Induction of Monocytic Tissue Factor Expression After Rewarming From Hypothermia In Vivo Is Counteracted by Heat Shock in c-Jun–Dependent Manner

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Objective—Triggering of tissue factor (TF)-mediated blood coagulation leads to the development of disseminated intravascular coagulation during rewarming from hypothermia. We studied post-rewarming TF levels, activity, and surface redistribution, along with the regulation of TF gene transcription in mononuclear cells (MNCs) obtained from an in vivo rat model.

Methods and Results—Rewarming after a 5-hour episode of 15°C hypothermia caused an increase in TF activity, protein levels, and externalization of TF antigen in rat MNCs. This was accompanied by a dramatic elevation of c-Jun and JNK phosphorylation, and the absence of EGR-1 and NF-κB activation. To search for a stimulus to counteract c-Jun–mediated induction of TF activity in MNCs from rewarmed rats, we applied heat shock pretreatment one day before the hypothermia/rewarming experiment. This restored post-rewarming TF activity, protein levels, and surface-to-total TF ratio in rat MNCs to normothermic levels. Furthermore, in heat shock-pretreated animals, rewarming failed to increase phosphorylated c-Jun and JNK levels. We attribute this to the profound overexpression of heat shock protein 70 and inhibition of JNK.

Conclusions—MNCs respond to rewarming from hypothermia by an induction of active TF antigen. This effect is dependent on c-Jun activation and is abolished by heat shock pretreatment. (Arterioscler Thromb Vasc Biol. 2006;26: 2401-2406.)

Key Words: heat shock proteins ■ hypothermia/rewarming ■ monocytes ■ rat ■ tissue factor

Decrease in body core temperature is associated with life threatening coagulation disturbances in humans.1,2 Humans sustain hypothermia in accidents or as a protective maneuver during certain types of surgery.2–4 Common to both is that body rewarming is a prerequisite for survival. The strategy of rewarming can lead to mobilization of large amounts of tissue factor (TF) from damaged tissues and, thus, can be responsible for the formation of fulminant disseminated intravascular coagulation (DIC).5,6

TF is an important glycoprotein that triggers FVIIa-mediated launching of the blood coagulation cascade. Among circulating blood cells, monocytes are the presumable source of TF.7 Characterization of monocytic TF expression during rewarming surprisingly has been overlooked. It is generally accepted that inducible TF gene transcription is mediated by NF-κB, EGR-1, and AP-1 transcription factors.8 Whereas NF-κB and EGR-1 are largely responsive to proinflammatory9 and growth10 stimulants, AP-1, which is a complex of two transcription factors c-Jun and c-Fos, is also activated by osmotic, oxidative and cold stress-es.11,12 In addition, activity of c-Jun was found to be associated with increased apoptosis.13

Several lines of evidence suggest that heat shock modulates both proinflammatory and stress-induced cell responses via induction of heat shock proteins (Hsp).14,15 The latter are molecular chaperons that protect cells by stabilization of protein folding, thus preventing aggregation.16 Overexpression of Hsp70, an inducible member of the heat shock protein family, prevents apoptosis via inhibition of signaling pathways responsible for activation of c-Jun.17

In our present study, we report an induction of TF activity, protein levels, and redistribution of TF antigen to the surface of mononuclear cells (MNCs) isolated from rats rewarmed from hypothermia. This increase was counteracted by a full body heat shock 24 hours before hypothermia via suppression of hypothermia-induced activation of c-Jun.

Materials and Methods

All experimental protocols were approved by the Norwegian Animal Research Authority (NARA) and conducted according to the Euro-
Rewarming From Hypothermia Induces Systemic Inflammatory Response

Plasma TNF-α concentrations were determined by using a PeliKine compact ELISA kit (R&D Systems Ltd., UK) according to manufacturer’s instructions. The minimal detectable TNF-α concentration was >5 pg/mL. The Light absorbance at 450 nm was measured on THERMOMax microplate reader (Molecular Devices Corp., Menlo Park, CA).

Western Blotting
Pellets of 10⁶ MNCs were sonicated on ice in 2X SDS buffer and centrifuged at 10 000g for 15 minutes at 4°C. Twenty µg of cell lysates were electrophoresed on 10% polyacrylamide gels and electrophoblotted onto nitrocellulose membranes (Amersham Biosciences, Inc). After blocking with 5% non-fat skimmed milk, membranes were sequentially probed with polyclonal antibodies, directed against Ser18-phosphorylated and total c-Jun, Thr202/Tyr208-phosphorylated and total c-Jun-N-terminal kinase (JNK), Ser73-phosphorylated p65, total IxB-α (all from Cell Signaling Technology, Inc), monoclonal antibodies against C terminus of human TF directed against Ser93-phosphorylated and total c-Jun, Thr202/Tyr208-phosphorylated and total c-Jun-N-terminal kinase (JNK), etc. Immunopositive bands were visualized by incubation of membranes with horseradish peroxidase–conjugated goat anti-mouse or goat anti-rabbit secondary antibody and a chemiluminescence-based detection system. For statistical analyses densitometrical data of immunopositive bands were analyzed using LumiAnalyst software (Boehringer Mannheim).

In-Cell Western Assay
We used In-Cell Western (ICW) assay to characterize surface and total mononuclear cell TF (MNC-TF) levels. This assay has been described in detail elsewhere. Isolated MNCs were briefly plated onto 8-well flat-bottomed chambers (8-well Laboratory-Tek chamber Slide Permanox, NUNC A/S), fixed in 4% paraformaldehyde, and permeabilized in an ice-cold methanol (if applicable). After blocking in PBS with 3% goat serum, the signal from mouse anti-TF monoclonal antibody was detected on 800 nm channel of Odyssey infrared imager (LI-COR Biosciences GmbH). Equal cell number was verified by immunostaining the permeabilized MNCs with rabbit polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which signal was detected on 700 nm channel. The minimal detectable TF concentration was 0.05 ng/mL. Statistical analyses densitometrical data of immunopositive bands were analyzed using LumiAnalyst software provided with the imager.

Blood Sampling and Experimental Design
At the end of the experiment venous blood was sampled from a right femoral vein catheter and distributed into sterile polystyrene tubes (Falcon, BD Biosciences Pharmingen) containing heparin (Sigma-Aldrich) at a final concentration of 10 U/mL.

Statistics
Values are presented as mean and SEM. Data were assessed by one-way analysis of variance followed by multiple comparisons if the F value was greater than critical. Differences between groups were analyzed by one-way ANOVA using Tukey post hoc test. A probability value below 0.05 was used as the measure of significance. For statistical analyses we used SPSS 11.0 (SPSS Inc).

Results
Rewarming From Hypothermia Induces Systemic Inflammatory Response
Plasma level of TNF-α is a general marker of systemic inflammation. Analyses of plasma TNF-α concentrations
revealed significantly elevated TNF-α levels from non-detectable in rats of NT group to 143±42 pg/mL in rats of HT/RW group (Figure 2A).

**TF Activity and Antigen Levels Are Elevated After Rewarming From Hypothermia**

Immunoblots of MNC lysates using mouse anti-TF monoclonal antibodies revealed one strong immunopositive band at 43 kDa. The representative immunoblot and densitometry data are shown in Figure 3A. Rewarming from hypothermia led to a 5-fold rise of TF protein levels in rat MNCs compared with the cells isolated from blood of normothermic rats. The intensity of the signal from immunopositive bands of β-actin displayed no differences between the groups.

The observation of elevated TF levels in MNCs after rewarming from hypothermia led us to carry out the assessment of intracellular and surface TF antigen levels in MNCs. For this purpose we chose an ICW assay of permeabilized and non-permeabilized cells. The relationship between intracellular and membrane located TF antigen in MNCs after rewarming is demonstrated in Figure 3B. Comparing intensities of total TF signal in MNCs from the HT/RW group with cells from the NT control group, we found significantly higher hypothermia/rewarming-induced accumulation of intracellular TF antigen compared with the normothermic controls (Figure 3B). At the same time, hypothermia/rewarming caused a 2-fold increase in surface MNC-TF signal intensity in the HT/RW group compared with the NT group. The ratio of surface-to-total TF antigen levels, which characterizes surface redistribution of TF antigen, was also higher after rewarming from hypothermia (Figure 3B, inset). The differences of MNC-TF levels in ICW assay could not be caused by variations in cell count, as equal amounts of cells were assessed and verified by immunostaining of permeabilized MNCs against GAPDH (Figure 3C).

To determine whether the increase in MNC-TF levels was functional, we assayed TF/FVIIa activity after rewarming from hypothermia. As illustrated in Figure 2B, MNC-TF activity and antigen levels are elevated after rewarming from hypothermia. Heat shock pretreatment attenuated post-rewarming induction of TF activity but had no effect on elevated TNF-α levels. The data are presented as mean and SEM; *P<0.05 as compared with NT group. ND indicates non-detectable; other acronyms are as in Figure 1.
activity was enhanced from $5.6 \pm 0.7$ mU/10$^6$/cells in NT group to $13.9 \pm 3.6$ mU/10$^6$/cells in HT/RW group ($P \leq 0.05$).

The Induction of TF Transcription After Rewarming From Hypothermia Was c-Jun Dependent

Because the induction of TF gene is regulated by inflammation- and stress-responsive pathways, we tested the activation of NF-κB, EGR-1, and AP-1. Surprisingly, we observed a stabilization of the regulatory IκB-α subunit in the HT/RW group as judged by a 6-fold increase in IκB-α levels compared with the NT group (Figure 4A). This indicates inhibition of the NF-κB transcription factor after rewarming from hypothermia. The lack of activation of this transcription factor was confirmed by the absence of increase in levels of Ser$^{276}$-phosphorylated p65 subunit of NF-κB in MNCs after rewarming (Figure 4B). We could not detect any significant increase in post-rewarming levels of EGR-1 protein in rat MNCs (Figure 4E), which rules out the involvement of this transcription factor in post-rewarming TF induction.

On the other hand, when we assayed the status of AP-1 activity by probing for phosphorylation status of c-Jun pathway members, we found a dramatic increase in levels of Ser$^{63}$-phosphorylated c-Jun (Figure 4C) and Thr$^{183}$/Tyr$^{185}$, phosphorylated c-Jun N-terminal kinase, a well-established activator of AP-1 complex of transcription factors. Therefore, the activation of TF gene transcription in rat MNCs after rewarming from hypothermia coincided with increased activity of AP-1, but not NF-κB or EGR-1.

Heat Shock Abolishes Rewarming-Induced MNC-Born TF via Inhibition of c-Jun Phosphorylation

As illustrated in Figure 2B, heat shock pretreatment 24 hours before hypothermia reduced rewarming-induced increases in MNC-TF activity, which was not significantly different from that for the normothermic control group. When heat shock was applied alone, it did not cause significant changes in MNC-TF activity ($4.4 \pm 0.8$ mU/10$^6$ cells in HS&NT group and $5.6 \pm 0.7$ mU/10$^6$ cells in NT group; Figure 2B).

By comparing TF levels in the lysates of MNCs from HS&HT/RW and NT groups, we determined that the absence of rewarming-induced MNC-TF activity in HS&HT/RW group was consistent with TF protein levels and was comparable to levels in the NT group (Figure 3A). Pretreatment with heat shock in the normothermic group led to reduction in TF levels to $12.4 \pm 5.7\%$ of normothermic controls (Figure 3A).

Data from TF ICW assay revealed that pretreatment of rats with heat shock weakened the induction of both intracellular and surface TF protein after rewarming from hypothermia (Figure 3B). Furthermore, heat shock attenuation of post-rewarming TF protein levels in MNCs was also extended to retard TF externalization onto the surface of MNCs. This was demonstrated by changes in surface-to-total TF ratio in HS&HT/RW group, which did not differ from the values observed in MNCs from normothermic control animals (Figure 3B, insert). These changes in TF protein levels were not caused by the variations in assayed number of cells, because

![Figure 4](http://atvb.ahajournals.org/)

Figure 4. Changes in regulators of TF gene induction in rat MNCs. Representative immunoblots of IκB-α (A) and Ser$^{276}$-phosphorylated p65 (B) demonstrate lack of NF-κB activation in post-rewarming MNCs. Post-rewarming activation of c-Jun pathway illustrated by an increased c-Jun (C) and JNK (D) phosphorylation. Immunoblot shows no significant induction of EGR-1 protein levels after rewarming (E). Heat shock led to a dramatic induction of Hsp70 protein levels (F), which were associated with reduced post-hypothermic phosphorylation of c-Jun and low levels of IκB-α. Densitometrical readings of immunopositive band intensities, normalized to total levels or β-actin, are presented as mean and SEM. *$P \leq 0.05$ as compared with NT group. Acronyms are as in Figure 1.
the levels of GAPDH did not differ between the groups (Figure 3C).

To understand the mechanisms behind the heat shock-mediated suppression of TF induction after rewarming from hypothermia, we evaluated the levels of activation of the common regulators of TF gene transcription: NF-κB, AP-1, and EGR-1 in MNCs from rats subjected to a brief whole body heat shock 24 hours before hypothermia. Lack of TF induction in MNCs from the HS&HT/RW group was accompanied by low levels of Ser63-phosphorylated c-Jun (Figure 4C) and JNK (Figure 4D). Absence of elevated phosphorylation of c-Jun and JNK was paralleled by a prominent overexpression of Hsp70 after pretreatment with heat shock in the HS&HT/RW group (Figure 4F). Levels of Hsp70 in the MNCs of normothermic rats pretreated with heat shock (HS&NT group) were also significantly higher than in the NT control group (Figure 4F). Only minor Hsp70 expression in MNCs from rats of both hypothermic and normothermic groups was observed (Figure 4F). Low IκB-α levels suggested degradation of inhibitory subunit and activation of NF-κB in MNCs from rats in the HS&HT/RW and HS&NT groups (Figure 4A). In addition, we found no changes in EGR-1 levels in MNCs after pretreatment with heat shock (Figure 4E).

The effect of heat shock pretreatment did not alter hypothermia/rewarming-induced TNF-α levels (Figure 2A). Persistently elevated plasma TNF-α in rats from the HS&HT/RW and HS&NT groups (Figure 2A) reflected the active state of NF-κB (Figure 4A).

**Discussion**

In this study we report an induction in TF activity, intracellular accumulation, and surface redistribution of TF antigen caused by rewarming from hypothermia in rat MNCs. We show that heat shock abolishes the expression of TF via inhibition of AP-1–mediated transcription of TF.

For the first time we demonstrate initiation of MNC-TF expression and proinflammatory response by rewarming from hypothermia in a rat in vivo model. In contrast, Johnson et al have shown that hypothermia-induced surface TF activity in TNF-α–stimulated human umbilical vein endothelial cells was completely reversed by rewarming. In the study by Lindenblatt et al it has been shown that hypothermia-accelerated microvascular thrombosis did not increase the risk of thrombus formation after rewarming.

The observation of high reactivity of rat MNCs was consistent with our finding from Western blotting, which revealed elevated levels of TF antigen in the rewarming phase but not under normothermic conditions. We distinguished the surface and intracellular pools of TF antigen by use of ICW assay and showed a clear induction of TF expression on the surface of MNCs along with significantly higher intracellular TF antigen accumulation after rewarming from hypothermia. In our study we observed the in vivo activation of MNCs after rewarming from hypothermia, signifying the role of MNC-born TF in the development of procoagulant conditions during the rewarming phase. Thus, we believe it to be clinically important that a therapeutic strategy be directed toward suppression of MNCs before rewarming from hypothermia. This would have noteworthy implications because hypothermia itself contributes to the development of coagulopathy in trauma patients, and current standard therapy recommends aggressive rewarming. This has its drawbacks as several case reports described fulminant coagulopathy within hours after initiation of rewarming in humans. In concordance with this, the data from our previous study demonstrated an increase in platelet count and a shortening of clotting time during rewarming from lethal hypothermia in a pig model.

We also questioned the mechanisms responsible for TF expression in MNCs during rewarming. We have found high levels of phosphorylated c-Jun protein, a member of the AP-1 complex known to be activated by stress-responsive signaling pathways. As was expected, c-Jun phosphorylation was accompanied with an increased phosphorylation of JNK in MNCs of the same experimental group.

Surprisingly, we observed downregulation of NF-κB as the levels of IκB-α were significantly elevated after rewarming. On the other hand, we were unable to detect differences in Ser286-phosphorylation of p65 between experimental groups. These findings indicate that NF-κB was not activated after rewarming from hypothermia and could not contribute to activation of TF gene transcription in rat MNCs. Furthermore, no increase in post-rewarming levels of EGR-1 transcription factor demonstrates that an induction of TF gene in MNCs after hypothermia/rewarming was not mediated by EGR-1 transcriptional activity.

Therefore, we speculated that NF-κB and EGR-1 had no or minor impact on the induction of TF transcription in MNCs after rewarming. On the contrary, a high level of c-Jun phosphorylation points to its importance for TF transcriptional regulation during rewarming from hypothermia. Interestingly, the whole body environment was indispensable for hypothermia-induced TF expression in MNCs as our unpublished data indicate that it is impossible to achieve an increase in TF activity and protein levels when effects of hypothermia were studied in an vitro whole blood system. Interestingly is the discrepancy between the effects of heat shock pretreatment on post-rewarming inductions of plasma TNF-α levels and TF antigen in rat MNCs. This could be connected to the differences in responsiveness of TF promoter to NF-κB and AP-1 activation attributable to higher number of AP-1 than NF-κB response elements. On the contrary, TNF-α gene is highly responsive to NF-κB activation rather than to AP-1.

Several previously published studies suggested that heat shock may alter both proinflammatory and stress-induced cell responses via induction of heat shock proteins.

In our present in vivo study, we found that heat shock attenuates hypothermia/rewarming-induced expression of TF in rat MNCs. These findings are in line with previously published data by Basi et al, where the reduction of TF mRNA expression, TF protein levels, and TF activity in endothelial cells challenged with LPS after heat shock was reported. We also found that a significant decrease of active TF antigen was accompanied by an induction of Hsp70 levels in MNCs of heat-shockedrewarmed animals. Several groups reported that Hsp70 could interfere with activation of c-Jun, which protected cells from damage caused by UV irradia-
tion,27 etoposide,28 and doxorubicin.29 It was clearly demonstrated that elevated levels of Hsp70 exhibited potent inhibitory effect on caspases30 and JNK.17,30 Our study shows that the increase in Hsp70 levels was followed by decline in phosphorylation of not only c-Jun but also its upstream kinase JNK. These data are in line with the previously published mechanism behind Hsp70-mediated inhibition of c-Jun activation, such as a direct binding of Hsp70 to JNK, which prevents phosphorylation of the latter by upstream mitogen activated protein kinase kinases 4/7 and increases the rate of JNK dephosphorylation.31–33

Taken together, our research shows an induction of TF activity and TF protein levels in MNCs from rats rewarmed from hypothermia. Heat shock pretreatment can attenuate post-rewarming expression and surface redistribution of TF antigen in MNCs in vivo. This effect was mediated via suppression of hypothermia-induced activation of c-Jun. We suggest that pharmacological inhibition of c-Jun activation could be a tempting strategy for the prevention of TF expression after rewarming from hypothermia.

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Disclosures
None.

References

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