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3 **A microfluidic cell culture system for monitoring of sequential changes in endothelial cells**
4 **after heat stress**

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6 **Short title: Microfluidic device for monitoring heat-shock responses**

7 **Key words:** Microfluidic cell culture system, microchip, endothelial cells, heat-shock response,

8 PAI-1

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10

1 **Abstract**

2 Endothelial damage induced by a highly elevated body temperature is crucial in some diseases
3 including viral hemorrhagic fevers. Here, we report the heat-induced sequential changes of
4 endothelial cells under shear stress, which were determined with a microfluidic culture system.
5 Although live cell imaging showed only minor changes in the appearance of heat-treated cells,
6 *Hsp70* mRNA expression analysis demonstrated that the endothelial cells in channels of the system
7 responded well to heat treatment. F-actin staining also revealed clear changes in the bundles of actin
8 filaments after heat treatment. Well-organized bundles of actin filaments in control cells disappeared
9 in heat-treated cells cultured in the channel. Furthermore, the system enabled detection of sequential
10 changes in plasminogen activator inhibitor-1 (PAI-1) secretion from endothelial cells. PAI-1
11 concentration in the effluent solution was significantly elevated for the first 15 min after initiation of
12 heat treatment, and then decreased subsequently. This study provides fundamental information on
13 heat-induced endothelial changes under shear stress and introduces a potent tool for analyzing
14 endothelial secretions.

1 **Introduction**

2 A highly elevated body temperature can damage endothelial cells, leading to diffusion of serum
3 proteins and blood cells and secretion changes of coagulation and aggregation inhibitors. Endothelial
4 damage is thus implicated in causing symptoms such as increased vascular permeability, internal
5 bleeding, and coagulation disorders. In particular, these symptoms are induced by some viral
6 hemorrhagic fevers such as Ebola virus infection, Crimean-Congo hemorrhagic fever, and dengue
7 hemorrhagic fever [1] (for review, [2, 3]). The pathogenic mechanisms underlying the endothelial
8 damage associated with these viral hemorrhagic fevers are very diverse and complex, including both
9 direct and indirect dysfunction of endothelial cells [2]. The direct effects of elevated temperature and
10 viral infection on endothelial cells are particularly important, and have been reviewed recently [4, 5].
11 Other studies using animal models have suggested that endothelial cells are early targets of heat
12 stress injury [6, 7]. Although the importance of heat injuries on endothelial functions in these viral
13 hemorrhagic fevers or other diseases that are accompanied by high fever has been long recognized,
14 only a few *in vivo* and *in vitro* studies have focused specifically on the effects of heat stress on
15 endothelial cells [8, 9, 10]. In addition, all previous *in vitro* reports were carried out using stationary
16 culture methods with standard culture flasks, wells, or transwell cell culture inserts. However, since
17 vascular endothelial cells *in vivo* are constantly subjected to shear stress associated with blood flow

[11], it is likely that their responses in conventional stationary cultures *in vitro* do not properly reflect their responses *in vivo*.

Therefore, in order to examine the effect of heat stress on endothelial cells cultured under shear stress, we constructed a microfluidic culture system for endothelial cells, and monitored their morphological and secretion changes during heat stress. This system enabled detection of the heat-induced sequential changes in cytoskeletal actin filaments and secretion of plasminogen activator inhibitor 1 (PAI-1).

Materials and methods

Cell culture in flasks

We used two endothelial cell lines: monkey RF/6A 135 cells (Cell Bank, RIKEN BioResource Center; Tsukuba, Japan) and human umbilical vein endothelial cells (HUVECs; Life Technologies). We used RF/6A 135 cells, which originate from monkey chorioretinal vessel endothelial cells, because overexpression of HSP70 is known to be easily induced in endothelial cells of chorioretinal vessels by increased temperature [12]. In addition, HUVECs were used to ensure the evaluation of monkey PAI-1 cells with the 'Human PAI-1 ELISA kit' employed in the present study. These cells were cultured in 75-cm² tissue culture flasks (Techno Plastic Products AG; Switzerland) at 37.5°C in a humidified 5% CO₂ atmosphere in RPMI 1640 medium (MP Biomedicals, Japan) supplemented

1 with 10% fetal bovine serum (FBS; Biological Industries; Kibbutz Beit Haemek, Israel), 50 IU/ml
2 penicillin (Meiji Seika Pharma Co., Ltd.; Japan), and 25 µg/ml streptomycin (Meiji Seika Pharma
3 Co., Ltd.; Japan) for RF/6A 135 cells, and in MCDB 107 endothelial basal medium (COSMO BIO
4 Co., Ltd.) supplemented with 10% FBS, 50 µg/ml endothelial cell growth supplement (Takara Bio,
5 Japan), 100 µg/ml heparin (heparin sodium, Wako Pure Chemical Industries, Ltd.; Japan), 50 IU/ml
6 penicillin, and 25 µg/ml streptomycin for HUVECs.

7 To analyze the expression of *Hsp70* mRNA and PAI-1 secretion from cells cultured in
8 flasks, RF/6A 135 cells were seeded into 12 culture flasks, six for controls (three for *Hsp70* mRNA
9 analysis and three for PAI-1 analysis) and the other six for the heat-treated groups. After the cells
10 were cultured at 37.5°C overnight, they were incubated for 1 h at 42.5°C (heat-treated group) or at
11 37.5°C (control group). Thereafter, mRNA or the media were collected and analyzed as described
12 below.

13

14 Cell culture in the channels of the microfluidic culture system

15 In this study, we used a microchip (Fig. 1A, Sumitomo Bakelite Co., Ltd.) with a parallel flow
16 channel made of polystyrene (PS), as preliminary studies determined that this was the optimal chip
17 material for culture of RF/6A 135 endothelial cells and HUVECs. A channel for fluidic cell culture
18 was formed in the interior of the base PS plate. The base and cover PS plates, both of which were

1 1-mm thick, were thermally laminated. Each microchip had 2 channels, which were 6-cm long,
2 300- μ m wide, and 100- μ m deep (Fig. 1B). Before the cells were introduced into the channel, the
3 microchip was sterilized with 70% ethyl alcohol and rinsed with sterilized distilled water. Then, each
4 channel was pre-coated with collagen type IV solution (Nitta Gelatin Inc.; Japan) as follows. First,
5 the channel was washed with 0.1 M NaOH, 70% ethanol, and sterilized distilled water in succession.
6 Next, the inner surface of each channel was coated by filling it with collagen type IV solution for 2 h.
7 The channels were then washed with sterilized distilled water five times and the chip platform was
8 connected with sterilized tubes, valves, and syringes (Fig. 1C).

9 After filling the channels with culture medium, the inlet was closed. The microchip was
10 then placed on the inserted heating plate (ITO temperature controller) set on the microscope stage
11 (Nikon; Tokyo, Japan) (Fig. 1D). The temperature of the heating plate was set to 37.5°C.

12 Subconfluent endothelial cells were dissociated with papain (Worthington Biochemical Corporation;
13 Lakewood, NJ, USA) and dispersed in the medium to prepare the cell suspension at a concentration
14 of 2×10^7 cells/ml. We then introduced 35 μ l of the cell suspension into the channel via the inlet.
15 When the cells passed through the channel, the same cell suspension was also inserted into another
16 channel. After seeding the cells, the inlet was closed and the microchip was immobilized for 2 h at
17 37.5°C to allow the cells to attach to the bottom of the channel. After 2 h, the valve was turned to the
18 medium port and perfusion was started at 0.2 μ l/min at 37.5°C. Then, after 1h perfusion, the

1 perfusion flow rate was gradually increased to 2μl/min.

2 To monitor cellular changes, we raised the temperature of the heating plate from 37.5°C to
3 42.5°C 1 h after the start of medium perfusion at 2μl/min, and the temperature was maintained for 1
4 h or more. Live-cell images of RF/6A 135 cells were captured before and during heat treatment
5 using phase-contrast microscopy (Axiovert 40C, Zeiss; Tokyo, Japan). The effluent solution was
6 collected to measure the concentration of PAI-1 secreted from RF/6A 135 cells and HUVECs before
7 and during heat treatment. At the end of the heat treatment, either RNA was extracted from the
8 RF/6A 135 cells in the channel, or the cells were fixed for immunocytochemistry. In the latter case,
9 the cells in the channel were fixed for 1 h with 4% paraformaldehyde in 0.1 M phosphate buffer (pH
10 7.3).

11

12 Real-time polymerase chain reaction (PCR) analysis for Hsp70 mRNA

13 Total RNA was extracted from cultured cells with RNAiso Plus (Takara Bio, Japan) and then
14 converted to cDNA using a reverse transcription kit (QIAGEN; Valencia, CA). The synthesized
15 cDNA was used as a template in subsequent PCR analysis. Quantitative real-time PCR analysis was
16 performed using a Thermal Cycler Dice Real Time System TP800 (Takara Bio) according to the
17 manufacturer's suggested protocol. Gene expression levels were normalized to ribosomal protein
18 L32 (*Rpl32*) mRNA expression, which was measured simultaneously. Primers used for gene

1 amplification were as follows: *Hsp70* FW primer: 5'-CGACCTGAACAAGAGCATCA-3', *Hsp70*
2 RV primer: 5'-AAGATCTGCGTCTGCTTGGT-3'; *Rpl32* FW primer:
3 5'-GCCCAAGATCGTCAAAAAGA-3', *Rpl32* RV primer: 5'-GTTGCACATCAGCAGCACTT-3',
4 *PAI-1* Fw primer: 5'-CGAAGTTGACCTCAGGAAGC-3', *PAI-1* Rev primer:
5 5'-TGACAGCTGGTGGATGAGGAG-3', *Bax* FW primer:
6 5'-AACTGGACAGTAACATGGAGCTG-3', *Bax* RV primer:
7 5'-CTGGCAAAGTAGAAAAGGGCGA-3', *Bcl-2* FW primer:
8 5'-TTCTTTGAGTTCGGTGGGGTC-3', *Bcl-2* RV primer:
9 5'-AGCCAGGAGAAATCAAACAGAGG-3'. Amplifications were carried out in a 20-µl volume,
10 with each reaction containing 1 µl of cDNA, 200 nM of each primer pair, and 1× SYBR Premix
11 Extaq (Takara Bio). The reaction mixture was subjected to 40 cycles of amplification, followed by
12 post-PCR fluorescence dissociation curve analysis to confirm the specificity of the primer sets.

13

14 *F-actin staining*

15 After fixation, cytoskeletal F-actin staining was performed with CytoPainter F-actin Staining kit
16 (Abcam; Cambridge, UK). First, cells cultured in the channels were incubated with 10 mM PBS
17 containing 0.1% Triton X-100 (Sigma-Aldrich) at room temperature for 5 min. After rinsing, cells
18 were treated with fluorescent phalloidin conjugate diluted with labeling buffer at room temperature

1 for 30 min. The cells were then washed with PBS and observed under a fluorescence microscope
2 (LSM5 Pascal, Carl Zeiss; Oberkochen, Germany).
3
4 Enzyme-linked immunosorbent assay (ELISA) for PAI-1
5 The cell culture effluent solution from each channel (n = 4 for RF/6A 135 cells and n = 3 for
6 HUVECs) was collected on ice 15 min before the heat treatment at 42.5°C and at 15-min intervals
7 during the heat treatment (0–15, 15–30, 30–45, 45–60, and 60–75 min after the start of heat
8 treatment). Then, the effluent solutions were centrifuged for 10 min at 3000 ×g at 4°C to remove
9 debris. Supernatants were subjected to a PAI-1 assay using Human PAI-1 ELISA Kit (ASSAYPRO;
10 St. Charles, MO, USA). The estimation of PAI-1 concentration was performed according to the
11 manufacturer's protocol. In brief, 20 µl of samples and 30 µl of the dilute mix included in the kit, or
12 50 µl of diluted human PAI-1 standard included in the kit were added into each well of the ELISA
13 plate. Then, the wells were covered with a sealing tape and incubated for 2 h. After each well was
14 completely washed five times with 200 µl of wash buffer, 50 µl of biotinylated human PAI-1
15 antibody was added to each well and incubated for 1 h. After washing, 50 µl of
16 streptavidin-peroxidase conjugate was added and incubated for 30 min. Following an additional
17 wash, 50 µl of chromogen substrate was added and samples were incubated until a blue color
18 developed. Then, 50 µl of stop solution was added into each well and the absorbance was

1 immediately read at a wavelength of 450 nm (microplate reader MTP-800, CORONA ELECTRIC
2 Co., Ltd., Japan). The mean value of the duplicate readings for each standard and sample was
3 calculated. Sample concentrations of PAI-1 were determined from the standard curve.

5 Statistical analysis

6 For statistical analysis, *p*-values were determined using the Student's *t*-test or the Student's *t*-test for
7 paired samples. *p*-values of less than 0.05 were considered significant.

9 **Results**

11 The heat-shock response of endothelial cells was first examined by comparing *Hsp70*
12 mRNA expression levels between heat-treated and control RF/6A 135 endothelial cells. As shown in
13 Fig. 2, the expression of *Hsp70* mRNA was significantly increased in heat-treated cells cultured in
14 flasks, compared to that of control cells ($p < 0.01$, Student's *t*-test; left columns in Fig. 2). The
15 expression of *Hsp70* mRNA was also significantly increased in the heat-treated cells cultured in
16 channels of the microfluidic culture system, compared to that of control cells ($p < 0.01$, Student's
17 *t*-test; right columns in Fig. 2). These results demonstrated that the microfluidic culture system is
18 capable of detecting cellular heat-shock responses.

1 Live images of control and heat-treated RF/6A 135 cells in the channel were captured 1 h
2 before the heat stress and every hour for 3 h during the heat-stress treatment. Heat treatment scarcely
3 caused morphological changes for 3 h, as shown in pictures 6–8 in the right column of Fig. 3,
4 compared to control cells (pictures 2–4 in Fig. 3) or cells before the heat stress (pictures 1 and 5 in
5 Fig. 3). However, cytoskeletal changes were already evident at 1 h after initiation of heat stress,
6 based on positive F-actin staining in RF/6A 135 cells cultured in channels (Fig. 4). In controls, actin
7 filaments were well organized into intracellular bundles, though some actin filaments were abundant
8 beneath the plasma membrane (Fig. 4A and C). On the contrary, the intracellular filamentous
9 organization of actin bundles almost disappeared in heat-treated cells, whereas strong actin staining
10 was found beneath the plasma membrane, especially at the junction between adjacent cells (arrows
11 in Fig. 4B and D). These observations clearly demonstrated heat-induced cellular cytoskeletal
12 changes.

13 We then studied the expression of *PAI-1* mRNA in control and heat-treated cells (Fig. 5).
14 The expression of *PAI-1* mRNA did not increase after exposure to higher temperature. Next, we
15 examined sequential secretion changes of PAI-1 from heat-treated RF/6A 135 endothelial cells, since
16 severe heat stroke is known to cause disseminated intravascular coagulation (DIC), which is
17 reportedly associated with significantly elevated plasma PAI-1 levels *in vivo* [13]. However, the
18 concentration of PAI-1 in the cell medium from conventional culture flasks 1 h after heat stress

1 tended to be decreased, compared to that from control flasks (Fig. 6, $p = 0.077$, control vs
2 heat-stressed group). On the contrary, heat-stress rapidly induced sequential changes of PAI-1
3 concentration in the effluent solution from RF/6A 135 cells cultured in the channel, measured at
4 15-min intervals (Fig. 7). The relative concentrations of PAI-1 secreted from endothelial cells were
5 significantly increased within the first 15 min and at 15-30 min after the start of heat stress compared
6 to the concentration before the heat stress ($p < 0.01$), and then gradually decreased, followed by a
7 significant decrease at 45–60 and 60–75 min after the stress ($p < 0.05$ and $p < 0.01$, respectively).

8 The PAI-1 ELISA kit used in the present study is suitable for analysis of human samples.
9 To examine whether the kit is usable to evaluate PAI-1 secreted from monkey cells, we examined
10 PAI-1 not only in monkey samples but also in human HUVEC samples under the same experimental
11 conditions (Fig. 8). Almost similar to the results obtained for RF/6A 135 cells (Fig. 7), the
12 concentration of PAI-1 in the effluent of HUVECs was significantly increased for the first 15 min
13 after the start of heat stress ($p < 0.01$). Then, it was gradually decreased and became significantly
14 reduced at 60–75 min compared to the concentration before heat stress ($p = 0.081$, before heat-stress
15 vs after heat-stress at 45-60 min; $p < 0.05$, before heat-stress vs after heat-stress at 60-75 min). These
16 studies clearly demonstrated that the heat stress induced a transient increase in PAI-1 secretion from
17 both monkey and human endothelial cells, followed by its suppression.

18

1 **Discussion**

2

3 Here, we showed that heat stress induced rapid changes in endothelial cells cultured under shear
4 stress conditions by using a microfluidic culture system. We confirmed a significant increase in
5 *Hsp70* mRNA expression 1 h after the initiation of heat stress. Furthermore, F-actin staining
6 demonstrated clear cytoskeletal changes in endothelial cells cultured in the channels, even within 1 h
7 after initiation of the heat treatment. We were also able to detect rapid heat-induced changes in PAI-1
8 secretion from endothelial cells. This is the first study to demonstrate stress-induced sequential
9 changes of PAI-1 secreted from cultured endothelial cells.

10 The wall shear stress under our present experimental condition was calculated as $\tau =$
11 $6\mu Q/\omega d^2$, where τ is the shear stress in dyn cm^{-2} , μ the fluid viscosity in dyn s cm^{-2} or Q the
12 volumetric flow rate in $\text{cm}^3 \text{s}^{-1}$ and ω and d are the channel width and depth in cm, respectively [14,
13 15]. When the volumetric flow rate was 2 $\mu\text{L}/\text{min}$ and the fluidic viscosity was 0.0145 dyn s cm^{-2}
14 [15], the shear stress was 0.7 dyn/cm^2 , which was lower than venous levels of shear stress (1
15 dyne/cm^2). Although we examined PAI-1 secretion under low shear stress in the present study, we
16 are currently studying it under different shear stresses.

17 It is well known that exposure to high temperature induces the expression of *Hsp70* mRNA
18 in various kinds of cells, including endothelial cells. For example, rapid increases in *Hsp70* mRNA

1 were observed when Jornot et al. [16] exposed HUVECs to 43–45°C, Fukao et al. [17] exposed
2 HUVECs to 43°C, and Nakabe et al. [18] exposed human arterial endothelial cells at 42°C. We first
3 confirmed that this indicator of a heat-shock response was well induced in endothelial cells cultured
4 in the microfluidic cell culture system, based on the initial increase in *Hsp70* mRNA expression (Fig.
5 2).

6 We then showed that heat stress at 42.5°C for 1 h disrupted the intracellular bundle
7 organization of actin filaments in endothelial cells cultured in the microfluidic cell culture system
8 (Fig. 4). Such heat-induced disruption of actin organization is consistent with previous observations
9 obtained from endothelial cells cultured in flasks [19, 20]. Romanov et al. [20] found that
10 short-lasting hyperthermia (1–4 h) triggered the disappearance of cytoplasmic actin filaments and
11 their redistribution to areas of cell-to-cell contact. DeMeester et al. [19] found that inflammatory
12 and/or heat shock treatments induced a dramatic cytoskeletal collapse in endothelial cells, suggesting
13 that cytoskeletal rearrangement is likely a critical event in the pathway to apoptosis. In order to
14 reveal the relationship between cytoskeletal collapse and apoptosis, we additionally examined
15 expression of *Bax* and *Bcl-2* mRNAs as indicators of pro- and anti-apoptotic gene expression,
16 respectively. After a 1-h heat treatment, although the expression of *Bax* mRNA was not changed
17 [*Bax* mRNA/*Rpl32* mRNA = 0.0955 ± 0.00522 (mean $\pm$ SD) in control cells (n = 3) and $0.0958 \pm$
18 0.0156 in heat-treated cells (n = 3)], the *Bcl-2* mRNA expression was significantly increased [*Bcl-2*

1 mRNA/*Rpl32* mRNA = 0.0964 ± 0.00615 in control cells (n = 3) and 0.122 ± 0.00607 in heat-treated
2 cells (n = 3; p < 0.001; Control *vs* Heat-treated)]. Thus, the heat treatment used in the present study
3 induced not a pro-apoptotic but an anti-apoptotic response. Combined with our data on the
4 cytoskeletal disruption and increase in *Bcl-2* mRNA, our results suggest that cytoskeletal
5 rearrangement induced by heat treatment is not directly related to the pathway to apoptosis.

6 One of the major advantages of a microfluidic culture system is the capability of
7 monitoring the sequential secretion of certain chemicals in the effluent solution. Our system enabled
8 monitoring of stress-induced sequential changes in PAI-1 concentration in the effluent for the first
9 time. Under physiological conditions *in vivo*, PAI-1 is considered to be released into the circulation
10 and the extracellular space by only a few types of cells, including liver cells, smooth muscle cells,
11 adipose cells, and platelets [21]. Severe sepsis, as well as other acute or chronic inflammatory
12 diseases, causes high PAI-1 plasma levels. Under these pathological conditions, several tissues,
13 including endothelial cells, are known to secrete quite large amounts of PAI-1 [21]. PAI-1 release
14 from endothelial cells is up-regulated by inflammatory cytokines and signal transduction pathways,
15 including the nuclear factor-kappaB and mitogen-activated protein kinase pathways (for review, see
16 [22]). Simultaneously, endothelial cells alter their secretory function in response to the shear stress
17 generated by flowing blood [23]. Therefore, in order to elucidate the precise regulatory mechanism
18 of PAI-1 in endothelial cells, their PAI-1 secretion should be examined under conditions of shear

1 stress.

2 In the present study, since PAI-1 increased quickly (within 15 min) upon exposure to
3 higher temperature, it is unlikely that the increase was mediated by enhanced protein synthesis. To
4 confirm this hypothesis, we measured *PAI-1* mRNA levels and revealed that the mRNA did not
5 increase after exposure to higher temperature (Fig. 5). Thus, the increase in PAI-1 antigen level was
6 most likely the result of enhanced secretion. Other mechanisms, however, may also be involved,
7 such as shedding of either cell-surface PAI-1 or matrix-bound PAI-1, as was shown by Iacoviello et
8 al. [24]. There are many reports showing that PAI-1 secreted from vascular endothelial cells plays
9 important roles under a variety of physiological and pathological conditions such as thrombolytic
10 resistance [25], endothelial cell death [26], and smooth muscle cell migration [27]. Thus, it is
11 possible that the sudden increase in PAI-1 secretion after exposure to high temperature plays
12 important roles under certain pathological conditions. In the present study, we measured PAI-1
13 antigen levels but not its activity, because we perfused a simple culture medium that did not contain
14 vitronectin, which binds PAI-1 and stabilizes its activity [28, 29]. We would like to evaluate active
15 PAI-1 level in the medium with vitronectin by using the microchip culture system in the near future.

16 The present study is the first to report sequential change of PAI-1 secretion from
17 endothelial cells during heat and shear stress. Elevation of body temperature is a common feature of
18 inflammatory reactions, and is also considered to enhance the body's defense mechanism. However,

1 outbreaks of viral hemorrhagic fevers are emerging as an increasing threat to human health
2 worldwide. Moreover, dengue virus-infected patients have a high plasma concentration of PAI-1, and
3 survival was shown to be significantly worse in patients with higher PAI-1 concentrations [30].
4 Elevated PAI-1 concentration in children suffering from dengue hemorrhagic fever was also reported
5 [3, 31, 32]. In addition, a recent study on Ebola virus infection showed that PAI-1 levels were
6 elevated in pediatric patients, and were strongly elevated in patients who died and in those with
7 hemorrhagic manifestations [33]. Although the mechanism underlying the increase of PAI-1 in these
8 patients remained unclear, Shyu et al. [34] suggested that domain III of the envelope glycoprotein of
9 dengue virus serotypes 2 (EIII) induces *PAI-1* gene expression via the MEK/ERK signaling
10 pathways. Our present study provides basic information and a new tool for further elucidation of this
11 mechanism. In addition, our system is expected to become a very useful tool for investigating many
12 other responses of endothelial cells under shear stress, since vascular dysfunction plays an important
13 role in various major diseases such as atherosclerosis [35], cancer [36], and diabetes [37]. Therefore,
14 our novel microfluidic culture system will be helpful to study the responses of endothelial cells
15 under sheer stress to various biological, chemical, or physical cues.

16

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3

1

2 **Legends**

3

4 Fig. 1. A. Schematic illustration of the chip platform with two channels. B. Schematic diagram
5 showing the partial cross-section of the channel inside the chip platform. C. Schematic illustration of
6 the overall experimental setup of the microfluidic culture system. D. Image of the chip platform
7 placed on the inserted heating plate set on the microscope stage.

8

9 Fig. 2. Expression of *Hsp70* mRNA (mean $\pm$ SD) in control cells and heat-treated (42.5°C for 1 h)
10 cells cultured in flasks (left columns) or in the channel of the microfluidic culture system (right
11 columns). The expression of *Hsp70* mRNA was normalized to the expression of the housekeeping
12 gene, *Rpl32* mRNA and y-axis indicates the relative expression of *Hsp70* mRNA vs. *Rpl32* mRNA.
13 Significant differences are denoted with asterisks (* p < 0.05, Student's *t*-test). ND = not detected.

14

15 Fig. 3. Representative images of cells cultured in the channel at 37.5°C (control cells, left column)
16 and at 42.5°C (heat-treated cells, right column). Images of control cells were taken every hour for 4
17 hours (1–4). Images of heat-treated cells were taken before the heat stress (5) and every hour after
18 the start of the heat stress (6–8). Scale bars = 200 μ m.

1

2 Fig. 4. Representative images of phalloidin staining for F-actin in control cells (A and C) and
3 heat-treated cells (B and D) in the channel one hour after the start of the heat stress. Regions marked
4 in white boxes in A and B are magnified and shown in C and D, respectively. Scale bars = 50 μm (A
5 and B), 25 μm (C and D).

6

7 Fig. 5. Relative expression of PAI-1 mRNA from control and heat-treated (1 h) endothelial cells
8 (RF/6A 135 cells) cultured in channels of the microfluidic culture system.

9

10 Fig. 6. Relative concentration of PAI-I secreted from control and heat-treated endothelial cells
11 (RF/6A 135 cells) cultured in flasks. The concentration of PAI-1 was analyzed in the culture medium
12 of control cells (incubated for 1 h at 37.5°C) and heat-treated cells (incubated for 1 h at 42.5°C).

13

14 Fig. 7. Relative concentration of PAI-1 secreted from endothelial cells (RF/6A 135 cells) cultured in
15 channels of the microfluidic culture system before and during the heat stress. Significant differences
16 are denoted with asterisks, compared to the concentration before the heat stress [Values represent
17 means $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$, Student's t -test (for paired samples)].

18

1 Fig. 8. Relative concentration of PAI-1 secreted from endothelial cells (HUVECs) cultured in
2 channels of microfluidic culture system before and during the heat stress. Significant differences are
3 denoted with asterisks, compared to the concentration before the heat stress [Values represent means
4 $\pm$ SD. * $p < 0.05$, Student's t -test (for paired samples)].

5

Figure 1

A.

B.

D.

C.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8