Water Immersion Stress Prevents Caerulein-induced Pancreatic Acinar Cell NF-κB Activation by Attenuating Caerulein-induced Intracellular Ca\(^{2+}\) Changes*

Antti J. Hietaranta‡, Vijay P. Singh, Lakshmi Bhagat, Gijs J. D. van Acker, Albert M. Song, Andreas Mykoniotis, Michael L. Steer, and Ashok K. Saluja§

From the Department of Surgery, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215 and Harvard Medical School, Boston, Massachusetts 02215

Prior stress ameliorates caerulein-induced pancreatitis in rats. NF-κB is a proinflammatory transcription factor activated during caerulein pancreatitis. However, the effects of prior stress on pancreatic NF-κB activation are unknown. In the current study, the effect of prior water immersion stress on caerulein and tumor necrosis factor-α (TNF-α)-induced NF-κB activation in the pancreas was evaluated. Water immersion of rats for up to 6 h prevents supramaximal caerulein-induced pancreatic IκB-α degradation and NF-κB activation in vitro. NF-κB activity is also induced in vitro in pancreatic acini prepared from water-immersed animals. TNF-α-induced NF-κB activation in pancreas or in pancreatic acini is unaffected by prior water immersion. Chelation of intracellular Ca\(^{2+}\) by 1,2-bis(2-aminophenoxy)ethane-N,N,N’N’-tetraacetate/acetoxymethyl ester has similar effects to water immersion in preventing caerulein but not TNF-α-induced NF-κB activation in pancreas. Both the spike response and the sustained rise in [Ca\(^{2+}\)]\(_i\), in response to supramaximal caerulein stimulation are reduced markedly in acini prepared from water-immersed animals as compared with normal animals. Our findings indicate that, in addition to Ca\(^{2+}\)-dependent mechanisms, Ca\(^{2+}\)‐independent signaling events also may lead to NF-κB activation in pancreatic acinar cells. Water immersion stress prevents supramaximal caerulein-induced NF-κB activation in pancreatic acini in vitro and in vivo by affecting intracellular Ca\(^{2+}\) homeostasis.

Doses of caerulein, the decapeptide analog of cholecystokinin, in excess of those that elicit a maximal rate of digestive enzyme secretion from the rat pancreas, elicit a reversible form of acute interstitial pancreatitis. This model of pancreatitis, referred to as secretagogue-induced pancreatitis, is associated with and possibly brought about by intra-acinar cell activation of digestive enzymezymogens including trypsinogen (1). Supramaximal stimulation of freshly prepared but otherwise normal pancreatic acini with caerulein also results in intra-acinar cell activation of trypsinogen and, subsequently, evidence of acinar cell injury in vitro (2).

We have shown recently that prior water immersion stress, under conditions that result in expression of heat shock protein 60 (HSP60), prevents caerulein-induced in vivo activation of trypsinogen in acinar cells and protects against this form of secretagogue-induced pancreatitis (3). Pancreatic acini prepared from prior water-immersed rats, which contain increased amounts of HSP60, are also protected against caerulein-induced in vitro activation of trypsinogen as well as caerulein-induced in vitro injury. The mechanism(s) responsible for this stress-induced protection against caerulein-induced injury has not been established.

Nuclear factor-κB (NF-κB) is a family of widely expressed transcription factors that acts to modulate inflammatory processes. Activation of NF-κB involves an intracytoplasmic kinase cascade that culminates in the phosphorylation of IκB proteins leading to their dissociation from NF-κB (4). As a result, the now activated NF-κB, which is comprised of dimers made up of various combinations of Rel homology-sharing subunits, can translocate to the nucleus and act to regulate expression of genes coding for various proinflammatory factors (5). The phosphorylated IκBs are, in parallel, degraded by proteasome.

Pancreatic acinar cell NF-κB activation has been reported to occur during the very early stages of caerulein-induced pancreatitis (6, 7) and evidence has been presented that suggests that prevention of caerulein-induced NF-κB activation in the pancreas can ameliorate the severity of caerulein-induced pancreatitis (6). Supramaximal stimulation of acini in vitro with caerulein also causes activation of NF-κB by a mechanism that has been shown to involve a rise in cytoplasmic free calcium levels ([Ca\(^{2+}\)]\(_i\)) and activation of protein kinase C (8–9).

In the present communication, we report studies that have evaluated the effects of prior water immersion stress, under conditions associated with up-regulated HSP60 expression, on the activation of pancreatic NF-κB. We show that prior water immersion stress prevents caerulein-induced in vivo NF-κB activation in the rat pancreas. Similarly, NF-κB activation in vitro is not observed when acini prepared from prior water-immersed animals are exposed to a supramaximally stimulating dose of caerulein. In contrast, NF-κB activation, both in vitro and in vivo, in response to TNF-α is not altered by prior

* This work supported in part by National Institutes of Health Grants DK-58694 and DK-31396. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

‡ Financially supported by the Finnish Academy of Sciences, Sigrid Juselius Foundation, Finnish Cultural Foundation, Maud Kuistila Foundation, Finnish Medical Association Duodecim, and Finnish Foundation for Alcohol Studies.

§ To whom correspondence should be addressed: Dept. of Surgery, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston, MA 02215. Tel.: 617-667-5369; Fax: 617-667-8679; E-mail: asaluja@bidmc.harvard.edu.

1 The abbreviations used are: HSP60, heat shock protein 60; NF-κB, nuclear factor-κB; IκB, inhibitory-κB; TNF-α, tumor necrosis factor-α; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetate/acetoxymethyl ester; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline.
either caerulein (0.1 mg/kg) and rat TNF-α were injected, and pancreatic tissue samples were collected immediately. After an additional 30 or 90 min of incubation, the acini were pre-incubated with BAPTA/AM (50 μM) for 30 min at 37 °C before adding the oligonucleotide probe. DNA-protein complexes were resolved in a 6% nondenaturing polyacrylamide gel in a TBE buffer (22.5 mM Tris, 22.5 mM boric acid, and 0.5 mM EDTA, pH 8.3) at 140 V for 2–3 h. Gels were dried and exposed to Kodak BioMax MR films at −70 °C. NF-κB bands from films were quantitated by using an HP Scanjet 4100 scanner and a Scion image analysis program.

Western Blot Analysis—Equal amounts of cytoplasmic protein extracts (5–10 μg) were diluted in Laemmli sample buffer with 5% mercaptoethanol. After boiling, the samples were resolved in 10% polyacrylamide gels in Tris-glycine-SDS buffer. The gels were transferred to nitrocellulose membranes, blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS), pH 7.5, containing 0.1% (v/v) Tween 20 (PBST-milk). Blots were then incubated with polyclonal rabbit anti-IκB-α antibody (sc-371, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 (v/v) dilution in PBST-milk at 4 °C overnight. The membranes then were washed in PBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG at 1:5000 (v/v) dilution in PBST-milk for 1 h. After washing, IκB-α protein bands in the membranes were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences). Analysis of Data—The results reported in this communication represent means ± S.E. of the mean values obtained from three or more separate experiments. In all figures, vertical bars denote S.E. values. Statistical evaluation of data was accomplished by analysis of variance, and p values of less than 0.05 were considered significant. All EMSA and Western blot gels shown are representative of at least three such gels prepared from independent experiments.

RESULTS

Effects of Water Immersion Stress on NF-κB Activation in Vivo—In preliminary experiments, rats were exposed to a supramaximally stimulating dose of caerulein (50 μg/kg) and sacrificed at various times thereafter. Activation of pancreatic NF-κB and degradation of IκB-α were observed to occur in a biphasic manner with an initial peak of 30 min (11.1 ± 1.8-fold control) after caerulein administration, a subsequent decline,
and then a second phase of increase that reached a maximal level between 90 min (9.4 ± 1.5-fold control) and 180 min (8.7 ± 1.7-fold control) after caerulein administration. These observations are in accord with those reported recently by Gukovsky et al. (6). In our subsequent experiments, NF-κB activation and the effects of water immersion stress on this process were evaluated at the selected times of 30 and 90 min after caerulein administration.

As shown in Fig. 1, prior water immersion profoundly inhibits the NF-κB activation and IκB-α degradation observed 30 as well as 90 min after caerulein administration. These observations are in accord with those reported recently by Gukovsky et al. (6). In our subsequent experiments, NF-κB activation and the effects of water immersion stress on this process were evaluated at the selected times of 30 and 90 min after caerulein administration.

As shown in Fig. 1, prior water immersion profoundly inhibits the NF-κB activation and IκB-α degradation observed 30 as well as 90 min after caerulein administration. The time course of the water immersion-induced blockade of caerulein-induced NF-κB activation is shown in Fig. 2. Half-maximal inhibition is observed after ~3 h of water immersion, and at that time water immersion-induced HSP60 expression is also approximately half-maximal (Fig. 2B). In contrast to these effects on caerulein-induced NF-κB activation, TNF-α-induced NF-κB activation and IκB-α degradation are not altered by prior water immersion (Fig. 3).

The observation that caerulein-induced NF-κB activation, but not TNF-α-induced NF-κB activation, is prevented by prior water immersion suggested the possibility that caerulein and

![Fig. 2. Time-dependent effects of water immersion on caerulein-induced NF-κB activation. A, NF-κB DNA binding activity as measured by EMSA. Nuclear protein extracts were prepared from pancreatic tissue samples collected after 30 min of supramaximal caerulein stimulation. Caerulein was administered after varying times of water immersion. B, time-dependent effects of water immersion on the inhibition of caerulein-induced NF-κB activation and the expression of HSP60. Black circles represent the relative inhibition of NF-κB DNA binding activity, shown in the right vertical axis, as a function of water immersion duration. NF-κB activation after 30 min of supramaximal stimulation in non-water-immersed animals represents 100%. The bars, defined in the left vertical axis, represent HSP60 expression as a function of water immersion duration (3).](http://www.jbc.org/)

![Fig. 3. Effect of water immersion on TNF-α-induced NF-κB activation. A, NF-κB DNA binding activity and IκB-α protein levels. Nuclear and cytoplasmic protein extracts were prepared from water-immersed and non-water-immersed animals 90 min after supramaximal stimulation with caerulein (C) or after stimulation with TNF-α (T, 10 μg/kg). Nonstimulated animals served as controls (Co). The upper panel represents EMSA, and the lower panel represents the corresponding IκB-α Western blot. B, densitometric quantitation of NF-κB binding activity. Water-immersed and non-water-immersed animals before (black bars) and after 90 min of either supramaximal caerulein stimulation (white bars) or TNF-α (10 μg/kg) stimulation (gray bars) were evaluated. The values represent fold increase over nonstimulated control animals. *, p < 0.05 when the water-immersed group was compared with the corresponding non-water-immersed group.](http://www.jbc.org/)

![Fig. 4. The subunits involved in caerulein- and TNF-α-induced activation of NF-κB. Nuclear protein extracts were prepared from non-water-immersed animals subjected to supramaximal stimulation with caerulein for 90 min or with TNF-α (10 μg/kg) for 90 min. Nuclear extracts were incubated for 30 min at 4 °C in the presence of 2 μl of either anti-p50, anti-p52, anti-p65, or anti-c-Rel antibodies before adding the labeled oligonucleotide probe.](http://www.jbc.org/)
TNF-α might activate different species of NF-κB. To evaluate this possibility, supershift assays were performed using antibodies to the p50, p52, p65, and c-Rel subunits of the NF-κB. As shown in Fig. 4 and in accord with results reported by others (6, 16), the caerulein- or cholecystokinin-induced activation of NF-κB involves p50/p50 and p50/p65 dimers. p50/p50 and p50/p65 dimers are involved also in the TNF-α-induced activation of NF-κB. These observations indicate that caerulein and TNF-α activate the same species of NF-κB in rat pancreas.

Effects of Prior Water Immersion on NF-κB Activation in Vitro—Acini were prepared from control rats and from rats immediately after 6 h of water immersion stress. Those acini then were exposed to a supramaximally stimulating concentration of caerulein or to TNF-α in vitro, and NF-κB activation was evaluated 30 or 90 min later. As shown in Fig. 5, caerulein-induced NF-κB activation and IκB-α degradation are not observed in acini prepared from prior water-immersed animals, but water immersion does not interfere with either TNF-α-induced degradation of IκB-α or activation of NF-κB.

Methods of Chelating [Ca2+]i on Caerulein- and TNF-α-induced NF-κB Activation—Freshly prepared acini obtained from control (i.e., non-water-immersed) animals were incubated with the Ca2+-chelator BAPTA/AM and then exposed to either caerulein or TNF-α. As shown in Fig. 6, chelation of intracellular Ca2+ with BAPTA/AM, which prevents the caerulein-induced rise in [Ca2+]i (11), prevents caerulein- but not TNF-α-induced NF-κB activation.

Effects of Prior Water Immersion on Caerulein-induced Changes in [Ca2+]i—Freshly prepared acini were loaded with Fura-2/AM, washed, and then incubated with a supramaximally stimulating concentration of caerulein. As shown in Fig. 7, when those acini were prepared from control animals, caerulein causes a large but transient rise in [Ca2+]i, which is followed by a sustained but lesser elevation of [Ca2+]i, that persists throughout the period of observation. The resting [Ca2+]i in acini prepared from water-immersed animals is lower than that observed in acini prepared from the control group, and both the peak and the sustained increases in [Ca2+]i, noted after caerulein addition are attenuated profoundly.

DISCUSSION

Otake et al. (17), in 1994, reported the results of studies that indicated that HSP60 expression in the rat pancreas was up-regulated by prior water immersion stress and that water immersion stress protected those animals from subsequent caerulein-induced pancreatitis. Recently, in studies designed to explore the mechanism(s) responsible for the protective effect of water immersion on pancreatitis, we found that caerulein-induced intrapancreatic trypsinogen activation, an early event in secretagogue-induced pancreatitis, was prevented by prior water immersion (3). We noted that this prevention of trypsinogen activation was correlated temporally with the up-regulation of HSP60 expression. Furthermore, we found that the effects of prior water immersion could be detected in acini studied in vitro. That is, intra-acinar cell activation of trypsinogen and cell injury were observed when acini from non-water-immersed animals were exposed to a supramaximally stimulating concentration of caerulein in vitro, but neither trypsinogen activation nor cell injury was observed when acini prepared from water-immersed animals were exposed in vitro to a supramaximally stimulating concentration of caerulein (3).

The currently reported studies were designed to examine further the mechanisms responsible for the protective effect of water immersion on secretagogue-induced pancreatitis.

NF-κB is a widely expressed transcription factor that in many systems has been shown to play a critical role in regulating inflammatory processes by modulating the expression of genes coding for inflammatory mediators including cytokines, chemokines, and adhesion molecules (5, 18). Studies from several groups have indicated that activation of pancreatic acinar cell NF-κB is an early event in secretagogue-induced pancreatitis. In a recent study, we showed that although caerulein-induced trypsinogen activation and NF-κB activation are closely related temporally, they are independent events in pancreatic acinar cells (19). Although still somewhat controversial (6, 7), the preponderance of evidence suggests that NF-κB activation is a proinflammatory event in this model of pancreatitis and that interventions that interfere with acinar cell NF-κB activation reduce the severity of secretagogue-induced pancreatitis (6, 16, 20).

Pancreatic acinar cell NF-κB activation by supramaximally stimulating doses of caerulein or cholecystokinin has been examined recently by several groups, and the results of their studies have shown that (a) activation depends on a rise in acinar cell [Ca2+]i, and activation of protein kinase C (8, 9), (b) activation is accompanied by degradation of IκB-α (8, 9), and (c) the NF-κB dimers activated in this process are composed of p50 and p65 subunits (6, 16). Pancreatic acinar cell NF-κB also can be activated by TNF-α (21), but this process has not been studied as extensively. Our own results (Figs. 3–5) indicate that TNF-α also promotes IκB-α degradation and translocation of...
The finding that caerulein-induced NF-κB activation is up-regulated and the time course of HSP60 expression after water immersion stress roughly correlates with that of prevention of NF-κB activation (Fig. 2). It is tempting, therefore, to speculate and simplify these observations by concluding that HSP60 mediates the process by which NF-κB activation is inhibited, but this conclusion can only be tentative because water immersion stress also might set in motion other as yet unidentified events that prevent NF-κB activation. The currently reported studies indicate that the activation of pancreatic acinar cell NF-κB by supramaximally stimulating doses of caerulein both in vivo (Figs. 1–3) and in vitro (Fig. 5) is inhibited by prior water immersion. Under these conditions, HSP60 expression is up-regulated and the time course of HSP60 expression after water immersion stress roughly correlates with that of prevention of NF-κB activation (Fig. 2). It is tempting, therefore, to speculate and simplify these observations by concluding that HSP60 mediates the process by which NF-κB activation is inhibited, but this conclusion can only be tentative because water immersion stress also might set in motion other as yet unidentified events that prevent NF-κB activation. Further studies will be needed before the relationship between HSP60 expression and prevention of caerulein-induced NF-κB activation can be defined more clearly and unambiguously.

Although the role of HSP60 in the events triggered by water immersion stress remains uncertain, the studies reported in this communication still provide some insights into the mechanisms by which water immersion stress affects NF-κB activation. Our studies indicate that water immersion stress interferes with caerulein-induced NF-κB activation, but it does not alter TNF-α-induced NF-κB activation either in vivo (Figs. 1 and 3) or in vitro (Fig. 5). This finding suggests that water immersion stress affects the activation process at a step that occurs before IκB kinase activation and IκB-α phosphorylation because, as noted above, these steps seem to be shared by both the TNF-α- and caerulein-induced NF-κB activation process. The finding that caerulein-induced NF-κB activation but not TNF-α-induced NF-κB activation can be blocked by chelation of cytoplasmic Ca²⁺ with BAPTA/AM (Fig. 6) indicates that the two pathways for NF-κB activation differ in their requirement for a rise in [Ca²⁺], and this observation suggested to us that prior water immersion stress might interfere with caerulein-induced NF-κB activation by interfering with caerulein-induced [Ca²⁺], changes in pancreatic acinar cells. To examine this possibility, [Ca²⁺], changes in acini prepared from control and water-immersed animals were evaluated (Fig. 7). Prior water immersion was found to reduce the resting [Ca²⁺], level in acini and to attenuate markedly the caerulein-induced rise in [Ca²⁺]. This observation leads us to conclude that prior water immersion prevents caerulein-induced NF-κB activation by interfering with caerulein-induced [Ca²⁺], changes and to suggest that this may at least in part explain the protection against caerulein-induced pancreatitis that is afforded by prior water immersion.

In summary, our studies indicate that supramaximally stimulating doses of caerulein and TNF-α activate the same species of NF-κB in pancreatic acinar cells but that they do so by different mechanisms. Caerulein-induced activation is a Ca²⁺-dependent process, whereas TNF-α-induced activation is independent of a rise in [Ca²⁺]. Prior water immersion stress induces HSP60 expression and also prevents caerulein-induced NF-κB activation. We suggest that prior water immersion stress prevents caerulein-induced NF-κB activation by inter-
ferring with the caerulein-induced rise in $[Ca^{2+}]_i$, which is critical to that event. Chelation of cytoplasmic Ca$^{2+}$ with BAPTA/AM can bring about the same effect. The reduction in caerulein-induced $[Ca^{2+}]_i$ rise and the activation of NF-$\kappa$B that follows water immersion stress is correlated with the rise in HSP60 expression that also follows water immersion stress, but whether HSP60 actually mediates the effects of water immersion stress on Ca$^{2+}$ dynamics and NF-$\kappa$B activation will require further studies.

REFERENCES

1. Hofbauer, B., Saluja, A. K., Lerch, M. M., Bhagat, L., Bhatia, M., Lee, H. S., Frossard, J. L., Adler, G., and Steer, M. L. (1998) Am. J. Physiol. 275, G352–G362
2. Saluja, A. K., Bhagat, L., Lee, H. S., Bhatia, M., Frossard, J. L., and Steer, M. L. (1999) Am. J. Physiol. 276, G835–G842
3. Lee, H. S., Bhagat, L., Frossard, J.-L., Hietaranta, A., Singh, V. P., Steer, M. L., and Saluja, A. K. (2000) Gastroenterology 119, 220–229
4. Karin, M. J. (1999) J. Biol. Chem. 274, 27339–27342
5. Grisham, M. R. (1999) Gastroenterology 116, 489–492
6. Gukovsky, I., Gukovskaya, A. S., Blinman, T. A., Zaninovic, V., and Pandel, S. J. (1998) Am. J. Physiol. 275, G1402–G1414
7. Steinle, A. U., Weidenbach, H., Wagner, M., Adler, G., and Schmid, R. M. (1999) Gastroenterology 116, 420–430
8. Tando, Y., Alguil, H., Wagner, M., Weidenbach, H., Adler, G., and Schmid, R. M. (1999) Am. J. Physiol. 277, G678–G686
9. Han, B., and Logsdon, G. D. (2000) Am. J. Physiol. 278, C344–C351
10. Powers, R. E., Saluja, A. K., Houlihan, M. J., and Steer, M. L. (1986) J. Clin. Invest. 77, 1668–1674
11. Dawra, R. K., Saluja, A. K., Runzi, M., and Steer, M. L. (1993) J. Biol. Chem. 268, 20237–20242
12. Saluja, A. K., Donovan, E. A., Yamanaka, K., Yamaguchi, Y., Hofbauer, B., and Steer, M. L. (1997) Gastroenterology 113, 304–310
13. Dyer, R. B., and Herzog, N. K. (1995) BioTechniques 19, 192–195
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
15. Saluja, A. K., Dawra, R. K., Lerch, M. M., and Steer, M. L. (1992) J. Biol. Chem. 267, 11202–11207
16. Han, B., and Logsdon, C. D. (1999) Am. J. Physiol. 277, C74–C82
17. Otaka, M., Itoh, H., Kuwabara, T., Zeniya, A., Fujimori, S., Otani, S., Tashima, Y., and Masumune, O. (1994) Int. J. Biochem. 26, 805–811
18. Pahl, H. L. (1999) Oncogene 18, 6853–6866
19. Hietaranta, A. J., Saluja, A. K., Bhagat, L., Singh, V. P., Song, A. M., and Steer, M. L. (2001) Biochem. Biophys. Res. Commun. 280, 388–395
20. Grady, T., Liang, P., Ernst, S. A., and Logsdon, C. D. (1997) Gastroenterology 113, 1966–1975
21. Gukovskaya, A. S., Gukovsky, I., Zaninovic, V., Song, M., Sandowal, D., Gukovsky, S., and Pandel, S. J. (1997) J. Clin. Invest. 100, 1853–1862
Water Immersion Stress Prevents Caerulein-induced Pancreatic Acinar Cell NF-κB Activation by Attenuating Caerulein-induced Intracellular Ca$^{2+}$ Changes
Antti J. Hietaranta, Vijay P. Singh, Lakshmi Bhagat, Gijs J. D. van Acker, Albert M. Song, Andreas Mykonìatis, Michael L. Steer and Ashok K. Saluja

J. Biol. Chem. 2001, 276:18742-18747.
doi: 10.1074/jbc.M009721200 originally published online February 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M009721200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 21 references, 3 of which can be accessed free at http://www.jbc.org/content/276/22/18742.full.html#ref-list-1