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THE DYNAMICS OF HEAT SHOCK SYSTEM ACTIVATION IN MONOMAC-6 CELLS UPON *HELICOBACTER PYLORI* INFECTION

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Immune system cells, particularly phagocytes, are exposed to direct contact with pathogens. Because of its nature - elimination of pathogenes - their cytoprotective systems supposed to be quick and forceful. Physiological consequence of phagocytosis for the phagocyte is the apoptotic death to prevent the eventual survival of bacteria as intracellular parasites. However, in some cases, defense systems used by the bacteria force the immune cells to prolong the contact with the pathogen for its effective elimination. Experiments were performed on Monomac-6 cells exposed to live CagA, VacA expressing *Helicobacter pylori* (*H. pylori*) over different period of time. Total cellular RNA, cytoplasmic and nuclear proteins were isolated for polymerase chain reaction, Western-blot and electrophoretic mobility shift assay, respectively. We found that Monomac-6 cells infection with *H. pylori* resulted in the translocation of the entire cellular content of the heat shock protein 70 (HSP70) into the cytoplasm, where its presence could protect cell against toxic products of engulfed bacteria and premature apoptosis. At the same time the nuclear translocation of heat shock factor 1 (HSF-1) and activation of HSP70 gene transcription was noticed. Action of HSP70 might to postpone monocyte apoptosis through protecting cytoplasmic and nuclear proteins from damaging effect of bacterial products, what could be the defending mechanism against the toxic stress caused by engulfed bacteria and provide the immune cell with the sufficient amount of time required for neutralization of the bacteria from phagosomes, even at the expense of temporary lack of the protection of nuclear proteins.

Key words: *monocytes, Helicobacter pylori, heat shock protein 70, heat shock factor 1, phagocytosis, apoptosis, cytotoxin-associated gene A protein*

INTRODUCTION

When in the early 80's of last century, Barry Marshall and Robin Warren described a spiral bacterium as a pathogen causing inflammation of the gastric mucosa and gastric cancer in humans, many researchers have expressed skepticism about this discovery (1). Over the next decades, papers supporting their hypothesis exceeded the number of 20,000. In 2005, for their spectacular discovery Warren and Marshall were awarded the Nobel Prize in medicine and physiology. Currently, *H. pylori* has been located on the list of possible carcinogens as the head of the pack and this fact no longer raises controversy. Although *H. pylori* infection is not questioned any more as a cause of the development of inflammation, ulcers and stomach cancer, the mechanism behind the induction of these changes is not fully understood.

Immune system is regarded as a key element participating in the development of gastric inflammation in response to the colonization of gastric mucosa with *H. pylori*. The infection leads to the activation of monocytes, neutrophils or lymphocytes and infiltration of the mucosa (2). This, very often, might be a natural consequence of the appearance of bacterial immune-modulators secreted by *H. pylori*, such as bacterial enzymes or neutrophil activating protein (NAP) (3-5). Epithelial cells, in response to bacterial toxins VacA and CagA actively introduced by the bacteria to the cell interior, also undertake the production of proinflammatory cytokines and chemokines leading to the recruitment of immune effector cells and enhancement of the inflammatory response (6-10).

For many years, the fact of phagocytosis of *H. pylori* bacteria by specialized immune cells *in vivo* remained unconfirmed and problematic. *In vitro* mixed cultures of bacteria and eukaryotic cells showed that the phagocytes such as a monocytes or granulocytes are capable of engulfment of live *H. pylori* bacteria (11). However, it was unclear whether during the infection of the stomach with *H. pylori*, contact between the pathogen residing in the lumen and the immune cells infiltrating the gastric wall occurs at all. Some observations made in the mid 80's indicated that phagocytes can attack *H. pylori* in the stomach in an active way and engulf them (12). Finally Zu *et al.* have demonstrated that in the case of chronic *H. pylori* infection of the stomach , there are evidences of a direct contact between the bacteria and neutrophils in the mucosa and the active removal of *H. pylori* by phagocytosis (13). Soon, other laboratories have confirmed this clinical fact (14). Detailed analysis of the interaction between *H. pylori* and phagocytes

revealed a series of reactions by which bacteria modulates immune cells to retard or even prevent phagocytosis and elimination from the mucosa. The resultant "equilibrium of power" leads to chronic penetration of the gastric wall by activated immune cells and maintenance of the continuous inflammatory reaction (15-19). Many known mechanisms are involved in the elimination of bacteria from phagosome. Contact with bacteria, and in particular their phagocytosis, alters the metabolism of phagocyte, causing eventually its apoptotic death, preventing from survival of the bacteria inside the phagosome in the form of "intracellular parasite" (20-23).

The heat shock system is one of the most important in regard to maintenance of cellular homeostasis. In certain situations, this system favors reactions leading to cell death, in others to the contrary, promotes cell survival. Such a "Janus attitude" is nothing new in biology and is represented by several molecular "switches". But in contrast to the heat shock proteins (HSP) these molecular "switches" act at strictly "quantized" states. As a signal integrating proteins they activate or deactivate certain cascades of reactions, while heat shock system, in response to various stimuli, triggers a series of processes which may lead to the contradictory effects. Studied extensively since the 70' of the last century, HSPs became well known, especially as the molecular chaperons. Most often that term refers to the ongoing, energy dependent binding reaction and stabilization of unstable conformers of other proteins to prevent them from aggregation and maintenance of the correct structure to ensure proper cellular localization. Most of the HSPs does not determine the structures of higher order of proteins, but make it easier for them to get the correct conformation. Only a few HSPs act as a "real catalysts of folding" increasing the efficiency of this process (24-28).

Based on the results presented in this paper, we propose the mechanism of heat shock system action in monocytic cells which had contact with the live *H. pylori* bacteria and this reaction contributes to the maintaining of proper conformation of the cellular proteins and the delay of apoptotic cell death. Such reaction might be beneficial as a defense mechanism against the toxic stress caused by engulfed bacteria and provide the immune cell with the sufficient amount of time required for neutralization of the pathogen from phagosomes.

MATERIALS AND METHODS

Bacterial culture

The selected strain 60190 of *H. pylori* (ATCC number 49503, LGC Promochem, UK) expressing CagA and VacA protein was grown on the Columbia solid agar medium supplemented with 5% of fresh horse blood (BioMerieux). Before the experiment bacteria were harvested and suspended in sterile PBS. The bacteria were densitometrically counted according to the McFarland scale before administration to the cell culture, and suitable dilutions were prepared.

Cell culture

Monomac-6, monocytic cell line was established from peripheral blood of a patient with monoblastic leukemia. Monomac-6 has been selected as the monocyte model due to its ability to constitutive phagocytosis of antibody-coated erythrocytes and expression of a specific for mature monocytes antigens like M42, LeuM3, 63D3, Mo2 and UCHMI (29).

Monomac-6 cells were grown in RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 10% foetal bovine serum (Biochrom GmbH, Germany), 2 mM L-glutamine, 1% of nonessential amino acids, 1 mM oxaloacetic acid, 0.45 mM pyruvate, 0.2 U/ml insulin and antibiotic cocktail (Sigma-Aldrich, USA). Cells were grown to the concentration of 0.1 - 1.0 $\times 10^6$ cell per ml. 24 h before the experiment 6×10^6 of Monomac-6 cells were seeded on a 100 mm dishes in RPMI 1640 with the addition of 1% foetal bovine serum, to which no antibiotics had been added. Cultures were infected with 1×10^8 of live *H. pylori* per dish (amount of bacteria were established based on the previous studies) reaching MOI value of 17. During the experiments any pH disequilibrium caused by the presence of the bacteria have never been encountered. All the experiments were repeated at least three times. The results presented here were taken from the most representative experiments. Both (S)-(+)-Camptothecin (C991) and Cycloheximide (C4859) were purchased in Sigma-Aldrich.

Real-time polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using TRIzol Reagent (Gibco-BRL) according to the manufacturer protocol. The synthesis of the first strand cDNA was performed with a Reverse Transcription System (Promega) using 1 µg of total cellular RNA. For polymerase chain reaction, 2 µl of cDNA and oligo primers were used. All PCR reactions were performed employing Promega PCR reagents. Specific primers, listed below, were synthesized by Sigma-Genosys (Pampisford, UK).

β-actin sense-5'-AGCGGGAAATCGTGCGTG-3',
antisense -5'-GGGTACATGGTGGTGCCG-3'; (307bp)
HSP70 sense -5'-TTTGACAACAGGCTGGTGAACC-3',
antisense -5'-GTGAAG ATCTGCGTCTGCTTGG-3'; (590bp)

Electrophoretic molility shift assay (EMSA)

Double-stranded probes were prepared from complementary single-stranded oligonucleotides obtained from Sigma-Genosys (Pampisford, UK), by melting at 95°C for 5 min followed by annealing for 3 h at ambient temperature. The nuclear extracts (10 µg per line) were prepared as follows. Cells were harvested and gently centrifuged (10 min at 700 rpm). Cell pellet was washed with ice-cold sterile PBS, centrifuged and resuspended in 200 µl of isolation buffer (10 mM Hepes pH 7.8; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM Na3VO4; 1 mM DTT, 0.2 mM PMSF, 10 µl/ml Complete Protease Inhibitor (Roche, USA) and incubated on ice for 10 min. After incubation 15ml of 10% NP-40 (Sigma-Aldrich, USA) was added and samples were vortex for 10 s and centrifuged for 3 min at 14,000 rpm. Supernatants containing cytoplasmic proteins were collected, aliquoted and kept frozen at –80°C. Nuclear pellets were re-suspended in 50 µl of nuclear proteins isolation buffer (20 mM Hepes pH 7.8; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM Na3VO4; 1 mM DTT; 20 µl/ml Complete Protease Inhibitor (Roche, USA) and incubated on ice for 15 min. Samples were centrifuged for 5 min at 14,000 rpm and supernatants were carefully transferred to new eppendorf tubes and frozen at –80°C for further analysis. The nuclear extracts (10 µg per line) were examined for band shift with 5 pmol of double-stranded biotinylated heat shock element (HSE) consensus containing DNA probe (5'-CTAGAAGCTTCTAGAAGCTTCT AGAA-3') obtained from Sigma-Genosys (Pampisford, UK) and labeled with Light Shift Chemiluminescence EMSA kit (Pierce Biotechnology, USA). Complexes were separated by electrophoresis in native 6% polyacrylamide gel and electro-blotted to the Hybond N^+ (Amersham Pharmacia Biotech, UK) membrane. After UV cross-linking, the membrane was subjected to procedures in accordance with the manufacturer's protocol, to detect bands. Unlabelled heat shock element oligo-probe was used in competition studies. Unlabelled AP-1 binding oligo-probe was used as a noncompetitor.

Immunoblotting

The nuclear and cytoplasmic protein extracts were prepared as described above. An equal load of protein in each sample was assessed using Quanti Pro BCA Assay Kit (Sigma, USA). Protein samples were boiled with western-blot sample buffer and loaded on the 12% SDS-polyacrylamide gel. After electrophoresis and electro-transfer of the samples, the PVDF membrane (BioRad, USA) was blocked with blocking buffer (5% non-fat dried milk in PBS) for 1 hour at room temperature. This blocking procedure was followed by a 1 h exposure to primary antibody (monoclonal anti heat shock factor 1 (HSF-1) (E4) Santa Cruz Biotechnology, USA, rabbit polyclonal anti-phospho-HSF-1 (Ser230) Santa Cruz Biotechnology, USA, mouse monoclonal HSP70 (C92F3A-5 Stressgen, Ann Arbor, USA) diluted 1:5000 and secondary antibody diluted 1:10000 in blocking buffer. After each antibody probing, the membrane was washed three times for 15 min in TBST buffer (0.1 M Tris pH 8.0; 1.5 M NaCl; 0.5% TritonX-100). Detection of membrane bound proteins was performed using BM Chemiluminescence Blotting Substance according to the producer protocol (Boehringer Mannheim, Germany). Mouse monoclonal anti HSP70 antibody (C92F3A-5 Stressgen) has been selected after extended comparison. The selection criteria were based on the declared by the producers specificity and lack of cross-reactivity with bacterial HSP70. To be sure that in our experiments we had been detecting eukaryotic HSP70, we have made control immunoblotting analysis of the pure protein isolates from heat shocked *Helicobacter pylori* bacteria and KATOIII cells. The results of immunoblotting reaction showed clearly that there is no cross-reactivity with procaryotic HSP70 in detection of eukaryotic HSP70. Using our set of antibody we have failed to detect *Helicobacter* originated HSP70 in the isolates (*Fig. 8*).

DNA fragmentation ladder assay

After induction of apoptosis, cells (6×10^6/sample, both attached and detached cells) were lysed with 150 ml hypotonic lysis buffer (edetic acid 10 mmol/l, 0.5% Triton X-100, Tris-HCl, pH 7.4) for 15 min on ice and were precipitated with 2.5% polyethylene glycol and 1 mol/l NaCl for 15 min at 4°C. After centrifugation at 13,000 × g for 10 minutes at room temperature, the supernatant was treated with proteinase K (0.3 g/l) at 37°C for 1 hour and precipitated with isopropanol at –20°C. After centrifugation, each pellet was dissolved in 10 µl of Tris-EDTA (pH 7.6) and electrophoresed on a 1.5% agarose gel containing ethidium bromide. The DNA ladder pattern was identified under ultraviolet light.

Immunofluorescence

Cell cultures were washed with PBS and fixed in 4% buffered formaldehyde for 15 min at room temperature. Fixative was aspirated and cells were rinsed three times in 1X PBS for 5 min. Then samples were blocked in blocking buffer (1X PBS/5% bovine serum albumin/0.3% Triton X-100) for 60 min. Primary antibody - mouse monoclonal anti-HSP70 (C92F3A-5 Stressgen, Ann Arbor, USA) has been applied in the dilution buffer (1X PBS/1% BSA/0.3% Triton™ X-100) for overnight incubation. Again cells were rinsed three times in 1X PBS for 5 min and specimens were incubated in fluorochrome conjugated secondary antibody anti-mouse IgG (H + L), F(ab')2 Fragment (Alexa FluorR 488 Conjugate, Cell Signaling Technology, USA) diluted in antibody dilution buffer for 1–2 h at room temperature in the dark. Samples were analyzed using an Axioscop microscope equipped with a Plan Neo Fluor 100x lens (Zeiss GmbH, Germany), and ZUS-47DE camera (Optronics, USA).

RESULTS

Dynamics of nuclear translocation of HSF1 in Monomac-6 cell culture in the presence of live Helicobacter pylori (H. pylori) bacteria

Monomac-6 cell cultures, as a laboratory model of human monocytes, have been incubated with live *H. pylori* bacteria. Cytoplasmic and nuclear protein fractions were isolated over 3, 6, 24 or 48 hours of culture. Immunoblotting has revealed a distinct differences in the intracellular distribution of heat shock transcription factor (HSF-1) dependent on time of incubation with bacteria. In sixth hour after inoculation of bacteria to the cell culture the amount of HSF1 in cytoplasmic fraction becoming untraceable (*Fig. 1A* line 2), while being noticeable increased in the nucleus at the same time (*Fig. 1B* lines 2) when compared the uninfected control (*Fig. 1A* line 1, *Fig. 1B* line 1). After 24 hours of culture in the presence of *H. pylori*, the initial

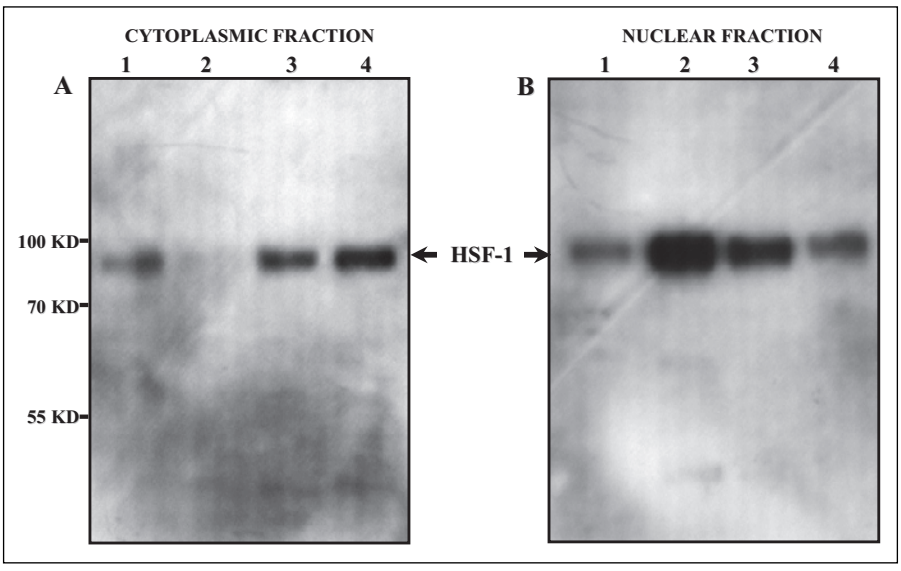

Fig. 1. Incubation of Monomac-6 cells with live *Helicobacter pylori* bacteria (strain 60190) resulted in translocation of HSF-1 between cytoplasmic and nuclear pool. Immunoblotting analysis of cytoplasmic (*A*) and nuclear (*B*) protein isolates from: line 1 - control Monomac-6 cells (cultures without *H. pylori*); Monomac-6 cells incubated with 1×10^9 *H. pylori* for 6 hours (line 2) 24 hours (line 3) and 48 hours (line 4).

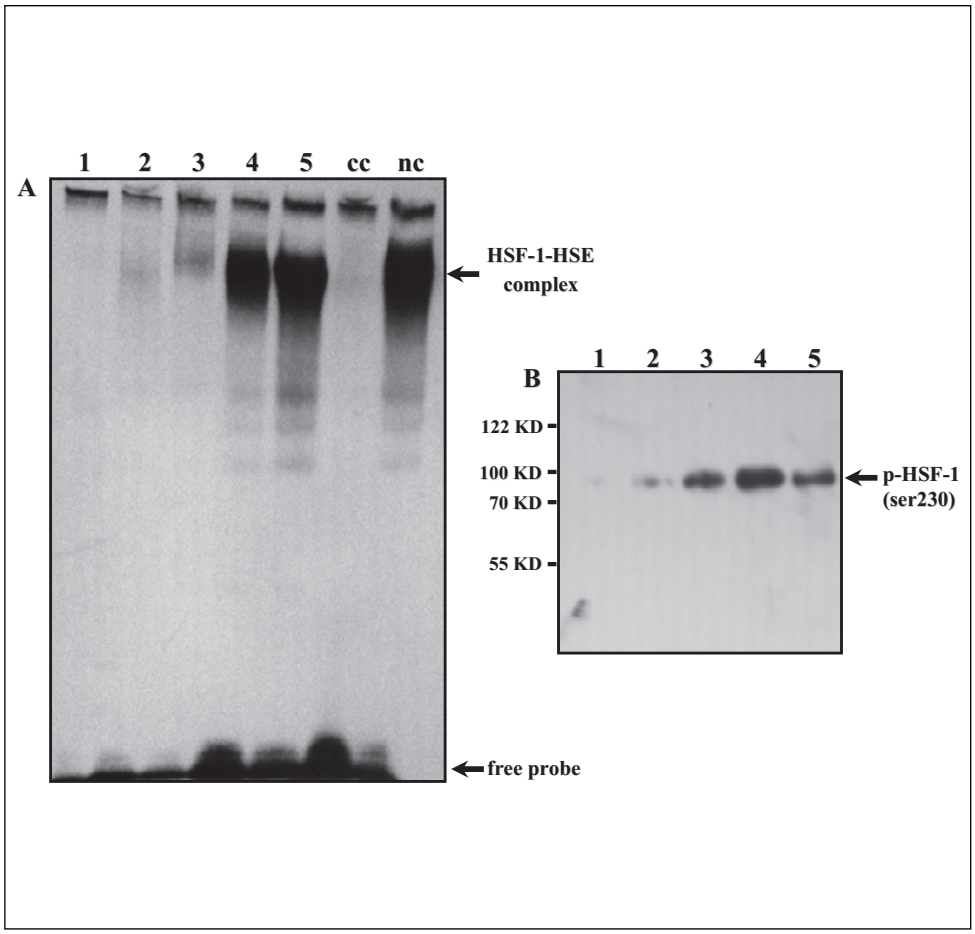

Fig. 2. Gel retardation assay has revealed that HSF-1 DNA binding in Monomac-6 cells was dependent on the duration of *Helicobacter pylori* presence in culture. Panel *A* shows the results of electrophoretic mobility shift assay reaction of 10 μg of nuclear proteins with 5 pmol of specific HSE sequence containing biotinylated probe. Specificity of the binding has been confirmed using competitor unlabeled HSE probe and non-competitor unlabeled AP-1 probe. Panel *B* presents immunoblotting analysis of nuclear protein fraction using antibody against HSF-1 phosphorylated on serine 230. Phosphorylation of serine 230 is required to evoke HSF-1 activation. Line 1 - control Monomac-6 cells (cultures without *H. pylori*); Monomac-6 cells incubated with 1×10^9 *H. pylori* for 3 hours (line 2), 6 hours (line 3), 24 hours (line 4) and 48 hours (line 5); cc - competitor reaction with 24 hours sample; nc - non-competitor reaction with 24 hours sample.

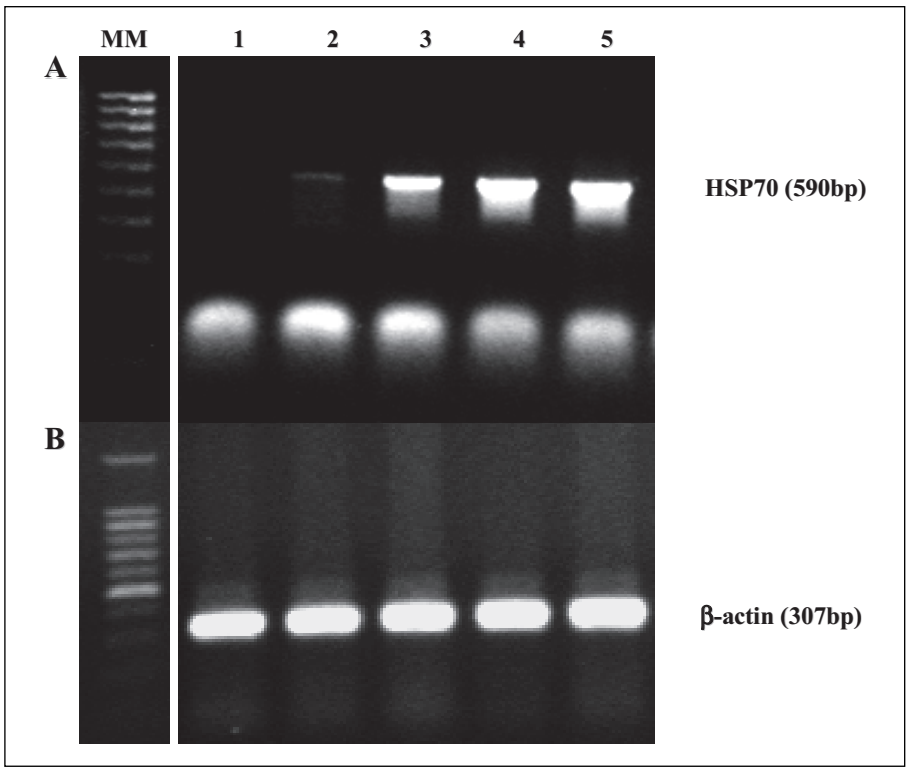

Fig. 3. RT-PCR analysis of HSP70 (panel *A*) and β-actin (panel *B*) genes expression in Monomac-6 cell cultures incubated with live *Helicobacter pylori* bacteria. Line 1 - control Monomac-6 cells (cultures without *H. pylori*); Monomac-6 cells incubated with 1×10^9 *H. pylori* for 3 hours (line 2), 6 hours (line 3), 24 hours (line 4) and 48 hours (line 5). MM - molecular marker.

cytoplasmic amount of HSF1 was recovered (*Fig. 1A* line 3) or even exceeded the initial level at 48 hour of culture comparing to the control (*Fig. 1A* line 4). In the nucleus, *H. pylori* induced increase of HSF1 abundance has been gradually declining to the level observed in not stimulated with bacteria control (*Fig. 1B* lines 3 and 4). However, the maximum of HSF1 nuclear

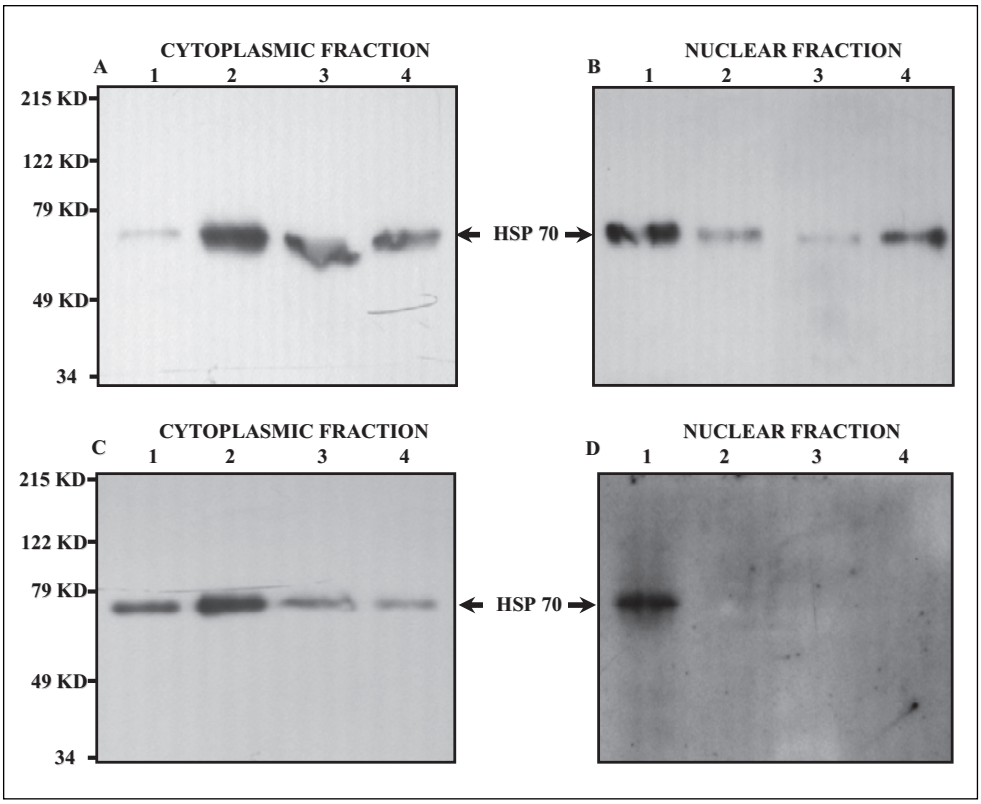

Fig. 4. Immunoblotting analysis of HSP70 in cytoplasmic (panel *A*) and nuclear (panel *B*) protein isolates from Monomac-6 cell cultures incubated with live *Helicobacter pylori* bacteria. Panel *C* represents the results of immunoblotting analysis of HSP70 in cytoplasmic and nuclear (panel *D*) protein isolates from Monomac-6 cell treated with cycloheximide (10 μg/ml) for 24 hours prior the inoculation of live *Helicobacter pylori* bacteria Line 1 - control Monomac-6 cells (cultures without *H. pylori*); Monomac-6 cells incubated with 1×10^8 *H. pylori* for 6 hours (line 2), 24 hours (line 3) and 48 hours (line 4).

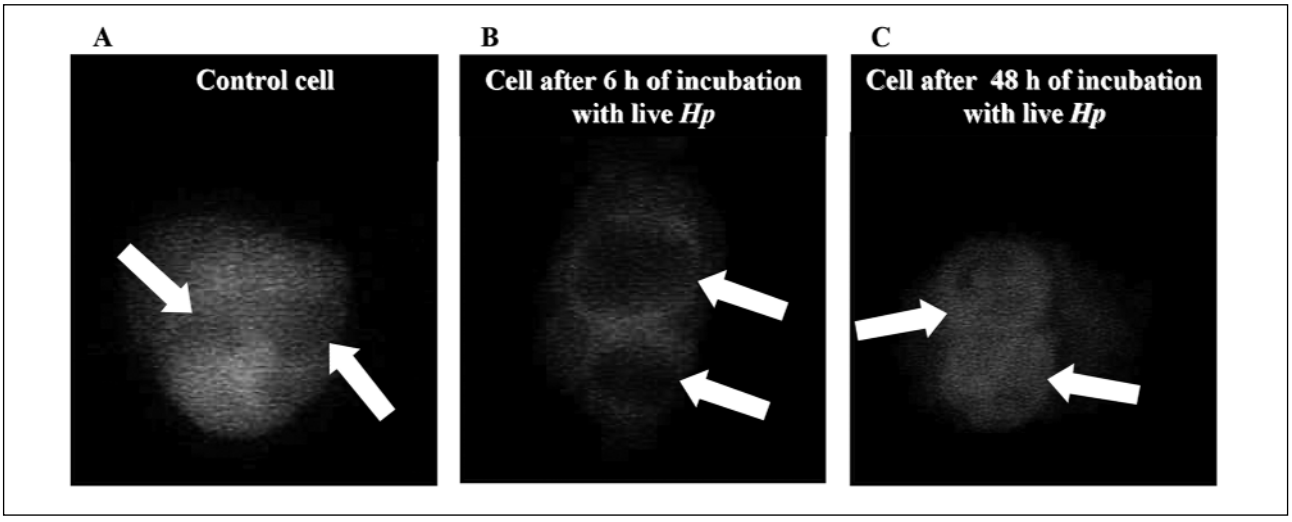

Fig. 5. Fluorescence microscopy analysis of the dynamics of HSP70 translocation in Monomac-6 cells exposed to live *H. pylori*. After 6 hours of incubation with *H. pylori* the displacement of nuclear HSP70 can be clearly seen (*B*). Recovery of nuclear HSP70 abundance was observed at 48 hours after the initial contact with live bacteria (Axioscop microscope, Plan Neo Fluor 100×, camera ZUS-47DE, Zeiss GmbH, Germany).

translocation have been observed in sixth hour of infection but formation of HSF-1-DNA specific complex became the most pronounced at 24 and 48 hours of experiment, as confirmed using EMSA (*Fig. 2A* lines 4 and 5). That delay in the acquiring of transcription competence of HSF1 might have been provoked due to the required activating phosphorylation on serine 230, which is indispensable for its full DNA binding property. Using specific antibody, we have been able to reveal the time course of the HSF1 activating phosphorylation on serine 230, showing the highest quantity of phosphorylated forms of that protein detected in the nucleus at 24 hour of culture in the presence of bacteria (*Fig. 2B* line 4), what is consistent with the results of EMSA analysis described above. However, even at six hours after administration of *H. pylori* to Monomac-6 culture some traces of phosphorylated forms of HSF1 may be found in the nucleus (*Fig. 2B* line 2).

Analysis of HSP70 expression in Monomac-6 cells in the presence of live Helicobacter pylori bacteria

As a consequence of the contact with live *H. pylori*, Monomac-6 cells have responded with activation of heat shock

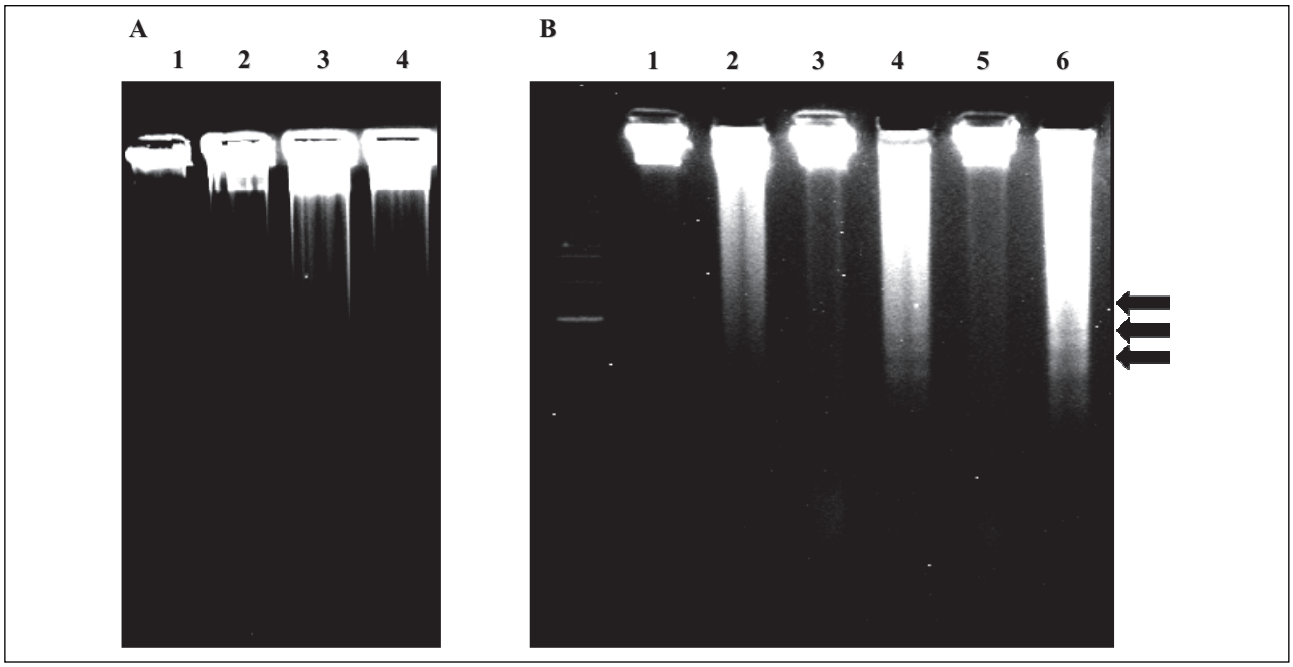

Fig. 6. DNA fragmentation ladder assay. Panel *A* represents analysis of samples isolated from the uninfected control cells cultures (line 1) or cultures incubated with live *H. pylori* for 12 hours (line 2), 24 hours (line 3) and 48 hours (line 4). No signs of DNA fragmentation were noted in the control cells (not treated with bacteria) as well as in cell cultures incubated with bacteria for 12, 24 or 48 hours. Panel *B* represents analysis of DNA integrity in the cultures of Monomac-6 cells treated with 3 μM of topoisomerase I inhibitor (S)-(+)-camptothecin for 12 (line 2), 24 (line 4) and 48 (line 6) hours. DNA fragmentation ladder assay exhibited massive fragmentation with a typical apoptotic pattern of intranucleosomal DNA cleavage at almost all experimental time points when compared to the untreated controls collected at respective time points (lines 1, 3, 5).

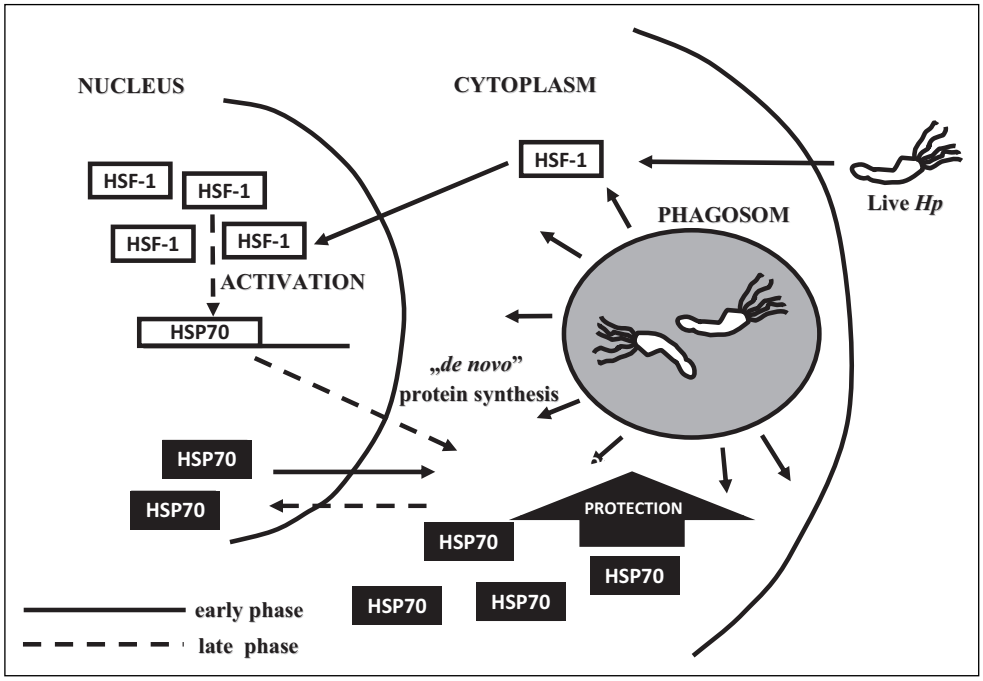

Fig. 7. Diagram presenting postulated mechanism of heat shock system activation in monocytes in response to the contact with live *H. pylori* bacteria. Solid lines represent the steps of early phase response, dashed lines represent the steps of late phase response.

system. Described above HSF1 trafficking and its DNA binding led to the increased expression of HSP70 gene, confirmed in RT-PCR reaction. Introduction of live *H. pylori* to the Monomac-6 cell culture resulted in gradual increase and accumulation of HSP70 transcript with noticeable maximum at 48 hours (*Fig. 3A* line 5) comparing to the uninfected with *H. pylori* control (*Fig. 3A* line 1). Unfortunately, immunoblotting analysis of cellular and nuclear proteins revealed other than expected HSP70 protein distribution. In uninfected control cell cultures the HSP70 protein in cytoplasmic fraction was barely detectable (*Fig. 4A*, line 1), while analysis of the nuclear fraction revealed the high level of its accumulation (*Fig. 4B*, line 1). At sixth hour of incubation with live *H. pylori*, abundance of cytoplasmic HSP70 noticeably increased and remained unchanged at later

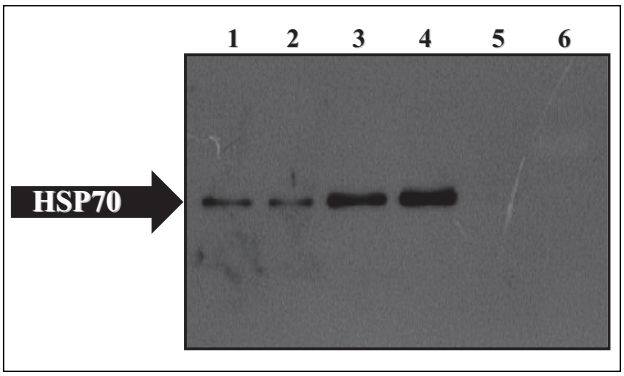

Fig. 8. Immunoblotting analysis of the pure protein isolates from heat treated *Helicobacter pylori* bacteria and KATO III cells to check specificity and cross-reactivity of with C92F3A-5 antibody with bacterial HSP70. Line 1 - control (not subjected to the heat shock) KATO III, line 2 - control (not subjected to the heat shock) KATO III, line 3 - 15 min heat shocked KATO III, line 4 - 30 min heat shocked KATO III, line 5 - control (not subjected to the heat shock) *H. pylori*, line 6 - 15 min heat shocked *H. pylori*.

experimental time points (*Fig. 4A*, lines 2-4). At the same time amount of HSP70 in the nuclear pool gradually declined till 24 hours from administration of live *H. pylori* to the culture (*Fig. 4B*, lines 2 and 3). This unexpected result, could not be explained by *de novo* synthesis of HSP70 and was not supported with RT-PCR (*Fig. 3*) or HSF-1 DNA binding analysis (*Fig. 2*). Immunofluorescence study also confirmed HSP70 depletion of cells nuclei at 6 hours (*Fig. 5B*) and its recurrence at 48 hour of culture (*Fig. 5C*). The only logical explanation of the observed phenomenon is the translocation of nuclear resident HSP70 to the cytoplasm at the early stage of infection with *H. pylori*. To confirm that hypothesis cycloheximide has been employed to block translation and in consequence *de novo* protein synthesis after the infection. Monomac-6 cell cultures were incubated for 24 hours with 10 μg/ml of cycloheximide prior to the application of live bacteria. At six hour after infection accumulation of HSP70 in cytoplasmic pool of cell cultures incubated with cycloheximide was identical with that observed before (*Fig. 4C*, lines 1 and 2). Also in this cell cultures depletion of HSP70 in nuclear pool (*Fig. 4D*, lines 1 and 2) comparing to the uninfected control has been noticed as previously described in cycloheximide free cultures. However, at the later time points, abundance of HSP70 in cytoplasmic fraction was gradually decaying reaching noticeably lower level after 48 hours than in the control (*Fig. 4C*, lines 3 and 4). Nuclear fraction remained deprived of HSP70 till the end of incubation with *H. pylori* at 48 hours (*Fig. 4D*, lines 2, 3, 4). Presented results suggest that in the early phase of infection of Monomac-6 cultures (6 hours from administration of bacteria) heat shock system engage the whole accessible pool of HSP70 in the cell (even these residing in nucleus) to maintain the equilibrium balanced by the infection and deleterious effects of bacterial products. Moreover, our results show that during the stress reaction nuclear fraction of HSP70 in Monomac-6 cell might be used in the cytoplasm but must be restored by the *de novo* protein synthesis only. In our opinion this reaction is necessary to delay the apoptotic cell death of phagocytes after contact and possible phagocytosis of *H. pylori* bacteria. Retardation of monocytes apoptosis might extent time required for bacteria annihilation inside the phagosome. To support that hypothesis DNA fragmentation ladder assay was performed in cell from cultures infected with live *H. pylori*. No fragmentation of DNA typical for ongoing apoptosis was detected at 12, 24 or 48 h after administration of live *H. pylori* bacteria to the culture (*Fig. 6A*, lines 2, 3, 4) comparing to the uninfected control (*Fig. 6A*, line 1). To confirm the susceptibility of Monomac-6 cells to induced apoptosis, cultures where exposed to 3 μM of topoisomerase I inhibitor (S)-(+)-camptothecin for 12, 24 and 48 hours. Analysis of DNA integrity exhibited massive fragmentation with a typical apoptotic pattern of intranucleosomal DNA cleavage at almost all experimental time points when compared to the untreated controls collected at respective time points (*Fig. 6B*).

DISSCUSION

Phagocytosis and elimination of pathogens invading into our body is the most important task of the immune system. For this purpose, monocytes and granulocytes are "armed" with the number of receptors and molecular mechanisms that enable them to engulf and kill the pathogens (30-32). In this duel the opponents, pathogenic microorganisms, are not "unarmed and defenseless". They are equipped with a several protective mechanisms eliciting the premature apoptotic or necrotic death of phagocytes (33-35). For many years *H. pylori* have been considered as a pathogen residing exclusively in the gastric mucosa with no direct contact with the immune system components. All observed pathological changes and mucosa lesions were thought to be attributed to the direct impact of cytotoxic agents produced by invading *H. pylori* or as the deleterious and damaging effect of the immune system response to the infection. It is now known that the action of phagocytic cells involves their direct interaction with the pathogen and its engulfment straight from the lumen of gastrointestinal tract (13, 14, 36).

H. pylori, like many other bacteria, utilizes multiple defense mechanisms to inhibit or postpone phagocytosis. Based on our previous results which might be concluded with the statement that regardless of the type of bacterial strain, CagA, VacA positive or negative we are dealing with, contact of eukaryotic cells with live *Helicobacter pylori* bacteria results always in down-regulation of HSP70 expression, we decided to conduct our experiments using only CagA, VacA positive strain of *H. pylori*. This kind of interaction were studied as an exemplar of infection - the most representative and exerting the most spectacular effect on the eukaryotic cell physiology.

Employing type IV secretion system *H. pylori* actively introduces several bacterial proteins encoded in Cag pathogenicity island (PAI) into the interior of phagocyte (37, 38). Cag A protein is immediately phosphorylated on tyrosine residues and then - unlike in case of epithelial cells - undergoes proteolytic cleavage into fragments of 100, 45 and 35 kDa. This kind of "treatment" of Cag A triggers its phosphatase-like activity leading to the dephosphorylation of many eucariotic proteins, what results in the commotion of phagocytosis, due to cell signaling pathways disturbances (39, 40). Similar effects were observed during phagocytosis of *Yershinia enterocolitica*. Bacterial protein YopH, a specific tyrosine phosphatase introduced into the phagocytes, evoked dephosphorylation of a series of signaling proteins in the phagocyte causing inhibition of phagocytosis (41).

Another protective mechanism used by *H. pylori* is the influencing of phagosome formation. Immediately after phagocytosis, *H. pylori* modify the behavior of a small phagosomes, leading to their merger, resulting in the formation of megaphagosomes, where *H. pylori* bacteria form larger clumps which allow them to survive inside up to 20 hours (42-44). In response to infection or more particularly, to the specific cellular and bacterial factors that occurs during contact of epithelial cells with bacteria, granulocytes and monocytes start to produce oxygen and nitrogen radicals, which oxidize bacterial

membrane destabilizing it what in consequence causes pathogen death (45-47). However, *H. pylori* actively counteracts to the deleterious effect of these reactive, oxidizing molecules, releasing free-radicals scavenging enzymes - catalase, superoxide dismutase and arginase, an enzyme that competes with eukaryotic nitric oxide synthase for substrate indispensable for NO synthesis. This kind of action effectively "shields" bacteria from phagocyte "chemical weapons" (48-51).

All described above mechanisms used by bacteria inhibit phagocytosis and promote apoptotic death of phagocytes. Therefore, preventing the premature death of immune system cells is an effective method of combating bacterial infection by leading to the prolongation of phagocyte contact with the pathogen. This task is accomplished by heat shock system. Guzik *et al.* showed that the elevated expression of HSP70 in monocytes prevents its apoptosis induced by *Staphylococcus aureus*, not affecting their phagocytic ability (52).

In Monomac-6 cells exposed to live *H. pylori* we have failed to demonstrate any signs of DNA fragmentation characteristic for ongoing apoptosis (*Fig. 6A*). But the expression of HSP70 mRNA was increased noticeably in this cells (*Fig. 3*). Moreover, the amount of available cytoplasmic HSP70 protein was also augmented 6 hours after introduction of bacteria to the cell culture (*Fig. 4A*). The results of our experiments indicate that at the initial stage of infection of Monomac-6 cells, the translocation of HSP70 from nucleus to the cytoplasm occurs, in order to provide maximum protection of the cytoplasmic proteins (*Fig. 4A* and *4B* lines 1 and 2).

This phenomenon observed in Monomac-6 cells might be an example of the eukaryotic cell adaptation to a bacterial originated stressors at the early phase of response. Ohgitani *et al.* has described identical "behavior" of HSP70 protein in cytomegalovirus-infected human fibroblasts. In these cells, HSP70 displacement between nuclear and cytoplasmic pool exhibited a biphasic, time dependent characteristics (53). Such dynamics of heat shock proteins translocation between the nucleus and the cytoplasm under stress conditions has been reported many times. However, the most frequent observation of HSP70 migration was reported from the cytoplasm to the nucleus of cell, particularly in response to heat or oxidative shock (54, 55). Nuclear matrix proteins are particularly susceptible to the damaging action of free radicals or high temperature. Maintaining of their proper state seems to be essential for sustaining basic functions of the nucleus (DNA replication, transcription and processing of hnRNA, DNA repair, etc.). Therefore, nuclear accumulation of HSP70 under thermal or oxidative stress should not be surprising. Moreover, HSP70 and HSP90 complex is involved in the introduction of steroid hormone receptors to the nucleus, what can have an effect on the observed nuclear accumulation of these proteins (56, 57). In case of phagocytic cells, bacterial pathogens and their cytotoxic products are the major source of cellular stress. Intra-cytoplasmic phagosome encloses engulfed but still live bacteria which pose a "serious threat" to the stability of cytoplasmic proteins. Therefore, displacement of chaperones - HSP70 - from the nucleus to the cytoplasm might become a priority for phagocyte. In the course of infection, pool of heat shock proteins is gradually reconstituted by *de novo* synthesis and this occurrence must be delayed at least a few hours in relation to previously described translocation.

Experiments in which synthesis of new proteins had been blocked with cycloheximide demonstrated that in Monomac-6 cell cultures infected with *H. pylori*, nuclear pool of HSP70 was reconstituted exclusively with the newly synthesized HSP70 protein 48 hours after the initial contact with bacteria (*Fig. 4B* and *D*). Cellular reaction for the stress stimuli have to be immediate but the synthesis of the new heat shock protein molecules takes time and requires its gene activation. This reaction is a multistage and time consuming process. Therefore, the majority of cells during the initial phase of a protective reaction "uses inventory" of the heat shock proteins, running at the same time their synthesis. Monomac-6 cells presented a rapid nuclear translocation of HSF-1 in response to the emerging *H. pylori* bacteria. At 6 hour of the infection, significant nuclear accumulation of HSF-1 was observed (*Fig. 1B* line 2). In contrast, binding to a regulatory element in the promoter of the HSP 70 gene was observed at 24 hours of infection (*Fig. 2A*, lines 4 and 5). The results obtained by Kowalczyk *et al.* in monocyte cultures infected with vaccinia virus, are in line with the dynamics of HSP genes activation process observed in Monomac-6. DNA binding of HSF-1 observed in peripheral blood monocyte cultures infected with the virus reached its maximum at 24 hours after infection, what is consistent with the results obtained in the experiments carried out on Monomac-6 cells infected with *H. pylori* (*Fig. 2A*, lines 4 and 5) (58).

Analysis of the results presented above entitle us to propose a model of the heat shock response in monocytic cells after infection with *H. pylori* bacteria. According to our data the heat shock reaction in Monomac-6 cells to *H. pylori* infection occurs in two stages: I - early and II - late. The early phase includes reactions occurring within a few hours after infection and is characterized by nuclear translocation of HSF-1 and HSP70 movement from the nucleus into the cytoplasm. Late phase addresses the phenomena occurring during tens of hours after infection and consist of HSF-1 binding to DNA, activation of heat shock protein genes as well as *de novo* synthesis of HSP70 and supplementation the nuclear pool of the protein (*Fig. 7*).

We hope that our paper has helped to shed some light on the increasingly reported by many researchers phenomenona of the effect of *H. pylori* or its components on biology of tissues other than only stomach epithelium. Recently published results of Krzysiek-Maczka *et al.* showing that *H. pylori* might be responsible for transdifferentiation of fibroblasts into myofibroblasts with markedly increased expression of the early carcinogenic marker HIF-1α, and inhibition of proapoptotic Bax expression, confirming that *H. pylori* should be analyzed as a pathogen capable of affecting the human body in a very comprehensive manner (59).

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