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Quercetin suppresses heat shock-induced nuclear translocation of Hsp72

Joanna Jakubowicz-Gil, Bożena Pawlikowska-Pawlęga, Tomasz Piersiak, Jarosław Pawelec and Antoni Gawron

Department of Comparative Anatomy and Anthropology, Maria Curie-Sklodowska University, Lublin, Poland

Abstract: The effect of quercetin and heat shock on the Hsp72 level and distribution in HeLa cells was studied by Western blotting, indirect immunofluorescence and immunogold electron microscopy. In control cells and after quercetin treatment, Hsp72 was located both in the cytoplasm and in the nucleus in comparable amounts. After hyperthermia, the level of nuclear Hsp72 raised dramatically. Expression of Hsp72 in cytoplasm was also higher but not to such extent as that observed in the nucleus. Preincubation of heated cells with quercetin inhibited strong Hsp72 expression observed after hyperthermia and changed the intracellular Hsp72 distribution. The cytoplasmic level of protein exceeded the nuclear one, especially around the nucleus, where the coat of Hsp72 was noticed. Observations indicating that quercetin was present around and in the nuclear envelope suggested an involvement of this drug in the inhibition of nuclear translocation. Our results indicate that pro-apoptotic activity of quercetin may be correlated not only with the inhibition of Hsp72 expression but also with suppression of its migration to the nucleus.

Key words Quercetin - Hsp72 - Indirect immunofluorescence - Electron microscopy

Introduction

Heat shock protein 72 (Hsp72) is a member of proteins called molecular chaperones. It is a highly conserved protein, controlling proper folding of newly synthesized polypeptides and their transport through cell membranes. Hsp72 also prevents protein aggregation and denaturation [3, 6, 25]. It is overexpressed in response to several stressors like hyperthermia, free radicals and chemotherapeutic agents. Its expression can also be modulated by many conditions leading to apoptosis which are associated with pathological processes such as ischemia, fever, inflammation, infections [24, 27] and cancer, where enhanced expression of Hsp72 has also been reported [14, 17, 23]. Hsp72 can also protect cells against apoptosis *via* several mechanisms: blocking of cytochrome c release from mitochondria [21], inhibition of procaspase 9 activation, apoptosome formation [2, 28] and kinase JNK phosphorylation [9, 10]. Our earlier experiments indicate that inhibition of Hsp72 expression makes tumour cells more vulnerable to pro-apoptotic action of quercetin (3,3',4',5,7-pentahydroxyflavone) [16]. In this study we analyse the effect of quercetin on localization of Hsp72 in HeLa cells using indirect immunofluorescence and immunogold electron microscopy.

Correspondence: J. Jakubowicz-Gil, Dept. Comparative Anatomy and Anthropology, Maria Curie-Sklodowska University, Akademicka 19, Lublin, Poland; e-mail: jjgil@biotop.umcs.lublin

Materials and methods

Cell culture. In the experiments, human negroid cervical carcinoma cell line (HeLa B, ECACC No 85060701) cultured in RPMI 1640 medium supplemented with 5% FBS (foetal bovine serum) (v/v) was used. Cells at a density of 1×10^6 cells/ml were seeded on coverslips (for indirect immunofluorescence) or in Falcon vessels (for immunogold electron microscopy and immunobloting), and incubated at 37˚C in humidified atmosphere with 5% CO_2.

Heat and drug treatment. In the experiments, quercetin dihydrate (Sigma, Germany) (15 μg/ml, 44 μM) dissolved in dimethyl sulfoxide (DMSO) was used. The final concentration of DMSO in culture medium did not exceed 0.1%, since, as indicated in preliminary experiments, it did not influence Hsp72 expression. Three variants of experiments were performed. In the first one cells were incubated with quercetin for 7.5 h at 37˚C. In the second one cells were heated at 42˚C for 1 h and transferred to 37˚C for 2.5 h. In the third variant cultures were preincubated with quercetin for 4 h at 37˚C, exposed to hyperthermia (42˚C for 1 h) and transferred to 37˚C for 2.5 h. As controls, cells were incubated with 0.1% DMSO at 37˚C.

The quercetin concentration was chosen on the basis of our earlier experiments and observations of other authors [12, 13, 15, 16].

Localization of quercetin in cells. HeLa cells were incubated with quercetin for 7.5 h at 37˚C. Autofluorescence of quercetin was analysed under fluorescence microscope (λ=458 nm). The relative level of pixel fluorescence was measured along chosen line passing through cytoplasm and nucleus using LSM5 Image Examiner software (Zeiss).

Indirect immunofluorescence. Cells were washed three times with PBS, fixed for 10 min in 3.7% paraformaldehyde in PBS, washed three times with PBS, treated with 0.2% Triton X-100 for 7 min, washed three times with PBS, all at room temperature. Subsequently, a blocking step of 30 min in 5% low fat milk at room temperature was included. Cells were then incubated with mouse anti-Hsp72 monoclonal antibody (SPA 810, StressGen) diluted 1:200. Anti-Hsp72-labelled cells were detected with FITC-conjugated goat anti-mouse antibody (Sigma) at 1:30 dilution.

Cells were analysed using Pascal 5 scanning head (Zeiss). Pictures were registered within fluorescence channel (λ=488 nm). The relative level of pixel fluorescence was measured along chosen line passing through cytoplasm and nucleus using LSM5 Image Examiner software. As controls, cells were incubated in the absence of primary antibody and were not labelled (not shown).

Immunoelectron microscopy. Cells were gently scrapped off flasks using cell scraper, fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer for 2 h and postfixed in 1% osmium tetroxide for the next 2 h, all at 4˚C. Then the cells were dehydrated in series of alcohol and acetone and embedded in Spurr resin. Ultrathin sections were cut with glass knife on RMC MT-XL microtome, collected on nickel grids, washed with PBS and incubated with anti-Hsp72 mouse monoclonal antibodies (StressGen, Canada) diluted 1:200 overnight at 4˚C. Following three washes with PBS, grids were incubated for 2 h with anti-mouse secondary antibody diluted 1:50, conjugated with 10-nm gold particles (Sigma). Grids were washed with deionised water, dried at room temperature and examined in LEO 912 AB electron microscope.

As controls, some grids were floated on the incubation mixture from which the primary antibody was excluded and then processed as above. Such omission resulted in no disposition of reaction product (not shown).

The gold particle density (number of gold particles per μm^2) over the cytoplasm, nucleus and 200 nm wide cytoplasmic zone surrounding the nucleus was calculated for eight cells coming from two independent experiments. The area of the respective cell compartments (cytoplasm, nucleus, perinuclear cytoplasm) was measured on micrographs using ESIVision analySIS 3.0 software. Significance levels were calculated using one-way ANOVA test.

Isolation of cell nuclei. Cells were gently scrapped off flasks using cell scraper and incubated for 15 min on ice in a buffer containing 10 mM Tris-HCl pH 7.9, 10 mM KCl, 0.2 mM EDTA, 2 mM β-mercaptoethanol, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A. Next, Triton X-100 was added to the final concentration of 1%. Samples were shortly shaken and centrifuged at 10000 × g for 1 min. The pellets were used for further experimental procedures.

Immunobloting. For Western blot analysis, nuclei isolated from HeLa cells and whole HeLa cells scrapped of flasks were lysed in hot SDS-loading buffer (125 mM Tris-HCl pH 6.8; 4% SDS; 10% glycerol; 100 mM DTT), boiled in water bath for 10 min, centrifuged at 10000 × g for 10 min and the supernatant was collected. The protein concentration was determined by the Bradford method [5] and samples of supernatants containing exactly 80 μg of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis [18]. Proteins were transferred onto Immmobilon P membrane (Sigma). Following transfer, the membrane was blocked with 3% low fat milk in PBS for 1 h, then incubated overnight with mouse monoclonal antibodies against Hsp72 (StressGen, Canada) diluted 1:1000. The membrane was washed 3 times for 10 min with PBS containing 0.05% Triton X-100 (Sigma) and incubated for 2 h with a 1:30000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). The membrane was visualized with alkaline phosphatase substrate (5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium, Sigma) in a colour development buffer (DMF, Sigma). Three independent experiments were performed.

Quantitative heat shock protein levels were assessed using Bio-Profil Bio-1D Windows Application V.99.03 software.

Significance levels were calculated using one-way ANOVA test.

Results

Localization of Hsp72 in HeLa cells at physiological temperature

Indirect immunofluorescence showed that in HeLa cells Hsp72 was located both in the cytoplasm and the nucleus. The relation of nuclear and cytoplasmic fluorescence was similar (1:1.3) (Figs. 1g, 2a). Electron microscopy observations indicated that Hsp72 was distributed uniformly both in cytoplasm and in nucleus, mainly in condensed chromatin (Fig. 1h; Table 1).

Localization of Hsp72 after heat shock treatment

Electron microscopy showed that 1-hour long hyperthermia at 42˚C increased positive immunocytochemical reaction towards Hsp72 in whole cells in comparison to non-heated ones (Fig. 1e; Table 1). Significant changes were observed especially in the nuclei where the level of Hsp72 exceeded the cytoplasmic one. As revealed by Western blotting of the isolated HeLa cell nuclei, the level of Hsp72 in heated cells was higher by 50% in comparison to controls (Fig. 3). Higher concentration of Hsp72 in nucleus rather than in cytoplasm was also observed under fluorescent microscope (Fig. 1b) and was confirmed by quantitative analysis (Fig. 2c).

The effect of quercetin on Hsp72 localization in nonheated and heated cells

Incubation of HeLa cells with quercetin for 7.5 hours resulted in slight inhibition of Hsp72 expression in the cytoplasm and nucleus in comparison to control cells (Table 1; Fig. 2b). Localization of Hsp72 after quercetin treatment (Figs. 1a,d) was similar to that in the control cells. Additionally, increased concentration of Hsp72 was observed in the cytoplasm surrounding nucleus and in the nuclear envelope where Hsp72 formed a kind of coat. In HeLa cells preincubated with quercetin and exposed to the heat shock, Hsp72 was detected both in cytoplasm and nucleus, but as observed under fluorescence and electron microscopes, the nuclear level was lower than the cytoplasmic one. A coat of Hsp72 around

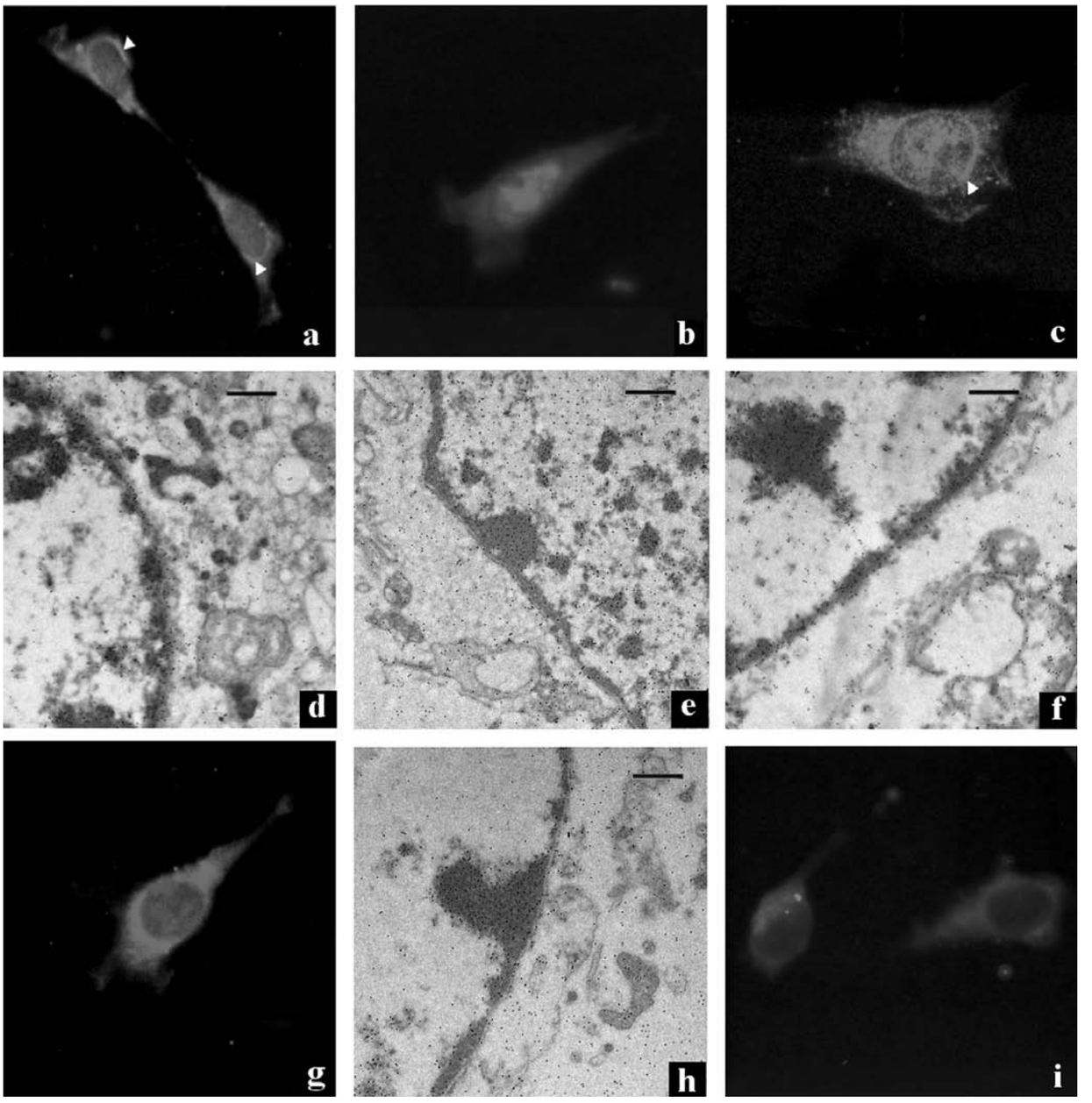

Fig. 1. Distribution of Hsp72 in HeLa cells after heat shock and quercetin treatment under fluorescence (**a, b, c, g**; magnification × 126) and electron (**d, e, f, h**; bar 500 nm) microscopes. Arrows show increased Hsp72 concentration around nuclear envelope after quercetin treatment. **a, d** - cells incubated with quercetin, **b, e** - cells exposed to hyperthermia, **c, f** - cells preincubated with quercetin and exposed to hyperthermia, **g, h** - control cells; **i** - control showing autofluorescence of quercetin.

and in the nuclear envelope was also observed (Figs. 1c,f, 2d; Table 1).

Localization of quercetin in HeLa cells

Quercetin shows autofluorescence what enables its simple detection within cells. The level of fluorescence of the studied drug was significantly lower than that of fluorescein connected with anti-Hsp72 antibodies. It suggests that the autofluorescence of quercetin did not influence significantly the level of the fluorescence of antibodies attached to Hsp72 (Fig. 2e). Fluorescence microscopy of HeLa cells incubated for 7.5 h with the studied drug showed its presence in the cytoplasm and around nuclear envelope (Figs. 1i, 2e). Only slight fluorescence was observed in the nucleus.

Table 1. The density of gold particles connected with anti Hsp72 antibodies per μm^2 on representative electron micrographs of control HeLa cells and cells after hyperthermia and quercetin treatment

Hsp72 concentration in HeLa cells ($gp/\mu m^2$)			
	Nucleus	Cytoplasm	Perinuclear cytoplasm
Control	38.88 ± 2.99	41.63 ± 4.69	40.38 ± 2.97
Quercetin	38.38 ± 3.46	40.38 ± 2.35	218.25 ± 10.7*
Heat shock	46.25 ± 3.41*	44.38 ± 3.99	42.25 ± 3.89
Quercetin + heat shock	40.88 ± 2.47	45 ± 4.1	180.13 ± 4.49*

$gp/\mu m^2$ - the number of gold particles per square micrometer; *$p<0.001$ represents a statistically significant difference between the values for the control and experimental treatments

The level of Hsp72 in HeLa cells after quercetin and heat shock treatment

The effect of quercetin on the level of Hsp72 in HeLa cells was studied in whole cell lysate (Fig. 4) and in isolated nuclei (Fig. 3). Incubation of HeLa cells with quercetin for 7.5 h resulted in slight, insignificant inhibition of Hsp72 expression in whole cells and in isolated nuclei. As revealed by Western blotting, the level of Hsp72 after hyperthermia was higher by about 50% in isolated nuclei and by about 33% in whole cells in comparison to control. In cells preincubated with quercetin and exposed to the heat shock, smaller amount of the studied protein (by about 40% in nuclei, 22% in whole cells) was detected in comparison to heat shock-treated cells.

Discussion

Quercetin is one of the most widely distributed flavonoids in the plant kingdom, having a broad range of activities within cells. It is a well known antioxidant [7, 20]. In tumour cells, it exerts antiproliferative effects by blocking cell cycle in check points G0/G1, G2/M, G1/S, leading to apoptosis [1, 22, 30, 31]. Pro-apoptotic activity of quercetin is also associated with inhibition of Hsp72 expression [11, 26]. It is known that blocking of *hsp72* gene expression by antisense oligonucleotides makes tumour cells more vulnerable to pro-apoptotic activity of quercetin [15]. In this study we present another possible explanation of such activity, considering the effect of quercetin on changes in Hsp72 localization in cells.

It is known that in physiological conditions Hsp72 shows basal levels of expression being present in both cytosol and nucleus, especially in nucleoli and in regions of heterochromatin [8]. Our results indicate that in untreated cells Hsp72 is distributed both in cytoplasm and

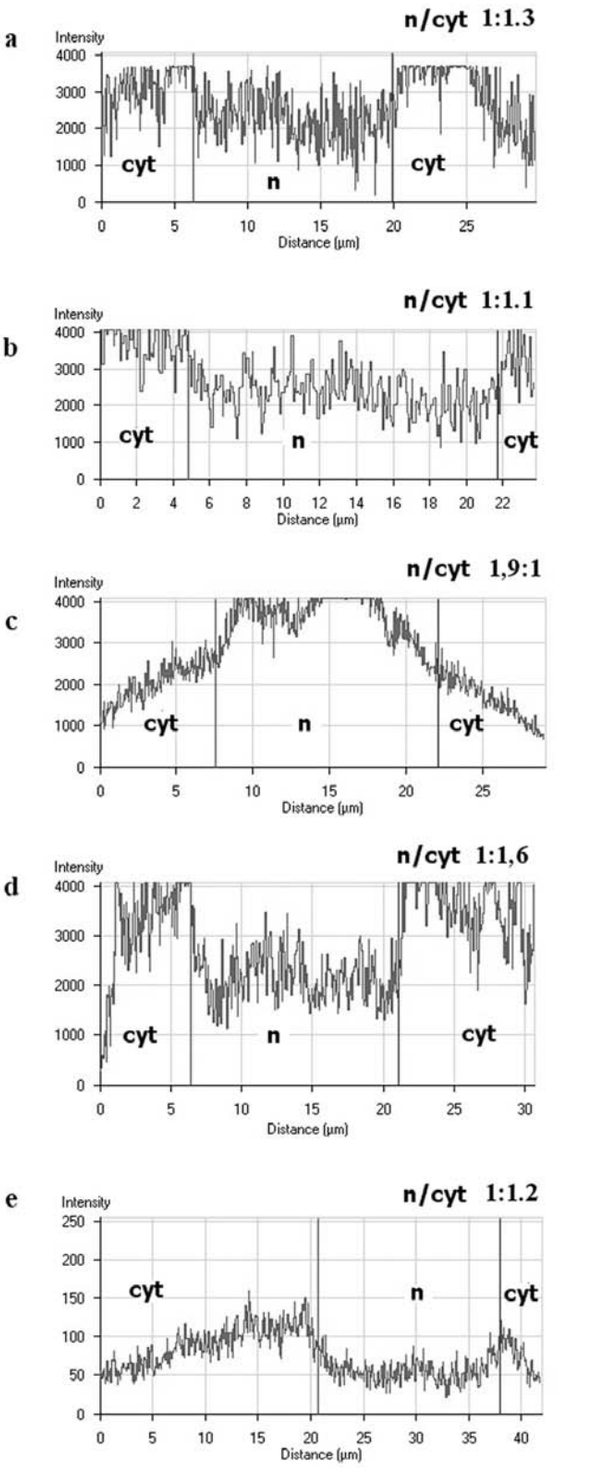

Fig. 2. The level of fluorescence of FITC conjugated with anti-Hsp72 antibodies and quercetin in the nucleus (n) and cytoplasm (cyt) of HeLa cells. **a** - control cells, **b** - cells incubated with quercetin, **c** - cells exposed to hyperthermia, **d** - cells preincubated with quercetin and exposed to hyperthermia, **e** - autofluorescence of quercetin, n/cyt - relation of the relative nuclear and cytoplasmic fluorescence measured along chosen line passing through the cell.

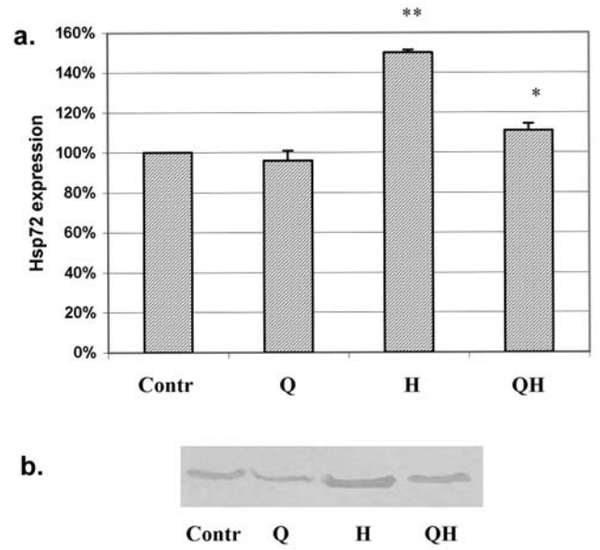

Fig. 3. The level of Hsp72 expression in the isolated nuclei of HeLa cells after quercetin and heat shock treatment expressed in percentage values. **a** - quantitative analysis; **b** - representative Western blot, Q - cells incubated with quercetin, H - cells exposed to hyperthermia, QH - cells preincubated with quercetin and exposed to hyperthermia, Contr - control; *p<0.05; **p<0.005 represent a statistically significant difference between the values for the control and experimental treatments.

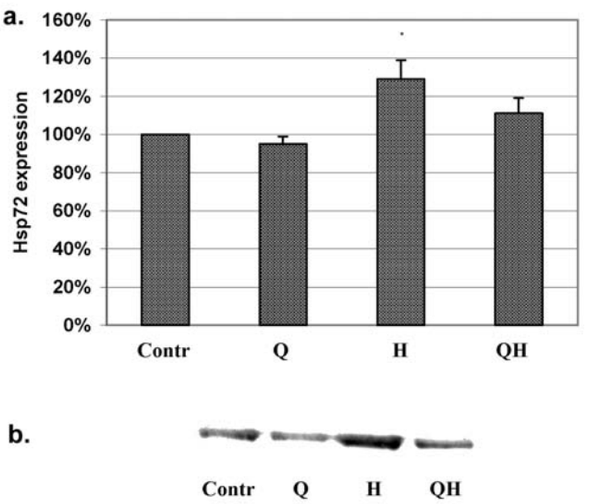

Fig. 4. The level of Hsp72 expression in HeLa cells after quercetin and heat shock treatment expressed in percentage values. **a** - quantitative analysis; **b** - representative Western blot, Q - cells incubated with quercetin for 7.5 h at 37˚C, H - cells exposed to hyperthermia for 1 h at 42˚C and incubated at 37˚C for 2.5 h, QH - cells preincubated with quercetin for 4 h at 37˚C and exposed to hyperthermia, Contr - control; *p<0.05 represents a statistically significant difference between the values for the control and experimental treatments.

in nucleus at the comparable levels. Hsp72 was located in the cytoplasm surrounding nucleus and in the nucleus, associated with condensed chromatin. Incubation of HeLa cells with quercetin for 7.5 hours did not influence significantly Hsp72 distribution within cells, except for increased concentration of the studied protein in the perinuclear cytoplasm and nuclear envelope.

As revealed by fluorescence and electron microscopy, after hyperthermia the positive immunocytochemical reaction towards Hsp72 in the nucleus raised dramatically. It was confirmed by Western blot analysis of isolated nuclei. Expression of Hsp72 in the cytoplasm was also higher but not to such extent. As discussed by other authors, Hsp72 after hyperthermia migrates from the cytoplasm to the nucleus to protect nuclear structures and transcriptional apparatus against heat shock damages [4, 19, 29].

Preincubation of cells with quercetin and exposition to the heat shock suppressed Hsp72 translocation from the cytoplasm to the nucleus. In isolated nuclei, the level of Hsp72 was by about 40% lower than that in nuclei obtained from cells treated with heat shock only. Weaker decrease (about 22%) of the amount of Hsp72 was detected in whole cell lysats. It may suggest that quercetin more effectively diminishes the nuclear level of Hsp72, probably by inhibiting its expression and nuclear translocation. Interestingly, a coat of Hsp72 around the nucleus was noticed, similarly to cells incubated only with quercetin.

Our observations indicate that quercetin blocks Hsp72 translocation from cytoplasm to the nucleus, probably at the level of nuclear envelope. Thus we present a novel mechanism of pro-apoptotic activity of quercetin *via* inhibition of not only synthesis of Hsp72 but also its nuclear translocation, making cells more vulnerable to apoptosis.

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