Heat shock protein hsp70 overexpression confers resistance against nitric oxide

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Abstract Heat stress is known to render rat islet cells resistant against the toxic effects of nitric oxide, reactive oxygen intermediates and the islet cell toxin streptozotocin. We report here for the first time that protection against nitric oxide is mediated by the major heat shock protein, hsp70, even in the absence of heat stress. The human *hsp70* gene was stably transfected into the rat insulinoma cell line RINm5F. Constitutive expression of hsp70 caused protection from NO-induced cell lysis which was of the same extent as seen after heat stressing cells. Our results identify hsp70 as a defence molecule against nitric oxide.

Key words: Diabetes; Hsp70; Nitric oxide; RIN cell

1. Introduction

Nitric oxide (NO) and reactive oxygen intermediates (ROI) are known to be major mediators of islet cell death during inflammation of pancreatic islets [1,2]. In vitro studies with isolated rat islet cells demonstrated that islet cells are highly susceptible to the toxic effects of reactive radicals [3,4] probably because they have a very low radical scavenging potential [5]. Both, NO and ROI, are known to induce DNA damage in islet cells which results in activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) [6–8].

Recently we demonstrated that heat shock confers protection against NO and ROI toxicity in rat islet cells [9]. Furthermore we showed that the protective heat shock response involves down-regulation of PARP activity which prevented NAD^+ depletion in radical-damaged cells.

Heat stress induces an array of different heat shock proteins among which the hsp70 family is the most conserved in evolution [10]. Heat shock proteins can be induced by various stresses such as heat, ethanol, arsenite, infections and heavy metals. Recent studies indicate a role for hsp70 in cytoprotection. It was shown that after transfection with hsp70 rat fibroblasts were more thermotolerant than control cells [11]. Further investigations proved that hsp70 transfected tumor cells were protected from the toxicity of tumor necrosis factor $\propto$ [12] as well as from the toxic effects of H_2O_2 [13,14]. Transfection of murine cells with hsp70 prevented cell death from metabolic stress [15,16]. In transgenic mice it was found that overexpressing hsp70 improved resistance of the heart against ischemic injury [16,17]. Finally, fusion of islet cells with hsp70-containing liposomes transiently protected the cells from the depressive effect of IL-1β on glucose-induced insulin secretion [18].

These findings led us to hypothesize that among the different stress proteins expressed in heat stressed islet cells, hsp70 may confer protection against NO toxicity. To test this hypothesis we transfected a rat insulinoma cell line, RINm5F, with a vector containing the complete coding region of the human *hsp70* gene [10]. Analysis of the stably transfected clones revealed that hsp70 confers resistance against the cytotoxicity of NO.

2. Materials and methods

2.1. Cell culture

The RINm5F cell line, a rat insulinoma cell line, was cultured at 37°C in a humidified air atmosphere with 5% CO_2 in RPMI-1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 25 mg/l ampicillin, 120 mg/l penicillin, 270 mg/l streptomycin (Serva GmbH, Heidelberg, Germany), 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 10 ml/l 100× non-essential amino acids (GIBCO BRL), 2 g/l $NaHCO_3$, 2.38 g/l HEPES (pH 7.3) and 10% fetal calf serum (FCS, GIBCO BRL).

2.2. Heat shock treatment

RIN cells were seeded in flat-bottom microtiter plates at 2×10^4 cells/120 µl per well. The cells were exposed to 42.5°C for 60 min.

2.3. Construction of eucaryotic expression vectors

pZEM-hsp70-tag was constructed by subcloning a 2.4 kb *Bam*HI–*Hin*dIII fragment from pSV-hsp70-tag containing the entire reading frame of human *hsp70* gene modified by adding sequences encoding human testis-specific lactate hydrogenase decapeptide immunotag in the carboxy terminus [12,19] into *Bgl*II cloning site downstream from metallothionin promoter in pZEM-neo eucaryotic expression vector. Prior to ligation, the overhangs created by restriction enzyme digestion were filled in by Klenow fragment of DNA polymerase I. pZEM-neo contains neomycin transferase gene-conferring resistance to G418.

2.4. Electroporation

DNA was introduced into RIN cells by electroporation. RIN cells (2.5×10^7) in logarithmic growth phase were trypsinized, washed with PBS and resuspended in 800 µl of PBS containing 50 µg plasmid DNA. Cells were given a double pulse (1 pulse: 330 V/cm, 1500 µF; 2 pulse: 100 V/cm, 900 µF resulting in a pulse time of 20 ms for the first and 13 ms for the second pulse). Cells were resuspended in 25 ml of culture medium and seeded in cloning plates (Greiner, Solingen, Germany). After 24 h at 37°C G418 was added (400 µg/ml; Boehringer-Mannheim, Mannheim, Germany) until resistant single-cell clones grew up.

2.5. Cytotoxicity assay

ROI were generated during the oxidation of hypoxanthine (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) by xanthine oxidase (Sigma). Nitric oxide was generated by the decomposition of sodium nitroprusside (Merck, Darmstadt, Germany) in the presence of 8 U/120 µl rhodanese (Sigma) and 5 mM $Na_2S_2O_3$ in order to inactivate cyanide ions. RIN cells (2×10^4/well) were exposed to ROI or NO for a distinct time. The cell viability was determined by Trypan blue

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exclusion assay. Cell death appeared to represent necrosis, since chromatin condensation as evidenced by Feulgen stain was observed in less than 5% of cells. At least 200 cells were evaluated microscopically per well. All tests were done in triplicate.

2.6. Western blot

Cells were washed twice with ice-cold PBS and scraped off culture dishes. After lysing in Laemmli sample buffer [20] cells were boiled for 5 min. Cells were electrophoresed in 10% SDS-PAGE and blotted onto a nitrocellulose filter (Amersham, Braunschweig, Germany). Filters were blocked for 30 min with Tris-buffered saline (TBS, pH 7.4) containing 5% dry milk and incubated for 1 h with a 1:1000 dilution of the following antibodies: (a) mouse monoclonal antibody directed against the inducible form of heat shock protein 70 (hsp70; clone C92F3A, Stress Gene, Victoria, B.C. Canada); (b) rat monoclonal antibody specific for the constitutive form of heat shock protein 70 (hsc70; clone 1B5, Stress Gene); (c) rabbit polyclonal antibody specific for heat shock protein 25 (hsp25; Stress Gene). The filters were washed with TBS containing 0.06% Tween-20 (Sigma) and incubated for 30 min with peroxidase-labeled anti-mouse, anti-rat or anti-rabbit antibody, respectively, diluted 1:50000 (Amersham). The detection step was performed with the ECL-detection reagent (Amersham). Air-dried filters were exposed to Hyperfilm-ECL (Amersham).

2.7. In situ nick translation

Cells were seeded on chamber slides (Nunc, Naperville, IL) at 3×10^4 per well. For the detection of DNA strand breaks in individual cell nuclei cells were analysed by in situ nick translation [21]. The cells were fixed in acetone (4°C) for 10 min and for 30 min in methanol containing 0.3% H_2O_2. The nick translation mixture was composed of 3 μM biotin-dUTP, 4 U/100 μl Kornberg polymerase, 3 μM each dGTP, dCTP, dATP, 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithiothreitol (all components from Boehringer Mannheim). The reaction was performed for 15 min at room temperature. After washing in PBS the cells were dehydrated in ethanol (30, 50, 70, 80 and 90%; 1 min each). Biotin-dUTP was visualized with streptavidin-biotin-peroxidase (Vector, Burlingame, CA) using diaminobenzidine as substrate. The percentage of stained nuclei was determined microscopically in at least 200 cells per well.

2.8. Statistical analysis

Statistical analysis was performed using the Student's 2-sided *t* test.

3. Results

3.1. Protection from cell lysis by heat shock

RIN cells were treated with heat shock and after a recovery period of 4 h at 37°C they were exposed to NO or ROI for 16 h. As shown in Fig. 1 heat shock reduced cell lysis induced by NO from 53 to 32% and ROI-induced lysis was reduced from 58 to 25%.

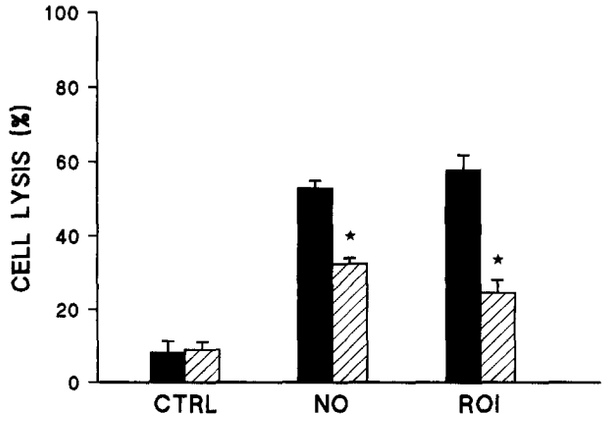

Fig. 1. Heat shock protects RIN cells from NO- and ROI-induced toxicity. RIN cells were sham-treated (CTRL) or exposed to NO (0.6 mM sodium nitroprusside) or ROI (9 mU xanthine oxidase, 0.25 mM hypoxanthine) for 16 h. Solid bars indicate non-heat shocked cells; hatched bars indicate heat stressed cells (60 min 42.5°C plus 4 h 37°C). Shown are means of 6 experiments ± SD; *$P < 0.001$ versus non-heat-shocked cells.

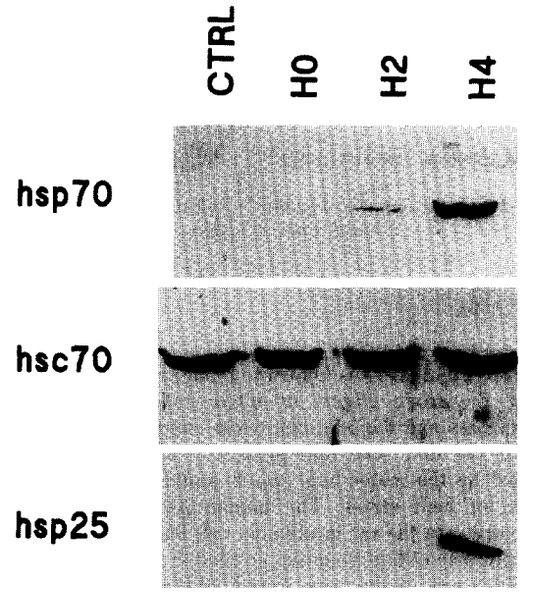

Fig. 2. Western blot analysis of heat shock protein expression in RIN cells after heat shock. Expression of hsp70, hsc70 and hsp25 of 10^5 RIN cells per lane was analysed. Shown are cells before heat stress (CTRL), directly after heat stress (H0) and after 2 or 4 h recovery at 37°C (H2 or H4, respectively).

Next we determined whether the protective effect of heat shock correlates with the expression of heat shock proteins in RIN cell lysates. Western blot analysis revealed that neither hsp70 nor hsp25 were constitutively expressed in RIN cells. Both proteins were, however, strongly induced 4 h after heat shock treatment (Fig. 2). The expression of the constitutive form of hsp70, hsc70, was not affected by heat shock.

3.2. Expression of transfected hsp70

RIN cells were transfected with pZEM-hsp70-tag containing the entire coding region of the human *hsp70* gene [19] or with pZEM-neo as control. Analysis of neomycin-resistant clones (Fig. 3) revealed that two clones transfected with pZEM-hsp70-tag stably expressed hsp70 (R70/3 and R70/20) whereas the clone RK/2 which was transfected with pZEM-neo did not. The expression of hsc70 was not affected by transfection. Clone R70/20 was selected out of primary clones obtained because it also stably expressed hsp25, although the little heat shock protein had not been transfected. This was observed only in clone R70/20 and not in any other clone. Hence, three RIN cell clones were available all differing in hsp expression. No effect of hsp70 transfection on cell growth was observed (data not shown).

3.3. Effect of hsp70 transfection on NO- and ROI-induced cell damage

To study the effect of stable hsp70 expression on NO and ROI toxicity we determined the cell lysis in the RIN cell clones after exposure to NO, generated during the decomposition of nitroprusside, or ROI, generated by the oxidation of hypoxanthine by xanthine oxidase. As shown in Fig. 4 NO-

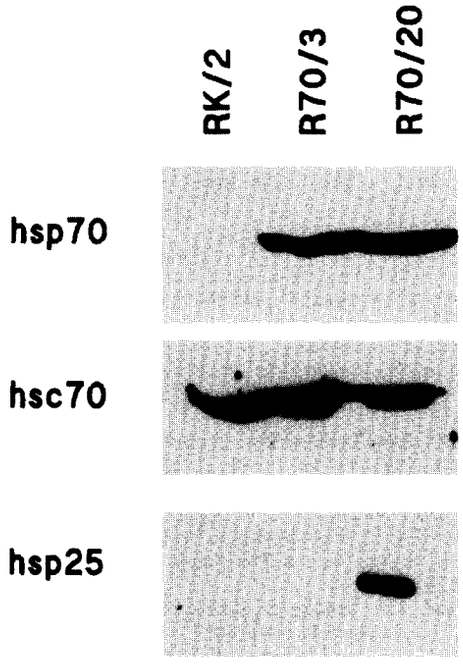

Fig. 3. Western blot analysis of hsp70 expression in transfected RIN cell clones. Expression of hsp70, hsc70 and hsp25 of 10^5 RIN cells per lane was analysed. Shown are two cell clones transfected with pZEM-hsp70-tag (R70/3; R70/20) and as negative control one cell clone transfected with pZEM-neo (RK/2).

induced cell lysis of 52% and ROI-induced lysis of 58% in the control clone RK/2 was reduced significantly in both hsp70 transfected clones by 60%. Furthermore cell lysis induced by the toxins was not influenced by transfection itself for the cell lysis in the control clone RK/2 (Fig. 4) was in the same range as in untreated RIN cells (Fig. 1).

As it is known that DNA strand breaks are important targets of reactive radicals, we determined the appearance of DNA strand breaks after exposure to ROI. Analysis of the transfected RIN cells by in situ nick translation revealed that

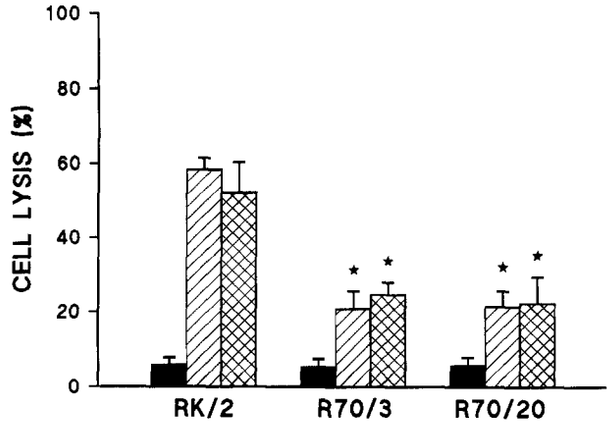

Fig. 4. Transfection with hsp70 protects from NO- and ROI-induced cell lysis. Control clone RK/2 and hsp70 transfected clones R70/3 and R70/20 were sham-treated (solid bars), treated with NO (0.9 mM nitroprusside) as indicated by hatched bars or treated with ROI (9 mU xanthine oxidase, 0.025 mM hypoxanthine) as indicated by cross-hatched bars for 16 h, respectively. Shown are means of 6 experiments ± SD; $*P < 0.001$ versus control clone RK/2.

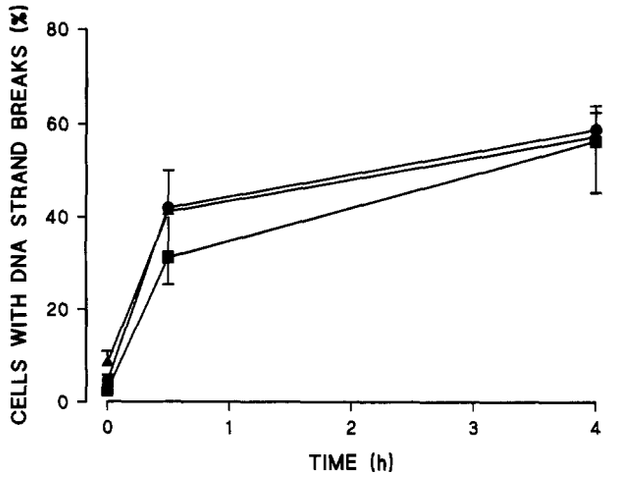

Fig. 5. Effect of hsp70 transfection on DNA damage. Control clone RK/2 (▲) and hsp70 transfected clones R70/3 (■) and R70/20 (●) were exposed to ROI (9 mU xanthine oxidase, 0.025 mM hypoxanthine). DNA strand breaks were determined microscopically by in situ nick translation. Shown are means of 4 experiments ± SD.

30 min after exposure to ROI 41% of the control transfected cells showed DNA strand breaks and after 4 h 57% of the cells were positive. Hsp70 transfection revealed no significant effect on the incidence of DNA strand breaks (Fig. 5).

4. Discussion

Overexpression of hsp70 led to resistance towards NO cytotoxicity, which was comparable to the protection induced against oxygen radicals. The observed inhibition of radical-induced cell lysis was of the same magnitude as seen after eliciting a complete stress response via heat shock. Interestingly, hsp25 was expressed constitutively in the clone R70/20 which may be a result of the stressful neomycin selection. Nevertheless overexpression of hsp25 did not have any additional protective effect. This result is consistent with observations of Jäättelä et al. [12] showing that the small human heat shock protein, hsp27, does not protect monocytes from tumor necrosis factor α toxicity.

The possible mechanism of protection by hsp70 was studied by analysing the integrity of nuclear DNA as a major target for both NO and ROI cytotoxicity [6–8]. When DNA strand breaks were determined in individual cell nuclei by in situ nick translation it became apparent that hsp70 overexpression did not reduce the number of radical-damaged nuclei. Hence, hsp70 does not exert its protective action by scavenging reactive radicals or by preventing cell damage to occur. Rather, hsp70 appears to modulate the cellular response to the toxic insult. This is supported by investigations of Simon et al. [14] showing that hsp70 does not inhibit UV light-induced O_2^- release but reduces O_2^--induced IL-6 production.

The finding that hsp70 overexpression renders RIN cells resistant against the toxicity of NO correlates to the protective action of heat shock in RIN cells as well as in rat islet cells. Since hsp70 is a prominent part of the stress protein response it may be assumed that much of the radical resistance induced by heat shock is carried out by hsp70. In this context it is of interest that human islet cells are much less affected by NO than rodent islet cells [22]. Concomitantly, recent results re-

vealed that human islet cells express 3–4-fold more hsp70 than rodent islet cells [23]. Hence, this pronounced difference between the two species may explain the better resistance of human islet cells towards NO. Another interesting aspect is that islet cells from diabetes-prone BB rats, a model of human type I diabetes, are deficient in hsp70 expression. Concomitantly, BB rat islet cells cannot be rendered resistant to NO toxicity by heat shock. Rather, an increase in susceptibility was seen (Bellmann et al., in preparation).

In conclusion, we have identified among heat shock-induced proteins hsp70 as a stress protein mediating the cellular defence against NO. Hsp70 overexpression does not appear to prevent radical caused cell damage but probably affects the cellular response to such insult.

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References

[1] Kolb, H. and Kolb-Bachofen, V. (1992) Diabetologia 35, 796–797.
[2] Corbett, J.A. and McDaniel, M.L. (1992) Diabetes 41, 897–903.
[3] Pipeleers, D. and Van De Winkel, M. (1986) Proc. Natl. Acad. Sci. USA 83, 5267–5271.
[4] Kröncke, K.-D., Brenner, H.-H., Rodriguez, M.-L., Noack, E., Kolb, H. and Kolb-Bachofen, V. (1993) Biochim. Biophys. Acta 1182, 221–229.
[5] Grankvist, K., Marklund, S.L. and Taljedal, I.-B. (1981) Biochem. J. 199, 393–398.
[6] Radons, J., Heller, B., Bürkle, A., Hartmann, B., Rodriguez, M.-L., Kröncke, K.-D., Burkart, V. and Kolb, H. (1994) Biochem. Biophys. Res. Commun. 199, 1270–1277.
[7] Heller, B., Bürkle, A., Radons, J., Fengler, E., Jalowy, A., Müller, M., Burkart, V. and Kolb, H. (1994) Biol. Chem. Hoppe-Seyler 375, 597–602.
[8] Heller, B., Wang, Z.-Q., Wagner, E.F., Radons, J., Bürkle, A., Fehsel, K., Burkart, V. and Kolb, H. (1995) J. Biol. Chem. 270, 11176–11180.
[9] Bellmann, K., Wenz, A., Radons, J., Burkart, V., Kleemann, R. and Kolb, H. (1995) J. Clin. Invest. 95, 2840–2845.
[10] Hunt, C. and Morimoto, R.I. (1985) Proc. Natl. Acad. Sci. USA 82, 6455–6459.
[11] Li, G.C., Li, L., Liu, Y.-K., Mak, J.Y., Chen, L. and Lee, W.M.F. (1991) Proc. Natl. Acad. Sci. USA 88, 1681–1685.
[12] Jäättelä, M., Wissing, D. and Bauer, P.A. (1992) EMBO J. 11, 3507–3512.
[13] Jäättelä, M. and Wissing, D. (1993) J. Exp. Med. 177, 231–236.
[14] Simon, M.M., Reikerstorfer, A., Schwarz, A., Krone, C., Luger, T.A., Jäättelä, M. and Schwarz, T. (1995) J. Clin. Invest. 95, 926–933.
[15] Williams, R.S., Thomas, J.A., Fina, M., German, Z. and Benjamin, I.J. (1993) J. Clin. Invest. 92, 503–508.
[16] Marber, M.S., Mestril, R., Chi, S.-H., Sayen, M.R., Yellon, D.M. and Dillmann, W.H. (1995) J. Clin. Invest. 95, 1446–1456.
[17] Plumier, J.-C.L., Ross, B.M., Currie, R.W., Angelidis, C.E., Kazlaris, H., Kollias, G. and Pagoulatos, G.N. (1995) J. Clin. Invest. 95, 1854–1860.
[18] Margulis, B.A., Sandler, S., Eizirik, D.L., Welsh, N. and Welsh, M. (1991) Diabetes 40, 1418–1422.
[19] Milarski, K.L. and Morimoto, R.I. (1989) J. Cell Biol. 108, 413–423.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Fehsel, K., Kolb-Bachofen, V. and Kolb, H. (1991) Am. J. Pathol. 139, 251–254.
[22] Eizirik, D.L., Pipeleers, D.G., Ling, Z., Welsh, N., Hellerström, C. and Andersson, A. (1994) Proc. Natl. Acad. Sci. USA 91, 9253–9256.
[23] Eizirik, D.L., Margulis, B., Borg, H., Jernberg Wiklund, H., Saldeen, J., Flodström, M., Mello, M.A., Andersson, A., Pipeleers, D.G., Hellerström, C. and Welsh, N. (1995) Mol. Med. 1, 806–820.