Discordant Activation of Stress-activated Protein Kinases or c-Jun NH$_2$-terminal Protein Kinases in Tissues of Heat-stressed Mice*

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Stress-activated protein kinases (SAPK) or c-Jun NH$_2$-terminal protein kinases (JNK) are believed to be crucial signal transducers between stress stimuli and genetic responses in mammalian cells. These kinases are activated in various types of in vitro cultured cells by heat shock, but a similar activation of SAPK/JNK in tissues in vivo has yet to be shown. In the present study, C57BL/6 mice were exposed to elevated ambient temperature for various time periods, and SAPK/JNK activities determined in protein extracts of brain, heart, liver, spleen, lung, and kidney using protein kinase assay and Western blot analyses. The time course and relative magnitude of the heat-induced SAPK/JNK activity differed among tissues of the same animal. Significant activation of SAPK/JNK was achieved in heart, liver, and kidney at 60 or 90 min of heat stress. This increased activity of SAPK/JNK kinases was demonstrated to result from activation or phosphorylation of existing proteins in tissues. The maximal activation of these kinases showed no direct relationship with the elevation in body temperature (38–40.5 °C). Interestingly, SAPK/JNK activation did not occur in lung, brain, or spleen of the same heat-stressed mouse. Although elevated body temperature (40.5 °C) did not result in SAPK/JNK activation in spleen and lung tissues, heat stress induced SAPK/JNK activation in cultured organs or fibroblasts derived from spleen or lung of C57BL/6 mice. Furthermore, activity and amount of SAPK/JNK proteins were the most abundant in brain among tissues examined. Thus, our findings demonstrated that heat shock-induced SAPK/JNK activation in vivo lacks much of the characteristic coordinate control of activation of cultured cell lines, and suggests that the mechanisms controlling SAPK/JNK activation are influenced by physiologic factors that cannot be studied in vitro.

Cells respond to extracellular stimuli by activating signal transduction pathways, which culminate in changes in gene expression. A critical component of eukaryotic signal transduction is the activation of protein kinases that phosphorylate a host of cellular substrates, including transcription factors controlling the induction of various genes. In mammalian cells, mitogen-activated protein (MAP) kinases are thought to play a pivotal role in transmitting transmembrane signals required for cell proliferation, differentiation and apoptosis (1, 2). MAP kinases comprise a ubiquitous family of tyrosine/threonine kinases and include stress-activated protein kinases (SAPK) or c-Jun NH$_2$-terminal kinases (JNK), extracellular signal-regulated kinases (ERK) and 38-kDa MAP kinase-related protein kinases (2–5).

SAPK, as implied by their name, are highly activated in response to stresses including UVC irradiation, heat shock, inflammatory cytokines, osmotic stress, and inhibitors of protein synthesis in cultured cells (6–8). JNK was named based on its ability to phosphorylate the c-Jun protein leading to its enhanced transcriptional activity (6, 9). Recently, they have also been shown to be capable of phosphorylating the transcription factor ATF2 (10, 11). Both ERK and SAPK/JNK can be activated by a variety of stimuli (1–4). However, activation of these kinases varies depending on the stimulating signals. For example, ERK appears to be more highly activated in response to mitogenic stimulation (12), while SAPK/JNK shows greater activation in response to cellular stresses (13, 14). Therefore, SAPK/JNK- and ERK-mediated signal pathways may play a key role in initiating cell proliferation, differentiation, and possibly apoptosis.

The pathways leading to SAPK/JNK and ERK activation have been extensively studied in cultured cells. Upstream activators of JNK/SAPK have been found involving SEK1/MKK4 (SAPK kinase 1/MAP kinase kinase 4), and the small GTP-binding proteins Rac1 and Cdc42 (15–17). Although the pathways involved in activating ERK and SAPK/JNK appear to share some signaling events, they also involve distinct regulatory molecules (2, 4). Activation of ERK in response to growth factors results from activation of membrane-associated receptor tyrosine kinases followed by sequential activation of Ras and Raf. Raf then phosphorylates MAP kinase/ERK kinase (MEK) (1), which, in turn, activates ERK (1–4). In addition, important cell type-specific differences dictate the activities of particular cellular factors involved in the signal transduction pathways (14, 18–21). This evidence has emphasized the complexity of the intracellular communication networks controlling the response to environmental stimuli.

It is known that in vitro exposure of cultured cells to 42 °C for 30 min causes SAPK/JNK activation (6–8). However, no systematic investigation of the kinetics of activation of SAPK/JNK has been conducted in multiple tissues of intact animals following heat stress. In addition, no data exist concerning the level of this kinase activity in a various organs in a defined

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1 The abbreviations used are: MAP, mitogen-activated protein; ATF, activating transcription factor; ERK, extracellular signal-regulated kinase(s); GST, glutathione S-transferase; JNK, c-Jun NH$_2$-terminal protein kinase(s); MBP, myelin basic protein; MEK, ERK kinase(s); SAPK, stress-activated protein kinase(s); DMEM, Dulbecco’s modified Eagle’s medium; Mops, 3-N-morpholino)propanesulfonic acid.

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animal model. In this report, we examined the SAPK/JNK activity in tissues of C57BL/6 mice exposed to various elevated ambient temperatures at various time intervals. The relative activity of SAPK/JNK and ERK kinases was assessed in protein extracts from heart, liver, spleen, lung, kidney, and brain. We provide evidence that the most abundant SAPK/JNK and ERK are present in brain tissue, and demonstrate that heat stress results in activation of SAPK/JNK in heart, kidney, and liver, but not other tissues examined. These results suggest that the mechanisms controlling this cellular stress response may have additional physiologic components that cannot be investigated using cultured cells.

MATERIALS AND METHODS

Animals and Treatment Conditions—Male C57BL/6 mice 8–10 weeks of age were maintained on a light/dark (12 h/12 h) cycle at 23 °C and received food and water ad libitum. All procedures were performed according to protocols approved by the Institution Committee for use and care of laboratory animals. The procedure for heat stress in the present study was similar to that described previously (22). Briefly, exposure to elevated temperature was achieved by placing animals in a positive forced air incubator for varying times and temperatures. The rectal temperature was monitored. After the desired time interval, following heat stress, animals were sacrificed and tissue specimens from heart, liver, spleen, lung, kidney, and brain were collected and immediately frozen in liquid nitrogen.

Organ and Cell Culture—Animals were sacrificed, and tissue specimens from liver, spleen, and kidney were cut into pieces (2 × 5 × 10 mm³), washed three times with DMEM (Life Technologies, Inc.) and cultivated in DMEM supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. The medium was changed every 3 days, and cells were passaged by treatment with 0.05% trypsin, 0.02% EDTA solution. Experiments were conducted on fibroblasts (passages 5–8) that had just achieved confluence after a 24-h culture in medium containing 0.2% serum for 8 min, the cells were harvested with buffer A (see below) for protein extracts.

Kinase Assay—The procedure used for protein extracts and kinase assay activity was similar to that described previously (24, 25), with a slight modification. Briefly, frozen tissues were homogenized using a Polytron homogenizer on ice with buffer A containing 20 mM Hepes, pH 7.4, 50 mM β-glycerophosphate, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% NaCl, 10% serum for 8 min, the cells were harvested with buffer A (see below) for protein extracts.

The activities of SAPK/JNK in the immunocomplexes were measured as described previously (18, 25). Briefly, immunocomplexes were precipitated by centrifugation and washed two times with buffers A, B, and C, respectively, as described for kinase assay. The proteins were then separated on an Immobilon-P transfer membrane. The membranes were processed with the antibody against SAPK/JNK (Santa Cruz Biochemicals). Specific antigen-antibody complexes were then detected with the ECL Western blot detection kit. Graphs of the results were obtained in the linear range of detection and were quantified for the levels of specific induction by scanning laser densitometry (PowerLook II, UMAX Data System Inc., Hsinchu, Taiwan) of autoradiographs.

Western Blot Analysis—Protein extracts prepared from the tissues as described above were separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P transfer membrane (28). For immunoprecipitation of proteins containing phosphotyrosine, 0.5 ml of the supernatant containing 0.5 mg of proteins was incubated with 10 μg of antibodies against phosphotyrosine (4G10; Upstate Biotechnology Inc.) for 3 h at 4 °C with rotation. Subsequently, 80 μl of protein A-Sepharose 4B suspension (Sigma) was added and rotating continued for 1 h at 4 °C. The immunocomplexes were precipitated by centrifugation and washed two times with buffers A, B, and C, respectively, as described for kinase assay. The proteins were then separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P transfer membrane. The membranes were processed with the antibody against SAPK/JNK (Santa Cruz Biochemicals). Specific antigen-antibody complexes were then detected with the ECL Western blot detection kit. Statistical Analysis—Statistical analysis was performed for multiple comparisons. The Mann-Whitney U test was used for comparison between two groups. A p value less than 0.05 was considered statistically significant.

RESULTS

Time Course of SAPK Activation—SAPK/JNK phosphorylate Ser-63 and Ser-73 of the NH₂-terminal transactivation domain of c-Jun in vitro and may be responsible for phosphorylation of c-Jun in vivo (29). We used GST-c-Jun (1–135) as a substrate in a kinase assay to identify the protein kinase that binds to and phosphorylates the NH₂-terminal domain of c-Jun.

SAPK/JNK activity was determined in protein extracts derived from tissues of mice exposed to elevated ambient temperatures (37 °C) for different lengths of time. Fig. 1 shows data from time course studies of SAPK/JNK activation in heart, lung, liver, brain, kidney, and spleen. Two control groups were employed in these experiments: 1) animals that were sacrificed immediately after removal from the housing facility as controls for local environmental changes, and 2) animals that were placed in the incubator in their original cages and exposed to room temperature for 90 min. Since no difference in SAPK/JNK activation was seen in tissues from the two control groups, the data in the figures are from the animals in control group 1. The basal level of SAPK/JNK activity showed great differences between tissues of the same untreated animal. The highest activity of SAPK/JNK was observed in brain tissue, whereas liver showed a low level of SAPK/JNK activation (Fig. 1). When animals were exposed to 37 °C, activation of SAPK/JNK was evident in heart, liver, and kidney, but not in lung, brain, or spleen. The maximal activities of SAPK/JNK appeared at 60 min in heart, and 90 min in liver and kidney after exposure of animals to elevated ambient temperature (Fig. 1).

Fig. 2A summarizes SAPK/JNK activity as determined by autoradiography of optical densities from autoradiograms of two experiments. Exposure of animals to 37 °C produced 6–8-fold changes in SAPK/JNK activity after 60 or 90 min in heart, liver, and kidney. Because the maximal activation of SAPK/JNK can be observed in cultured cells exposed to heat shock for 30 min, we measured the rectal body temperature of mice to determine any relationship between body temperature and...
SAPK/JNK activity. The rectal temperature reached 40 °C at 60 min and decreased to 39 °C at 90 min after heat stress (Fig. 2). Changes in body temperatures in response to elevated ambient temperatures was consistent with those described previously (22). Interestingly, the maximal activation of this kinase showed no direct relationship with elevation in body temperatures (Fig. 2), suggesting that discordant activation of SAPK/JNK in tissues of heat-stressed mice may reflect additional factors present in individual organs participating in the process of kinase activation or inactivation.

Effect of Ambient Temperature on SAPK Activation—To further study the effect of different ambient temperatures on SAPK/JNK activation, mice were exposed to 35, 37, or 40 °C and SAPK/JNK activity in protein extracts from various types of tissues was measured. Likewise, activation of SAPK/JNK was observed in heart, liver, and kidney tissues, but not the other tissues examined (Fig. 3). The statistical data shown in Fig. 4 indicate a 4–8-fold induction of SAPK/JNK activation in these three types of tissues in mice treated at 37 or 40 °C. Ambient temperature of 35 °C did not result in SAPK/JNK activation in any organs examined, although the body temperature of the mice was higher than 38 °C (Fig. 4). Furthermore, no activation of SAPK/JNK was detected in tissues of spleen, brain, and lung, even though mice exposed to 40 °C for 1 h had an elevated body temperature up to 40.5 °C. These results further suggest that additional factors are involved in controlling the activation of these kinases.

To determine whether the observed increases in SAPK/JNK activity are due to increased expression of SAPK/JNK or due to activation of existing proteins by phosphorylation, the amount of SAPK/JNK proteins were analyzed in tissues of animals exposed to heat stress. Data summarized in Fig. 5 show Western blot analysis of SAPK/JNK proteins in tissues of heart, lung, kidney, liver, spleen, and brain of animals with or without exposure to heat stress. No significant difference was found in the amount of SAPK/JNK proteins between tissues derived from heat-stressed and untreated animals. Since it is established that tyrosine phosphorylation of SAPK/JNK proteins is necessary for their activation in cells’ response to en-
environmental stimuli (1–4), proteins containing phosphotyrosine were immunoprecipitated from protein extracts of heart and kidney tissues of untreated or heat-stressed animals and analyzed by immunoblotting with the antibodies against SAPK/JNK. Data shown in Fig. 5B indicated that tyrosine phosphorylation of SAPK/JNK proteins occurred in tissues of animals in response to heat stress. Taken together, our findings demonstrated that the increased activity of SAPK/JNK was regulated by phosphorylation of existing proteins in various tissues.

**Heat Stress-induced SAPK/JNK Activation in Cultured Organs and Lung Fibroblasts**—Depending on the heat stress conditions, cultured cells often require a short periods of time following heat exposure before maximal SAPK/JNK activation (6–8). Because of differential activation of SAPK/JNK in various types of tissues, we examined the SAPK/JNK activation in cultured organs with or without heat stress (40 °C) for 30 min. Fig. 6 summarizes SAPK/JNK activities as determined by quantification of optical densities from autoradiograms of three experiments. Exposure of cultured organs to 40 °C produced 5–8-fold changes in SAPK/JNK activity in liver, kidney, and spleen, suggesting that cells of three organs have a similar ability of SAPK/JNK activation in response to elevated temperatures ex vivo.

To further verify ability of SAPK/JNK activation in vitro, experiments were performed with fibroblasts cultivated from lung tissues of mice. Fig. 7A shows SAPK/JNK activation induced by elevated temperatures. A significant increase in SAPK/JNK activity was seen in fibroblasts exposed to 38 °C, although the maximal activation of SAPK/JNK was found at 42 °C for 30 min. Heat shock-activated ERK2 to a lesser degree, whereas serum strongly stimulated ERK2 activation in serum-starved fibroblasts (Fig. 7A). These results indicate that SAPK/JNK activation in response to heat shock was similar between fibroblasts primarily cultured from lung tissue and the cell lines used by others (6–8).

Both SAPK/JNK and ERK2 kinases are activated by dual phosphorylation of tyrosine and threonine residues in response to stress or mitogenic stimuli (1–4). The molecular weight of ERK2 shows a difference between activated and inactivated forms, whereas SAPK usually does not show this alteration. We performed a Western blot analysis using protein extracts from the fibroblasts and the antibody recognizing SAPK/JNK and ERK2. As expected, the amounts of SAPK/JNK proteins showed no changes in protein extracts from heat-stressed or untreated cells, and no significant shift of SAPK/JNK in the electrophoretic mobility (Fig. 7B). On the other hand, the activated (phosphorylated) form of p42 was identified based on its slower electrophoretic mobility compared with the unmodified (nonphosphorylated) form (Fig. 7B). Heat shock did not induce a significant shift of ERK2 in the electrophoretic mobility of p42 protein seen on Western blots.

**Abundant SAPK in Brain Tissue**—As seen in Figs. 1 and 3, very high levels of SAPK/JNK activity were observed in brain tissue. We further analyzed the activity and amount of SAPK in brain tissue from normal mice. Fig. 8 shows the activities (A)
and amounts (B) of SAPK/JNK in brain and liver determined by the kinase assay and Western blot analysis. Activity of SAPK/JNK was 100 times higher in brain than in liver tissues (Fig. 8A), and SAPK/JNK proteins appeared much more abundant in protein extracts from brain (Fig. 8B). Two bands was observed in the Western blot representing two isoforms of SAPK/JNK, i.e. p54 and p46. No other bands were seen in the blot, verifying the specificity of the SAPK/JNK antibody used in this study.

Again, this phenomenon does not occur in cultured cells, since no significant difference in SAPK/JNK activity was found between untreated fibroblasts and PC12 cells derived from brain tissue (data not shown). In the present report, we provide the first evidence of the abundant SAPK/JNK present in brain tissue, although any additional role of SAPK/JNK in brain function has yet to be proven. The search for answers to this question may pose a challenge to researchers in neuroscience.

**ERK2 Activity in Tissues of Heat-stressed Mice**—Both SAPK/JNK and ERK can be activated by a variety of stimuli, including different types of cellular stresses, cytokines, and growth factors in vitro (1–8). However, these kinases are activated to varying degrees depending on the stimulating signals. Therefore, activities of ERK2 were simultaneously determined using MBP as a substrate in protein extracts from tissues of mice exposed to elevated ambient temperatures (data shown in Fig. 9). Activities of ERK2 were not significantly altered in all tissues of mice treated with 37 °C ambient temperature for various time periods, an observation consistent with that seen in cultured cells (Fig. 7; Refs. 6–8). In addition, ERK2 activity in brain tissue appeared much higher than that in other tissues (Fig. 9), further suggesting the importance of MAP kinases in brain.

**DISCUSSION**

Various stress stimuli that control cell growth and apoptosis use kinase cascades to transmit signals from the cell surface to the nucleus. Activation of the SAPK/JNK is believed to be critical for the cell’s response to environmental insults, such as heat stress. Evidence obtained from study of cultured cells indicates that SAPK/JNK is involved in mediating cell growth or hypertrophy via activation of transcription factor activator protein-1 (AP-1) (30, 31), and in cell apoptosis (32, 33). Our findings demonstrated that heat stress selectively induces SAPK activation in tissues of intact animals, suggesting a physiological relevance of these protein kinases in response to
energized. p42ERK2 proteins were immunoprecipitated from the pro-
tin samples from heart, lung, liver, spleen, brain, and kidney were harvested and
homogenized. The time course of ERK2 activities in tissues of heat-
stressed mice. Mice were sacrificed at the indicated time points after exposure to elevated ambient temperature (37 °C), and tissue samples from heart, lung, liver, spleen, brain, and kidney were harvested and homogenized. p42ERK2 proteins were immunoprecipitated from the pro-
tin extracts (0.5 mg in 0.5 ml of kinase buffer A), and their kinase activities were measured based on phosphorylation of MBP. Note that no significant changes in ERK activities were found in various types of tissues.

Elevated Ambient Temperatures

Possible Mechanisms of SAPK/JNK Activation—Although it is established that heat stress activates SAPK/JNK kinases in cultured cells in vitro, the initiating signals responsible for such activation remain to be clarified. In the present study, we have demonstrated SAPK/JNK activation in heart, liver, and kidney, but not in spleen, lung, or brain in intact animals exposed to heat stress. This selective activation in various organs may reflect differential physiological responses because SAPK/JNK activity did increase in organ and cell cultures from spleen and lung after exposure to elevated temperatures. Likewise, cells or organs per se have ability to activate these kinases in response to thermal stress. Thus, our findings support the notion that additional factors should be involved in heat-in-
duced SAPK/JNK activation or inactivation in different organs in vivo.

In heart, considerable activation of these protein kinases was observed 60 min after heat stress (Fig. 1). In response to elevated body temperatures, peripheral blood vessels become di-
lated, resulting in an increased output from the heart, a process,
resulting in cardiomyocyte overload. Evidence indicates that overloading or mechanical stretching of cultured myocytes can activate SAPK/JNK kinase (34). In addition, acute hypertension can also activate SAPK/JNK in the arterial wall (25). Our data demonstrated that maximum activity of SAPK/JNK in heart was achieved as late as 60 min after heat stress. These findings suggest that kinase activation following elevated body temperature may be due to heat as well as overloading or mechanical stimulation to the cardiomyocytes.

In kidney, it is established that a marked increase in SAPK/ 
JNK activity is observed after ischemia followed by reperfusion (35). Osmotic stress results in SAPK/JNK activation in cul-
tured cells (6–8). We have previously shown that vasopressin selectively induces heat shock protein expression in renal tubular cells (36). In the present report, we demonstrated that the maximal activity of SAPK/JNK is seen 90 min after heat stress. Elevated body temperature alone is not a plausible explanation for this SAPK/JNK activation in kidney, since it declined 60 min following elevated ambient temperatures (Fig.

2B). Additional factors participating in SAPK/JNK activation in the kidney might be ischemic and osmotic stresses. During elevations in body temperature, the osmolarity of blood may change stimulating the neurone to release vasopressin and other hormones into circulation (37). They, in turn, bind to arteriole smooth muscle cells and/or tubular epithelial cells in the kidney, resulting in contraction of the glomerulus arteriole and water reabsorption in tubular cells. These responses lead to decreased blood perfusion to the kidney and increased osmo-
larity around the tubular lining cells (38). Thus, heat stress combined with ischemic and osmotic stress may contribute to induction of SAPK/JNK activation in the kidney.

In the liver, 3–4-fold induction of SAPK/JNK activity occurs 90 min after elevated ambient temperature (Figs. 1 and 2A). Again, we speculate that other factors, such as metabolite accumulation in the liver, might be important for kinase activation. Liver is a center for metabolism of production that may increase during elevated body temperatures. In this process, SAPK/JNK can be activated via a combination of heat stress and metabolite accumulation. As described above, exposure of animals to elevated temperatures is known to cause the release of several stress hormones, including epinephrine, norepineph-
rine, renin-angiotensin system, and vasopressin, into the peripheral circulation of mammals (37). This observation, combined with the results presented in this study, suggests that there is a multilevel response to heat stress in vivo. Activation of neural stress centers in the brain with subsequent release of stress hormones occurs in response to stress at the organismal level, while SAPK/JNK activation occurs as a response to stress at a cellular level. Elucidation of the interactions between the stress response at these two levels may provide important insights into the physiologic control of cellular function and organic homeostasis.

Possible Physiological Relevance of SAPK/JNK Activation—As described above, JNK/SAPK kinase can phosphoryl-
ate or activate several transcription factors, including c-Jun, 
ATF2, and Elk-1 (11, 39). ATF2 can dimerize not only with 
c-Jun, but also with itself and some other members of the ATF 
family, including ATF3, CREB, and the closely related NF-
κB. Elk-1, together with serum response factor, controls transcription from the serum response element. These transcription factors regulate gene expression, including matrix metallopro-
teinase, adhesion molecule E-selectin, NO synthase, interleu-
kin-8, and proliferating cell nuclear antigen (31). These genes have been demonstrated to play a key role in cell growth and cellular homeostasis. Thus, our findings of SAPK/JNK activation in heart, liver, and kidney could be important to our understanding of the mechanism controlling expression of these genes during physiological stress.

Recent studies have provided evidence that activation of MEKK, a SAPK/JNK kinase, and SAPK/JNK may be involved in the process leading to cell apoptosis (32, 33). It is conceivable that some cells in the organs of animals exposed to elevated ambient temperatures may die. Given the fact of selective activation of SAPK/JNK in tissues of animals exposed to high temperatures, we suggest a role of SAPK/JNK in mediating apoptosis in vivo in response to heat stress. A recent study concerning heat-related deaths during a heat wave (July 1995) in Chicago demonstrated that people at greatest risk of dying from the hot weather are those with cardiovascular diseases (40). Thus, a combination of heat stress and ischemia might initiate myocyte apoptosis, which leads to cardiac dysfunction. During this process, SAPK/JNK-mediated signal pathways could be involved in vivo.

In the present study, we found that relatively small in-
creases in body temperature can elicit the SAPK/JNK activa-
tion. Animals heated to 37 °C for 90 min showed only a modest rise in body temperature, yet they displayed appreciable induction of SAPK/JNK activation in heart, liver, and kidney. These results indicate that relatively slight increases in body temperature are sufficient to activate SAPK/JNK in vivo. Elevations of body temperature to these levels are well within the range known to occur with physiologic stress conditions such as exercise, environmental heat, and fever. The observation of known to occur with physiologic stress conditions such as exercise, environmental heat, and fever. The observation of

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