BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 6–9 (1997)
ARTICLE NO. RC977067

Cyclophilins Are Induced by Hypoxia and Heat Stress in Myogenic Cells

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Received June 26, 1997

This is a novel study demonstrating that cyclophilins are heat and stress inducible proteins in eukaryotic myogenic cells. We investigated the expression of cyclophilins in embryonal rat heart derived H9c2 myocytes following heat stress and chronic hypoxia. We report here that cyclophilins, the proteins capable of catalysing the interconversion of cis and trans isomers (PPIses) in proteins and peptides, are heat and stress inducible, and are involved in the complex stress response, as their level is significantly elevated after heat stress and hypoxia. A time course analysis showed the gradual increase in expressed levels of cyclophilin after heat stress of cells, with maximal expression as measured by Western blot at 48 hours after the actual treatment. Rat myogenic cells exposed to chronic hypoxia followed by 5 hours reoxygenation resulted in approximately threefold expression of PPIases. The results showing that cyclophilins are heat and stress inducible suggest a multiple role for cyclophilins in ischemia: a potential functional association with the different heat shock proteins, with the established protective role in ischaemic injury, as well as the possible involvement of cyclophilins in the protein folding in cooperation with molecular chaperones.

The family of proteins, termed cyclophilins were defined on the basis of their binding to a potent immunosuppresant drug, cyclosporin A (CsA) which inhibits their activity. These proteins are capable of catalysing the interconversion of cis and trans isomers of peptidyl-prolyl bonds in peptides and proteins (1). *In vitro* studies with purified cyclophilin showed that they form a family of peptidyl-prolyl trans isomerases (PPIases), which are crucial for folding and isomerisation of proteins *in vitro.* The PPIases are ubiquitous and abundant proteins present in the cytosol, endoplasmic reticulum and mitochondria both as free species and anchored to the membranes. Several PPIases have been recently identified as heat inducible proteins in widely divergent species. It has been reported that two PPIases of *Saccharomyces cerevisiae,* one in endoplasmic reticulum and one in the cytosol (Cyp1 and Cyp2 respectively), are heat inducible proteins and the presence of at least one of them is necessary for maximal survival of yeast after heat shock (2). Mutations in one class of PPIases jeopardise the organisms' tolerance to stress. In *Saccharomyces cervisiae* mutations in the mitochondrial cyclophilin, Cpr3, make the cells unable to grow on lactose at 37°C (3). Yeasts possess at least eight different immunophilin genes, the products of which are located in various cellular compartments (4). It has been suggested that a high level of redundancy of their function is required for cell viability. According to the literature, mammalian genomic DNA contains at least 20 copies of sequences of different cyclophilins as revealed by Southern blotting experiments (4, 5).

We have previously isolated heart mitochondrial PPIase active fraction, which we suggest to be part of the CsA receptor during mitochondrial pore blocade by CsA (6, 7, 8). It has been speculated that the mitochondrial inner membrane contains a large channel, approximately 2nm in diameter, which opens under conditions of high matrix [Ca^{++}] and oxidative stress (9). Since a correlation has been established between the ischemia- reperfusion injury and pore opening in isolated mitochondria, it has been implied that irreversible opening of the mitochondrial pore might be the cause of the reperfusion injury and cell death. We also suggested a functional association of mitochondrial PPIases with the machinery which imports mitochondrial proteins in the unfolded state, and that the pathological pore may be a distortion of the so-called general insertion pore for protein import (6). Another mitochondrial cyclophilin, Cyp20, present in the matrix has been studied in pre-protein translocation across the mito-

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0006-291X/97 $25.00

chondrial membranes and it has been concluded that Cyp20 is a component of the protein folding machinery in mitochondria and functions in co-operation with molecular chaperones HSP60 and HSP70 (10). Mechanisms of cytoprotection of HSPs during ischemia are far from being fully defined.

Therefore, this study was designed to examine the level of expressed cyclophilins following heat shock and hypoxia-reoxygenation in eukaryotic myogenic cells; and whether cyclophilins would react in a manner similar to that of the molecular chaperones, as cyclophilins are functionally linked with HSP70 and HSP60. This is the first step in the series of experimental work aimed at defining the functional role of cyclophilins in the cell response to ischemia and the possible role of cyclophilins in protective effect of heat preconditioning.

MATERIALS AND METHODS

Experimental design. H9c2 cells were obtained from the American Type Culture Collection (CRL-1446, ATCC, Rockeville, MD, USA) and were routinely cultured in Dulbecco's modified Eagle medium (DMEM) containing glucose and supplemented with 10% (v/v) fetal calf serum, 1% penicillin-streptomycin, in an atmosphere of 5% CO_2. For the experimental work, H9c2 cells were grown in 75cm^2 flasks and in 6 well plates to confluence. For the heat stress experiments, flasks and plates were sealed in parafilm and floated in a water bath maintained at 43C° for 1 hour, after which cells were returned to 37 °C in an atmosphere of 5% CO_2 and then harvested for Western blot analysis 12 hours, 24 hours and 48 hours later. To induce hypoxia, cells were transferred to a specially designed chamber (Billups-Rothenberg, Del Mar, California) and a hypoxic gas mixture (95% NO_2, 5%CO_2) was infused continuously for 18 hours. Cell plates and flasks were then returned to an atmosphere of 5% CO_2 for a 3 hour period of reoxygenation and harvested for the Western blot. Cell viability assay was performed at the same time points.

Western blot analysis. Cells were harvested in 100μl of concentrated sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (20% glycerol, 6% SDS in 0.12 mol/L Tris at pH 6.8). Protein estimation was performed using the BioRad protein assay kit (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Proteins were separated by SDS-PAGE on 0.8 mm thick, 10% polyacrilamide gels according to Laemmli. The protein concentration and adequacy of sample preparation were determined by visualisation of proteins using Coomassie Brilliant Blue stain. For Western blotting following electrophoresis, proteins from the gel were transferred electrophoretically onto nitrocellulose membranes (Hybond C, Amersham, Buckinghamshire, UK) at 4C° overnight at 30V. The transfer of proteins to the membrane was verified by Ponceau staining, the membranes were then washed with Tris buffered saline (pH −7.4) with 3% BSA and 0.05% Tween 20 to block non-specific binding sites. After washing, membranes were incubated at room temperature for 1 hour with anti-rabbit IgG to human cyclophilin 20 (Calbiochem, Novabiochem U.K.) at 1:1000 dilution in 2% BSA solution. The secondary antibody was anti-goat IgG at a dilution 1:1000. Blots were developed using an alkaline phosphatase detection system. Western blots were scanned using GS-700 BioRad model imaging densitometer. Analysis was performed with BioRad Molecular Analyst Software.

Cell viability assay. This assay was performed using Promega cell proliferation assay (Madison, WI, USA) based on the use of the novel tetrazolium compound MTS, (Owen's reagent) which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The assay was performed by adding a small amount of the provided reagent directly to the culture wells and incubated for 1 to 4 hours. The quantity of formazan product was measured by the absorbance at 490 nm using a 96 well plate reader.

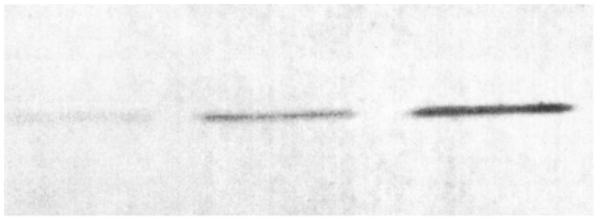

FIG. 1. Western blotting of H9c2 cells harvested 24 and 48 hours after heat treatment for 1 hour at 43°C. Control was experimental cells without treatment. Blots were treated with antirabbit IgG to human cyclophilin 20 and staining was performed as described under Materials and Methods.

Trypan Blue exclusion assay. After myocytes were subjected to either the heat stress or hypoxya, cells were washed with phosphate buffered saline (PBS) and then resuspended in a 0.8% trypan blue in PBS. The number of dead cells was estimated by counting the cells incapable of excluding the dye.

Data analysis. All the data were expressed as mean ±SEM. Analysis of variance and Student *t*-test were used for statistical evaluation, and all the differences between groups were considered to be significant at $p<0.05$.

RESULTS

Heat shock resulted in a significant induction in the amount of expressed cyclophilin. The accumulation of the cyclophilin occurred gradually as determined by Western blotting (Fig. 1). The relative cyclophilin expression in H9c2 was maximal after 48 hours of recovery at 37 °C. The difference between the expressed amounts of cyclophilins 24 hours and 48 hours after heat treatment was not significant, but the tendency, according to the relative analysis of the protein expression, was a gradual rise of expressed cyclophilin with time (Fig. 2). The observed accumulation has been compared with the results of the cell viability assay; which in turn, demonstrated the increase in the viability of the analysed cells, which peaked at 24 hours after heat stress. The number of cells capable of excluding Tryphan Blue did not change after 24 hours nor after 48 hours following heat shock, which indicates that the H9c2 cell line is quite resistant to heat shock and the heat treatment has more of a preconditioning effect according to the results of the MTS assay. (Fig. 3). The assay demonstrated that following heat treatment cells became metabolically more active compared to control H9c2 cells which did not have any treatment. To investigate whether these proteins are also involved in the complex cell response during other physiological stresses, we subjected H9c2 cells to experimental hyp-

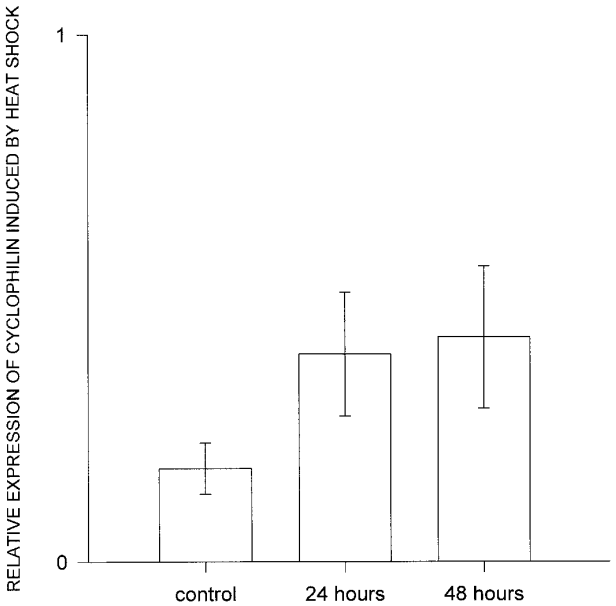

FIG. 2. Time course of relative cyclophilin expression after heat treatment of H9c2 cells for 1 hour at 43 °C.

oxia for 18 hours followed by 5 hours of reoxygenation. Experimental hypoxia using constant gas flow in a specifically designed sealed chamber (see Materials and Methods), showed a significant increase in expressed cyclophilin after 5 hours of reoxygenation, as determined by Western blot (Fig. 4A). MTS assay demonstrated a decrease in the amount of viable cells after 5 hours of reoxygenation (Fig. 4B), which was confirmed by the results of the Trypan Blue exclusion assay. The amount of dead cells, which could not exclude the Trypan Blue was approximately 20%.

DISCUSSION

The present study demonstrates that peptidyl -prolyl cis-trans isomerases (PPIases or cyclophilins) are stress and heat inducible proteins in myocardial cells. This is a novel study in eukaryotic cells, as previous work has only demonstrated that cyclophilins are heat inducible proteins in prokaryotic cells (2). Cyclophilins are abundant and highly conserved; moreover, they exist in a multitude of cellular isoforms and it has been proposed that one or more of cyclophilins are involved in the cellular response to stress (2, 5, 6).

The present study demonstrating that cyclophilins are induced by heat shock and hypoxia gives us the possibility to propose a role for cyclophilins in the preconditioning effect. We also suggest a functional association between cyclophilins and specific heat inducible proteins, and their involvement in the complex mechanisms of ischemia-reperfusion injury in the myocardium. One possible justification for the functional association of cyclophilins and proteins acting as molecular chaperones and their part in ischemic-reperfusion injury can be that the opening of the inner mitochondrial membrane under conditions of high [Ca^{++}] and depleted adenine nucleotides is the pathological transformation of the so-called general insertion pore used for protein transportation through the mitochondrial membrane under normal conditions. The molecular identity of the components involved in the formation of this [Ca^{++}] induced pathological mitochondrial pore has not been established, but the involvement of the cyclophilin in the pore regulation has been proved experimentally (11, 12, 13). As cyclophilins catalyse the peptidylprolyl bonds in peptides and proteins, they are crucial for the folding and isomerisation of the proteins. Moreover they operate as a coregulatory subunits of molecular chaperones, which are, in turn, heat shock proteins with a direct protective role against ischaemia-reperfusion injury (14, 15, 16, 17). Several studies describe the inverse relationship between the expression of major heat shock proteins in the heart and the severity of injury resulting from coronary occlusion (17, 18). This correlation is supported by investigations in which either heat shock or transient ischemia was used as a preconditioning stimulus (19). A cytoprotective function of the heat shock proteins in the ischaemic heart has been suggested because of the induction of the expression of HSP70 during or following ischemia and the apparent analogy between the thermotolerance induced by the prior heat shock and the ischemic preconditioning phenomenon. Cyclophilins accelerate the slow refolding processes of those released proteins that

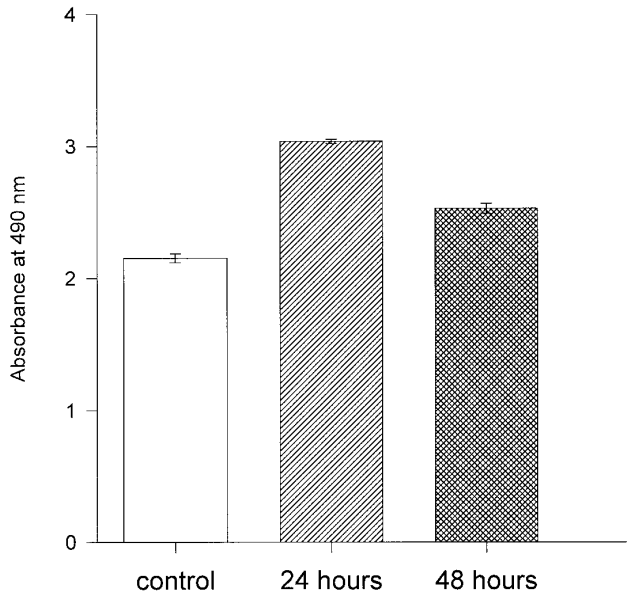

FIG. 3. MTS cell viability assay 24 and 48 hours after heat stress at 43°C for 1 hour. Control was confluent cells without any treatment. Values represent mean ± SEM, $p<0.05$.

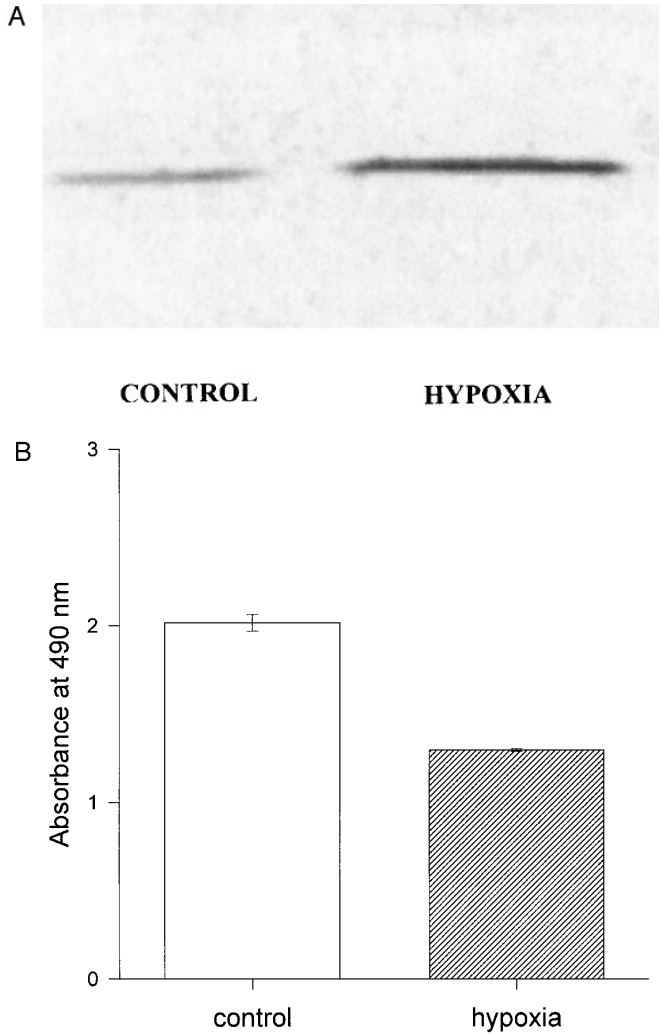

FIG. 4. (**A**) Western blotting of H9c2 cells harvested after 18 hours of incubation in the hypoxic chamber with a 5 hour reperfusion period in the atmosphere of 10% CO_2. Control was confluent H9c2 cells without treatment. (**B**) MTS cell viability assay of the H9c2 cells harvested after 18 hours of incubation in the hypoxic chamber (see Materials and Methods). Control was confluent H9c2 cells without treatment. Values represent mean ± SEM, $p<0.05$.

require peptidyl-prolyl isomerization. The lack or reduced activity of PPIases retards the folding and thus favors rebinding to the chaperones HSP70 and 60. Every binding release cycle of HSP70 and HSP60 requires the hydrolisis of additional ATP (16). Cyclophilin function is proposed to save metabolic energy by increasing the rate of folding of some proteins (1).

We suggest that the present observation that cyclophilins are heat and stress inducible proteins, will offer us a new approach for the understanding of the mechanism implicated in cell damage during ischemia in the myocardium and preconditioning protective phenomena and therefore in determining possible pharmacological targets for the prevention of reperfusion injury.

ACKNOWLEDGMENTS

The authors are grateful to James Clark and Dr. Roberta Forresti for technical assistance.

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