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Pre-exposure to heat shock inhibits peroxynitrite-induced activation of poly(ADP) ribosyltransferase and protects against peroxynitrite cytotoxicity in J774 macrophages

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Abstract

The reaction of nitric oxide (NO) with superoxide yields the cytotoxic oxidant peroxynitrite, produced during inflammation and shock. A novel pathway of peroxynitrite cytotoxicity involves activation of the nuclear enzyme poly(ADP) ribosyltransferase, and concomitant ADP-ribosylation, NAD^+ consumption and exhaustion of intracellular energy stores. In the present report we provide evidence that pre-exposure of J774 macrophages to heat shock reduces peroxynitrite-induced activation of poly(ADP) ribosyltransferase and protects against the peroxynitrite-induced suppression of mitochondrial respiration. The protection was significant at 8 h after heat shock, but was absent at 24 h after heat shock. Thus, the protection showed a temporal correlation with the expression of heat shock protein 70, the expression of which was maximal at 8 h. Exposure to heat shock did not alter the expression of poly(ADP) ribosyltransferase over 24 h. In summary, the heat shock phenotype or heat shock proteins may protect against peroxynitrite induced cytotoxicity.

Keywords: Nitric oxide (NO); Superoxide; Mitochondrial respiration; Shock; Inflammation; Endotoxin

1. Introduction

During inflammation and shock, endotoxin induces the release of pro-inflammatory cytokines, which trigger the expression of a multitude of cellular responses, including expression of the inducible isoform of nitric oxide (NO) synthase and production of oxygen derived free radicals (Schiller et al., 1993; Leeson and Morrison, 1994; Szabó, 1995). The simultaneous production of NO and superoxide in shock and inflammation produces peroxynitrite, a toxic oxidant species (Miller et al., 1995; Kaur and Halliwell, 1994; Wizemann et al., 1994; Szabó et al., 1995a,b).

We recently proposed an indirect mechanism for peroxynitrite cytotoxicity in which peroxynitrite induces DNA single-strand breakage (Inoue and Kawanishi, 1995; Salgo et al., 1995; Szabó et al., 1996a), which then triggers a futile, energy-consuming repair cycle by activating the nuclear enzyme poly(ADP) ribosyltransferase (Szabó et al., 1996a; Zingarelli et al., 1996a).

Activation of poly(ADP) ribosyltransferase, a protein-modifying and nucleotide polymerizing enzyme, cleaves NAD^+ into ADP-ribose and nicotinamide. Poly(ADP) ribosyltransferase covalently attaches ADP-ribose to various proteins and in turn, rapidly depletes the intracellular concentration of its substrate, NAD^+, slowing the rate of glycolysis, electron transport, and therefore ATP formation, resulting in cell dysfunction and cell death (Szabó et al., 1996a; Zingarelli et al., 1996a).

The heat shock phenotype is a highly conserved stress response characterized by the rapid expression of a specific group of proteins (heat shock proteins) that provides cellular adaptation to a variety of thermal and nonthermal cytotoxic stimuli (Minowada and Welch, 1995). Pre-exposure to heat shock has been demonstrated to provide cytoprotection against various forms of oxidant injury including NO (Kapoor and Lewis, 1987; Jaattela and Wissing, 1993; Bellmann et al., 1995). Studies involving stably transfected cells indicate that heat-shock protein 70, in particular, is a critical component of heat-shock-mediated cytoprotection (Li et al., 1992).

In the present study, in cultured J774 macrophages, we have investigated (i) whether the heat-shock phenotype is

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protective against the peroxynitrite-induced suppression of mitochondrial respiration; (ii) studied the potential temporal correlation between expression of heat-shock protein 70 and the extent of protection over a 24 h period after heat shock; and (iii) investigated whether the protection by heat-shock pre-exposure against the effects of peroxynitrite is associated with changes in the expression of poly(ADP) ribosyltransferase, or with changes in the degree of direct activation of poly(ADP) ribosyltransferase by peroxynitrite.

2. Materials and methods

2.1. Cell culture

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium with 4×10^{-3} M L-glutamine and 10% fetal calf serum as described (Szabó et al., 1994). Cells were cultured in 96-well plates with 200 μl culture medium until they reached 60–80% confluence.

2.2. Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Szabó et al., 1994). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg/ml for 60 min). Culture medium was removed by aspiration and the cells solubilized in dimethylsulfoxide (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550} using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA, USA) (Szabó et al., 1994). Formazan production was expressed as rate of production ($\mu g/10^6$ per min) or as a percentage of the values obtained from untreated cells.

2.3. Measurement of poly(ADP) ribosyltransferase activity

After a 10 min exposure to peroxynitrite (1 mM), the culture medium in 12-well plates was replaced with 0.5 ml of 56 mM HEPES buffer, pH 7.5 containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin and 125 nmol NAD^+ spiked with 0.25 μCi [^{3}H]NAD^+. Poly(ADP) ribosyltransferase activity was then measured as described (Szabó et al., 1996a). Digitonin was used to permeabilize plasma membranes. The permeabilized cells were incubated for 5 min at 37°C, and the protein that was ribosylated with [^{3}H]NAD^+ was precipitated with 200 μl of 50% TCA. After two washes with TCA, the protein pellet was solubilized in 2% SDS in 0.1 M NaOH, incubated at 37°C overnight, and the radioactivity was determined by a Wallac 1450 Microbeta Plus scintillation counter (Wallac, Gaithersburg, MD, USA).

2.4. Heat-shock protocol

Cells were subjected to 43°C heat shock for 60 min, followed by recovery at 37°C for various times (2, 8 and 24 h). At these time points, cells were treated with peroxynitrite (1 mM) in the absence or presence of the poly(ADP) ribosyltransferase inhibitor 3-aminobenzamide for 10 min (for the measurement of poly(ADP) ribosyltransferase activity) or for 1 h (for the measurement of mitochondrial respiration). We have previously established that these exposure times to peroxynitrite are optimal in this cell type (Szabó et al., 1996a).

2.5. Western blotting for heat-shock protein 70 and for poly(ADP) ribosyltransferase

Cells were lysed in ice-cold buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100 and the protease inhibitor phenylmethylsulfonylfluoride. Protein concentration was determined using the Bradford assay (BioRad, Hercules, CA, USA). Whole cell lysate was boiled in equal volumes of loading buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol and 10% 2-mercaptoethanol) and 25 mg of protein then loaded per lane on an 8–16% Tris-glycine gradient gel (Novex, San Diego, CA, USA). Proteins were separated electrophoretically and then transferred to a nitrocellulose membrane (Novex) using the Novex Xcell Mini-Gel system. For immunoblotting the membrane was blocked with 10% non-fat dried milk in Tris-buffered saline for 1 h. Primary antibodies used were (1) a murine monoclonal IgG specific towards the inducible isoform of the heat-shock protein 70 family (SPA-810, Stressgen, Victoria, British Columbia, Canada) at a dilution of 1:2500 for 3 h and (2) a rabbit polyclonal antibody against calf poly(ADP) ribosyltransferase (a kind gift of Prof. Ernest Kun, San Francisco State University), used at a dilution of 1:1000 for 3 h. After washing three times in TBS containing 0.1% Tween 20, secondary antibody (peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, respectively, both from Sigma) was applied at a 1:2500 dilution for 1 h. The blot was then washed in 0.1% Tween 20 in Tris-buffered saline three times over 30 min, incubated in commercial enhanced chemiluminescence reagents (ECL, Amersham, Amersham, UK) and exposed to photographic film.

2.6. Materials

All chemicals were from Sigma (St. Louis, MO, USA). Peroxynitrite was a kind gift of Dr. H. Ischiropoulos (University of Pennsylvania).

2.7. Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean of n observations,

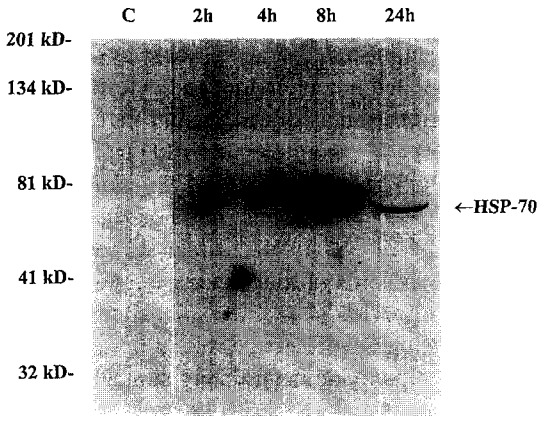

Fig. 1. Western blot analysis demonstrating expression of heat-shock protein 70 at various time points after heat shock in J774 macrophages.

where n represents the number of wells studied (6–12 wells from experiments performed on 2–3 experimental days). Student's unpaired t-test was used to compare means between groups. A P value less than 0.05 was considered significant.

3. Results

Heat shock induced the expression of heat-shock protein 70 in a time-dependent fashion, with maximal expression at 8 h after heat shock (Fig. 1). The expression of heat-shock protein 70 was minimal at 2 h after heat-shock exposure, and returned to low levels at 24 h after heat-shock exposure. Therefore, in a time-course study, cells were exposed to peroxynitrite at 2 h, 8 h or 24 h after heat shock.

Peroxynitrite (1 mM) markedly suppressed mitochondrial respiration (Figs. 2 and 3), and triggered the activation of poly(ADP) ribosyltransferase (Fig. 4). Heat shock *alone* caused a slight decrease in mitochondrial respiration (8 ± 4, 13 ± 4 and $9 \pm 2\%$ reduction at 2, 8 and 24 h, respectively, $n = 6$) (Fig. 2) and caused a slight increase in the activity of poly(ADP) ribosyltransferase at 8 h (Fig. 4).

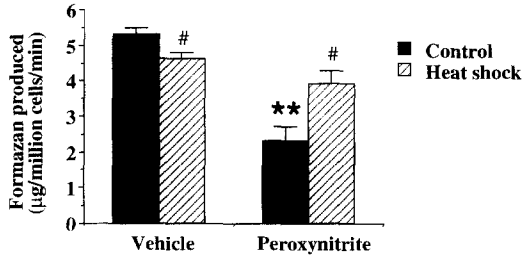

Fig. 2. Mitochondrial respiration at 1 h after exposure to 1 mM peroxynitrite in cultured J774 macrophages; protection against the suppression of the respiration by heat shock. ** Significant decrease in the respiration when compared to controls ($P < 0.01$), # Significant difference between control and heat-shock groups either before or after peroxynitrite exposure ($P < 0.05$); $n = 6$–12 wells.

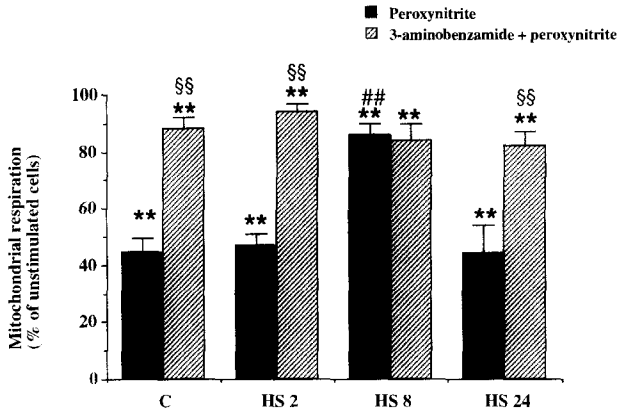

Fig. 3. Mitochondrial respiration (expressed as percent of respiration of unstimulated control cells) at 1 h after exposure to 1 mM peroxynitrite in cultured J774 macrophages, under control conditions (C), and at various time points (2 h, 8 h and 24 h) after heat shock (HS). For comparison, the effect of inhibition of poly(ADP) ribosyltransferase activity by 3-aminobenzamide (1 mM) on the peroxynitrite-induced changes in mitochondrial respiration is also shown. ** Significant decrease in the respiration in response to peroxynitrite ($P < 0.01$), ## Significant protection against the peroxynitrite-induced suppression of mitochondrial respiration by heat shock ($P < 0.01$); §§ Significant difference in the mitochondrial respiration in the presence of 3-aminobenzamide, when compared to the absence of 3-aminobenzamide, in control cells, or at various times after heat shock ($P < 0.01$); $n = 6$–12 wells.

In cells previously exposed to heat shock, peroxynitrite caused a less pronounced suppression of mitochondrial respiration, when compared to control cells at 8 h (Figs. 2 and 3). However, at 2 h and at 24 h after exposure to heat

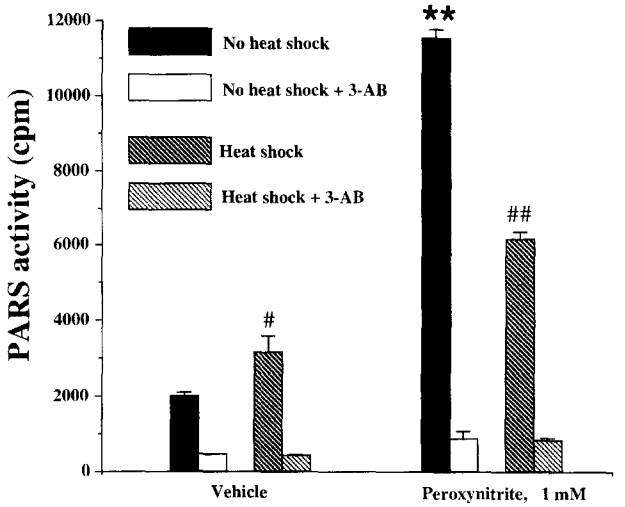

Fig. 4. [^{3}H]NAD$^+$ incorporation into proteins as an indicator of poly(ADP) ribosyltransferase activity in control in J774 macrophages in control conditions, after heat shock and after exposure to 1 mM peroxynitrite, in the presence or absence of the poly(ADP) ribosyltransferase inhibitor 3-aminobenzamide (3-AB) (1 mM). ** Significant increase in the poly(ADP) ribosyltransferase activity in response to peroxynitrite ($P < 0.05$ and $P < 0.01$, respectively), ## Significant difference in poly(ADP) ribosyltransferase activity between control and heat-shocked groups ($P < 0.01$). In all instances, 3-aminobenzamide (1 mM) caused a significant suppression of poly(ADP) ribosyltransferase activity ($P < 0.01$); $n = 6$ wells.

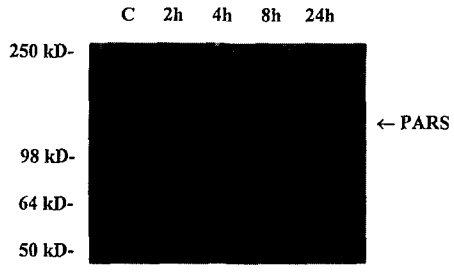

Fig. 5. Western blot analysis demonstrating expression of poly(ADP) ribosyltransferase at various time points after heat-shock in J774 macrophages.

shock, no protection was seen against the cytotoxic effects of peroxynitrite (Fig. 3). Thus, the degree of protection against peroxynitrite toxicity by heat shock showed a good temporal correlation with the degree of the expression of heat-shock protein 70 in these cells (Figs. 1 and 3). In agreement with recent data (Szabó et al., 1996a), the poly(ADP) ribosyltransferase inhibitor 3-aminobenzamide (1 mM) largely reduced the suppression of mitochondrial respiration elicited by peroxynitrite (Fig. 3).

There were no notable differences in the expression of poly(ADP) ribosyltransferase at 2–24 h after heat shock, when compared to control cells (Fig. 5). At 8 h after heat-shock exposure, the activation of poly(ADP) ribosyltransferase in response to peroxynitrite was reduced, when compared to the activation of this enzyme by peroxynitrite in cells not exposed to heat shock (Fig. 4). In contrast, at the other time points studied, there was no difference in the degree of activation of poly(ADP) ribosyltransferase by peroxynitrite between control cells and cells previously exposed to heat shock (not shown). For instance, at 2 h, in the cells previously exposed to heat shock, the degree of poly(ADP) ribosyltransferase activation by 1 mM peroxynitrite amounted to $107 \pm 5\%$ of the activation seen in cells not pre-exposed to heat shock ($n = 6$).

4. Discussion

In various experimental conditions, NO has been proposed to be an important cytotoxic mediator. However, recent data challenge the prevailing dogma that NO is independently toxic. Accumulating evidence suggests that much of the NO-related injury may be due to the generation of peroxynitrite. This proposition is supported by theoretical considerations regarding the levels of superoxide and the rate of peroxynitrite formation in biological systems (Pryor and Squadrito, 1995; Squadrito and Pryor, 1995) as well as by recent observations demonstrating that peroxynitrite is more cytotoxic than NO or superoxide in a variety of experimental systems (Szabó and Salzman, 1995; Brunelli et al., 1995; Hausladen and Fridovich, 1994; Castro et al., 1994) and that oxygen radical neutralization protects against NO-mediated cell injury in various cells (Burkart et al., 1995; Dawson, 1995). Careful in vitro studies have demonstrated that, in some systems (experiments using isolated mitochondrial and cytosolic aconitase, for example) NO itself is not toxic, but peroxynitrite is a potent inhibitor of aconitase activity (Hausladen and Fridovich, 1994; Castro et al., 1994). Similarly, peroxynitrite, and not NO, is a potent initiator of DNA strand breakage (Szabó et al., 1996a; Zingarelli et al., 1996a). The proposal that peroxynitrite is a major cytotoxic mediator would also provide a cogent explanation for previous data demonstrating the protective role of both NO synthase inhibitors and superoxide neutralizing strategies, in stroke, inflammation and shock (Szabó, 1996).

Peroxynitrite exerts its cytotoxic effects by a variety of pathways including lipid peroxidation, inhibition of mitochondrial enzymes and membrane pumps, and by causing intracellular glutathione depletion (Radi et al., 1991; Hu et al., 1994; Guzman et al., 1995; Phelphs et al., 1995). However, a recent, additional mechanism has been shown to be related to activation of poly(ADP) ribosyltransferase and subsequent energy depletion (see Section 1).

Although the cytoprotective properties of the heat-shock phenotype were originally described in cells undergoing heat stress (Li and Werb, 1982), recent data clearly demonstrate that heat shock also confers cytoprotection against non-thermal cytotoxic stimuli, including oxidants and endotoxin (Kapoor and Lewis, 1987; Jaattela and Wissing, 1993; Bellmann et al., 1995; Villar et al., 1994). In pancreatic islet cells, prior exposure of the cells to heat shock protected against the cytotoxic effects of exogeneous NO with concomitant decreases in poly(ADP) ribosyltransferase activation (Bellmann et al., 1995). Despite the recent abundance of descriptive data demonstrating these cytoprotective effects, relatively little is known regarding the mechanisms by which the heat-shock phenotype confers cytoprotection. Experiments using cell lines stably transfected to overexpress human heat-shock protein 70 suggest a critical role for this particular heat-shock protein (Li et al., 1992). One well known function of heat-shock proteins is their ability to stabilize, refold, and transport damaged and nascent polypeptides (molecular chaperones) (Lindquist, 1986). Accordingly, it has been hypothesized that heat-shock-mediated cytoprotection is related, in some way, to these molecular chaperone properties. An additional cytoprotective mechanism may involve the characteristic inhibitory effects of the heat-shock phenotype on non-heat-shock protein gene expression (Lindquist, 1986). In this regard, it is notable that the heat-shock phenotype has recently been demonstrated to inhibit the expression of inducible NO synthase in response to interleukin-1 (Wong et al., 1995).

The present data demonstrate that the cytoprotective properties of the heat-shock phenotype during oxidant stress can now be extended to peroxynitrite-mediated cytotoxicity. Moreover, the present data suggest that the protection may be related to the prevention by heat shock of

poly(ADP) ribosyltransferase activation. Whether heat shock inhibits signals for poly(ADP) ribosyltransferase activation (for example, oxidant injury to DNA or glutathione depletion) remains to be elucidated. However, our data clearly show that the protection by heat-shock pre-exposure against the peroxynitrite toxicity is not due to changes in the expression of poly(ADP) ribosyltransferase (Fig. 5), and not due to reduction of the basal activity of the enzyme (Fig. 4). In fact, we have observed a slight increase in the basal activity of poly(ADP) ribosyltransferase at 8 h after exposure to heat shock (Fig. 4). The mechanism of the increase in basal poly(ADP) ribosyltransferase activity in response to heat shock remains to be investigated.

NO has been suggested to be a potent activator of poly(ADP) ribosyltransferase in brain slices and in pancreatic islet cells. Recent data demonstrating that heat shock protects against NO-mediated cytotoxicity and poly(ADP) ribosyltransferase activation in islet cells (Bellmann et al., 1995) may also be relevant to our data since, in pancreatic islet cells, oxidant neutralization protects against cellular injury in response to NO donors (Burkart et al., 1995). Thus, it is likely that peroxynitrite, formed by the reaction of NO with endogenous superoxide, importantly contributes to the cytotoxicity in cells exposed to NO donors (Szabó et al., 1996a; Zingarelli et al., 1996a; Burkart et al., 1995).

There was a small decrease in the mitochondrial respiration in cells exposed to heat shock (Fig. 2), which may be related to the small increase in the activity of poly(ADP) ribosyltransferase after heat shock, or possibly due to a slight cytotoxic effect of the heat-shock protocol itself. However, our data support the view that the protection against the peroxynitrite-induced cytotoxicity by pre-exposure to heat shock is a specific mechanism, and it is not due to a damaged state of the cells in general: after a 2 h exposure to heat shock, no protection against the peroxynitrite toxicity was found. The good temporal correlation between the expression of heat-shock protein 70 and the protection against peroxynitrite-induced cytotoxicity suggests, but does not prove, the role of this protein in the cytoprotection. Future experiments using stable transfected cell lines with heat-shock protein 70 should provide a definitive answer to the role of this specific protein in the protection.

The measurement of reduction of MTT by the mitochondria is a useful assay to investigate the effect of the general metabolic status of the cell on the mitochondrial respiration. Although the reduction of MTT appears to be mainly by the mitochondrial complexes I and II, it also may involve NADH- and NADPH-dependent energetic processes that occur outside the mitochondrial inner membrane (Berridge and Tan, 1993; Sung and Dietert, 1994). Thus, this method cannot be used to separate the effect of free radicals, oxidants or other factors on the individual enzymes in the mitochondrial respiratory chain, but is useful to monitor changes in the general energetic status of the cells.

Taken together, our data suggest that pre-exposure to heat shock protects against the peroxynitrite-induced suppression of cellular energetics, and this may be due to the ability of heat shock (or possibly heat-shock proteins) to prevent the peroxynitrite-induced activation of poly(ADP) ribosyltransferase and the subsequent energy depletion. Endotoxin shock results in peroxynitrite formation (Wizemann et al., 1994; Szabó et al., 1995a,b), activation of PARS and subsequent energy depletion (Zingarelli et al., 1996a,b; Szabó et al., 1996b). Interestingly, the heat-shock phenotype has been shown to induce protection against endotoxin shock (Villar et al., 1994). It is conceivable that this cytoprotection is, in part, a consequence of reduced poly(ADP) ribosyltransferase activation and diminished cytotoxicity in response to LPS-induced production of NO, superoxide and peroxynitrite.

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