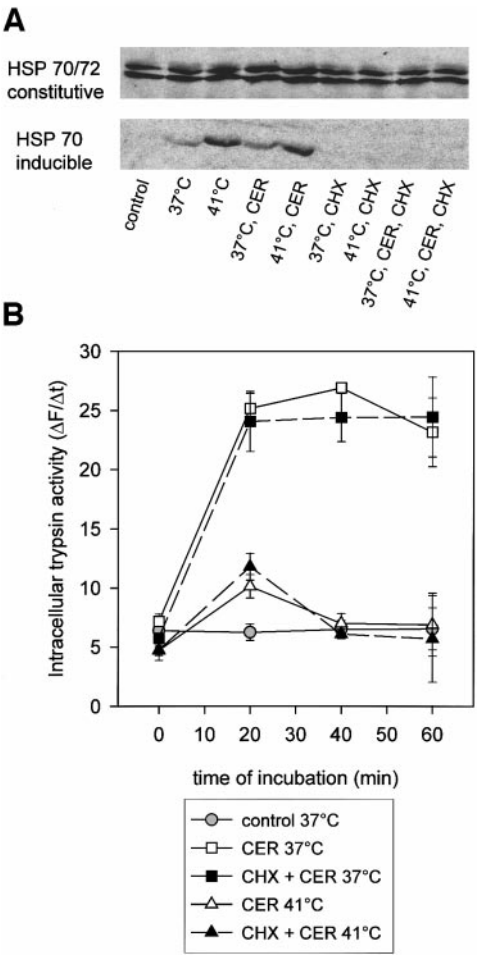

FIG. 6. Protein synthesis and the effect of *in vitro* hyperthermia on secretagogue-induced trypsinogen activation in pancreatic acini. To exclude that the *de novo* synthesis of heat shock or chaperone proteins plays a role in the effect of hyperthermia on caerulein-induced intracellular trypsinogen activation, we performed experiments as described under the Fig. 2 legend, but added the protein synthesis inhibitor cycloheximide (CHX 300 μM) during the 30 min preincubation, as well as during exposure to 37 and 41°C or caerulein (CER). (A) Acini were homogenized in Ripa-buffer and, after SDS–gel-electrophoresis blotted with antibodies directed against constitutive HSP-70/72 (top lane) or inducible HSP-70 (bottom lane). Note that preincubation with cycloheximide completely prevented the upregulation of inducible HSP-70, but had no effect on constitutive HSP-70/72 expression. Conversely, in the activation studies shown in (B) the preincubation with cycloheximide had no effect on the hyperthermia-induced prevention of trypsinogen activation in living acini which indicates that this event is independent of the synthesis or upregulation of heat shock proteins. Data represent means ± SEM of triplicates, representative for three experiments.

ulation had, however, no detectable effect on intracellular trypsinogen activation (Fig. 6B). The result of this experiment conclusively rules out that the synthesis or upregulation of hyperthermia-induced stress response proteins is a critical factor in mediating the protective effect on intracellular zymogen activation in the pancreas.

DISCUSSION

The manner in which the exocrine pancreas responds to stress appears to have a profound effect on the onset and course of acute pancreatitis. When, for example, a generally mild and oedematous variety of experimental pancreatitis is induced in rats by injection of a supramaximal concentration of the secretagogue caerulein and the animal is simultaneously exposed to exogenous stress by water immersion, the resulting pancreatitis is much more severe than after application of the secretagogue alone (20). When, alternatively, the animal is exposed to the same exogenous stress before the induction of experimental pancreatitis a marked protection against the disease onset has been reported (9, 21, 22). From a variety of studies that have addressed this issue it can be concluded that the manner in which the systemic stress is experimentally applied remains immaterial because water immersion (22), prolonged fasting (23), or controlled hyperthermia (9) have all resulted in a protection against pancreatitis when induced prior to the disease onset. The reason for this protective effect has remained unclear but a number of stress response proteins (also called heat shock proteins) have been implicated in this context (9, 19, 22). These proteins have complex functions as chaperones for misfolded or malfunctioning proteins (24) and are known to be upregulated in response to hyperthermia or other exogenous types of stress to the cell. In the exocrine pancreas the heat shock proteins (HSP) 27, 60, 70, 85, 90, and 100 have not only shown to be expressed, but also to be upregulated under a variety of physiological and pathological conditions (17, 19, 25, 26). It was therefore suggested that they may play a role in the protection against pancreatitis which can be observed after the induction of exogenous stress.

Recent studies have shown that the earliest intracellular event that precedes pancreatic necrosis in the course of pancreatitis and which determines whether or not pancreatic damage develops is the premature and intracellular activation of digestive enzymes (4, 5, 6). We have therefore investigated whether the protection against pancreatitis by hyperthermia affects this initial cell biological event or later steps in the disease process. Using a model system in which we can image and quantitate the intracellular activation of protease zymogens (8, 16), we found that both, *in vitro* and *in vivo* hyperthermia directly inhibited the premature and intracellular activation of trypsinogen that is induced by supramaximal caerulein stimulation of pancreatic acini. This effect could have been due to a depletion in pancreatic trypsinogen content or to an interference with trypsin activity. In several control experiments we could rule out these possibilities and found that trypsin is, in fact, more active at temperatures of up to 41°C. We could further exclude that controlled hyperthermia interfered with acinar cell stimulus response coupling or that it had damaged acinar cells in a manner that would have precluded intracellular zymogen activation.

Surprisingly, the inhibition of protein synthesis in pancreatic acini had no effect on the prevention of trypsinogen activation by controlled hyperthermia. Although this observation excludes upregulation of heat shock proteins as the underlying mechanism through which hyperthermia prevents trypsinogen activation it can not completely rule out that one of these chaperones is involved in the process. Rather than an upregulation on the protein level, which our data excludes, a conformational change or an event involving protein phosphorylation (24, 27) in one of the heat shock proteins could account for the effect of hyperthermia on intracellular trypsinogen activation. For HSP 27 a similar effect involving protein phosphorylation and affecting the function of the acinar cell cytoskeleton has already been demonstrated (28).

We conclude that controlled hyperthermia, which has previously been shown to protect the pancreas against a subsequently induced variety of experimental pancreatitis, has a direct effect on the intracellular activation of trypsinogen within pancreatic acinar cells. This stabilising effect appears to be completely independent of the synthesis or upregulation of heat shock proteins or of the stablility and catalytic activity of trypsin. To what extend this effect can be employed clinically in patients who are at risk of developing acute pancreatitis warrants further investigation.

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