The Impact of Intermittent and Repetitive Cold Stress Exposure on Endoplasmic Reticulum Stress and Instability of Atherosclerotic Plaques

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Key Words
Cold stress • Atherosclerotic plaque • Instability • Endoplasmic reticulum stress

Abstract
Background: The incidence of acute coronary syndrome caused by the rupture of atherosclerotic plaque and subsequent arterial thrombosis increases as the weather gets colder. However, the association between cold stress and atherosclerotic plaque rupture is currently unknown. Methods: An atherosclerotic plaque model was established in rabbits by balloon injury and a high-fat diet with or without cold stress (4°C, 1 hour per day, 20 weeks) at the onset of modeling. Additionally, oxidized low-density lipoprotein (ox-LDL) was applied to induce the formation of macrophage foam cells in vitro. Results: Serum lipid profiles and inflammatory cytokines (ox-LDL, high-sensitivity C-reactive protein, and interleukin-8) were significantly higher in cold stress–exposed rabbits than in controls (P<0.05). Animals with atherosclerotic lesions that were exposed to cold stress had increased macrophages, foam cells, intima-media thickness, and neovascularization in the plaque, along with significantly thinned plaque fibrous caps. Moreover, we found that cold stress induced more apoptotic cells in the atherosclerotic plaques and up-regulated endoplasmic reticulum stress (ERS)-associated proteins CHOP, GRP78, and p-JNK (P<0.05). In addition, tunicamycin treatment promoted ox-LDL–induced apoptosis, expression of CHOP and GRP78, and the p-JNK level in macrophage foam cells, while JNK inhibitor sp600125 reduced cell apoptosis and the p-JNK level. The three main ERS sensors sensors phosphorylated extracellular signal-regulated

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kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme1 (IRE1) declined significantly after ox-LDL treatment. **Conclusions:** Cold stress may enhance the instability of atherosclerotic plaques through activating ERS and enhancing cell apoptosis. Up-regulated CHOP levels mediated by PERK and ATF6 and the activated IRE1-XBP1-JNK pathway contributed to the apoptosis of foam cells.

**Introduction**

Atherosclerosis is a dynamic degenerative disease of the arterial wall with acute cardiovascular manifestations. It is generally triggered by a local arterial occlusion by a thrombus overlying a pre-existing atherosclerotic plaque, which eventually increases the risk of unheralded events such as acute coronary syndrome (ACS), myocardial infarction, and stroke [1, 2]. Patients with ACS consistently present with unstable angina, acute myocardial infarction, and sudden coronary death [3]. Postmortem studies have established plaque rupture to be the cause of up to 75% of ACS episodes [4]. The transition from stable atherosclerotic plaque to vulnerable plaque is associated with the sudden rupture of plaque and subsequent thrombosis. Accumulated evidence has revealed that internal or external mechanical triggers exceeding plaque tensile strength in vulnerable regions result in plaque rupture [5, 6]. Over the past several decades, a great deal of effort has been expended toward creating a definition of and a detection method for “vulnerable plaque” so ACS can be better predicted. Vulnerable plaque was initially defined as a nonobstructive, silent coronary lesion that suddenly becomes obstructive and symptomatic. A more thorough definition proposed in 2003 by Naghavi et al. [7] included active inflammation; a thin cap (<100 μm) with a large lipid core (>40% of the plaque’s total volume); endothelial denudation with superficial platelet aggregation; and fissured cap or severe stenosis. The presence of at least one of these major criteria may indicate a higher risk for plaque complication. The size of the necrotic core and the thickness of the fibrous cap have been highlighted as major structural determinants of vulnerability [2].

Epidemiological studies have revealed close correlations between season or month and the rate of hospital admissions for ACS as well as the morbidity and mortality of ACS; these correlations are presumably due to weather and climate variations [3, 4]. It has been suggested that ambient temperature is an important predictor of acute myocardial infarction mortality, which is significantly higher in the cold weather of winter than in summer [5, 6, 7]. Cold stress is hypothesized to have adverse impacts on plaque stability, but the correlations between these factors have not yet been clearly defined [8].

With regard to the modulation of plaque stability, a wealth of evidence has implicated endoplasmic reticulum stress (ERS), which has been proposed to contribute to the progression of atherosclerotic plaques [9]. ERS appears to play a critical role in lipid metabolic disorder, activation of inflammatory reactions, and cellular apoptosis [10]. Recent reports have demonstrated that apoptosis of macrophages may contribute to the vulnerability of atherosclerotic plaques through ERS [10-12]. The ERS response is an adaptive mechanism by which cells react to perturbations in endoplasmic reticulum (ER) homeostasis through the unfolded protein response (UPR) and the up-regulation of ER-resident chaperones such as glucose-regulated protein (GRP78), which is the principal biomarker for the onset of UPR. If ER function is severely impaired, however, the organelle spreads apoptotic signals via C/EBP homologous protein (CHOP). In the present study, we hypothesized that cold stress may have a deleterious effect on atherosclerotic plaque stability via aggravation of cellular ERS. A series of in vivo experiments were designed and performed to test this hypothesis.
Materials and Methods

Animals

Forty male New Zealand white rabbits, each 3 months of age and 2.5–3.0 kg, were provided by the Fourth Military Medical University Animal Center and housed in animal care facilities in individual cages under moderate temperatures (20°C–24°C) and humidity levels (54%–66%) and changing light conditions (12:12 hour light–dark cycle, light beginning at 8:00 a.m.) with access to food and clean water. All tests were performed during the light phase of the cycle. All protocols were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University, and in compliance with the Guidelines for the Care and Use of Laboratory Animals.

The atherosclerotic animal model was established as previously described [13] with minor modifications. In brief, all rabbits were fed with an atherogenic diet (cholesterol 0.5 g/kg, lard 0.5 ml/kg, and 3% yolk powder) for 2 weeks before arterial injury. To induce arterial injury, each rabbit was anesthetized and a 4F sheathing balloon catheter was introduced into the abdominal aorta. A 4.0 mm × 15.0 mm balloon dilation catheter (Medtronic, Inc., USA) was then guided into the abdominal aorta with guidewire assistance. The balloon catheter was inflated to 16 atmospheres, and withdrawn to the common iliac artery bifurcation five times to produce endothelial denudation. The cut-down sites and the skin wounds were repaired.

One week after arterial injury, the rabbits were randomly divided into two groups: a control group (n=20) and a cold stress group (n=20). The cold stress group was exposed to cold conditions (4°C) for 1 hour per day for 20 weeks, while the control group was kept at 20°C–24°C at all times. Both groups continued to receive the atherogenic diet for the 20 weeks following arterial injury. Five rabbits (two in the control group and three in the cold stress-exposed group) died of diarrhea or intestinal obstruction during the experiment; the remaining rabbits completed the entire study.

Cell culture

Murine macrophage RAW264.7 cells were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM medium (Life Technologies, Grand Island, NY, USA) supplemented with 2 mM glutamine, antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin), and 10% heat-inactivated fetal bovine serum and maintained in a 37°C humidified incubator containing 5% CO₂. Cells were incubated with 100 mg/L oxidized low-density lipoprotein (ox-LDL) for 24 hours to induce the formation of foam cells.

Biochemical measurements

At the end of the 20th week, a 10-ml blood sample was collected from the marginal ear vein after the rabbit had been fasted for 12 hours. After settling for 20 minutes, blood samples were centrifuged at 2000 r/min for 15 minutes. The supernatants were collected and stored at ~80°C. The serum lipid concentrations of total cholesterol, triglycerides, and LDL were determined using diagnostic enzyme assay kits (WAKO Pure Chemical Co., Osaka, Japan). The serum inflammatory factors (ox-LDL, hs-CRP, and IL-8) were detected by enzyme-linked immunosorbent assay kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s instructions.

Ultrasoundographic studies

At the end of the 20th week, all rabbits were placed under sufficient anesthesia and given an abdominal aortic ultrasound examination using an Acuson Sequoia 512 ultrasound scanner (Siemens, Munich, Germany) with a 15L8 linear array and an 8- to 15-MHz transducer. Aortic intima-media thickening and vascular cavity-filling defects were regarded as atherosclerotic plaque. The average plaque area of each rabbit was determined from 10 cross-sections. We then injected Sono-vue (0.05 ml/kg) (BRACCO, Beijing, China), followed immediately by a 2-ml normal saline flush. A punctate appearance of the contrast agent from the vascular adventitia to the plaque or linear enhancement indicated the formation of neovascularization.

Histopathology, immunohistochemistry and western blotting

All rabbits were euthanized by intravenous overdose of pentobarbital, and the aortas were dissected. Tissue samples taken from the abdominal aorta were fixed in 4% formaldehyde overnight. Some segments were embedded in paraffin and cut into 5-μm-thick sections for staining with hematoxylin and eosin (HE)
or Masson trichrome, while other sections were cut into 6-μm-thick sections and stained with Sirius red and Oil-red O (Sigma-Aldrich).

Immunohistochemical staining was performed according to the standard techniques. In brief, after deparaffinization and hydration, sections were incubated with 3% H$_2$O$_2$ in distilled water for 10 minutes to quench the endogenous peroxidase activity. After incubation in a goat serum blocking solution for 30 minutes, the sections were reacted with mouse monoclonal antibodies to macrophage MAC387 or CD31 JC/70A (1:100 dilution, Abcam, Cambridge, UK) overnight at 4°C. After a phosphate-buffered saline wash, the sections were incubated with the biotinylated secondary antibody for 30 minutes at 37°C. Immunoreactions were visualized with a diaminobenzidine kit and counterstained with hematoxylin.

Histopathological slides were analyzed with a computer-assisted morphometric analysis system (Image-Pro Plus 6.0, Media Cybernetics, Rockville, MD, USA). The fibrous cap thicknesses and intima-media thicknesses (IMTs) of aortic plaques were measured at 10 equidistant points around the cap in each slice; three slices per segment were measured and the values were averaged. The positive stain area for macrophages and CD31 was expressed as a percentage of the stained area divided by the plaque area of the aortic plaque in at least 10 high-power fields.

Expression of GPR78, CHOP and JNK/pJNK in the control (n=6) and cold-stress group (n=6) were analyzed by Western blotting using commercially available antibodies from Santa Cruz Biotechnology, Inc. (CA, USA). The cell lysates were subjected to sodium dodecyl sulfate (SDS)-PAGE and subsequently transferred to a PVDF membrane. Blots were visualized using Amersham Western blot detection reagent (GE Healthcare, Piscataway, NJ, USA).

Apoptotic cell analysis by in situ TUNEL labeling
Cells undergoing apoptosis in rabbit abdominal aortic plaque tissue were identified by the terminal dUTP nick end-labeling (TUNEL) method with the ApopTag In Situ Cell Apoptosis Detection Kit I (Wuhan BOSTER Co., Ltd, Wuhan, China).

Reverse-transcription (RT)-PCR
Total RNA was extracted and purified from lesioned rabbit abdominal aorta tissue with AS plaques in the control (n=6) and cold-stress group (n=6) or RAW264.7 cells using an RNeasy Plus Universal Kit according to the manufacturer’s instructions (Qiagen, Santa Clara, CA, USA). After RNA purity was assessed, 2.5μg of RNA was reverse transcribed into cDNA using Superscript III (Invitrogen). The cDNA was used as a template in RT-PCR amplifications performed using a SYBR Green Real-Time PCR Master Mix kit (Takara Biotechnology, Dalian, China) based on the manufacturer’s instructions. The primer sequences (Shanghai Biological Engineering Co., Ltd, China): GADD153(CHOP): sense 5′-TGCTTTCTCGTGCTGGTGAC-3′, antisense 5′-CTGTTTCCCTGCCCTGC-3′; GPR78: sense 5′-CTGCTCC ATCGCCTCACTTT-3′, antisense 5′-CTAACCCTGATACGCTG-3′; ATF4: sense 5′-CCACAC ACATGACCGAGAT-3′, antisense 5′-CTCATCTGGGAT GGTCTCC-3′; ATF6: sense 5′-CAGACGGTTTTGCTGTCTCAG-3′; antisense 5′-ACCATTT CATTTGTAGGG-3′; XBPI (unspliced XBPI [205 bp] and spliced XBPI [179 bp] ) : sense 5′-GAA CCA GGA GTT AAG AAC AGC-3′; antisense 5′-CAG CAG GTG CAG GCC AG TT-3′; GAPDH: 5′-AGGTATCCACGACCACCTTCC-3′; 5′-GGAGTTTCC-GTCTCAGCTC-3′. Amplification cycles were as follows: 94°C for 3 minutes, then 33 cycles at 94°C for 1 minute, 58°C for 1 minute, 72°C for 1.5 minute, followed by 72°C for 15 minutes. Aliquots of the PCR product were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining.

Statistical analysis
Data are presented as mean ± SD. Differences were compared by analysis of variance (ANOVA) followed by the LSD post hoc test. A P value <0.05 was considered statistically significant. All statistical tests were performed using SPSS, v. 13.0 (SPSS Inc., Chicago, IL, USA).

Results
Effects of cold stress on serum lipid profiles and inflammatory markers
To determine the central roles played by lipid metabolism and inflammation in atherosclerosis development and plaque characteristics, we evaluated levels of serum
inflammatory markers and certain lipids as biochemical parameters in animals with and without cold stress exposure. As shown in Table 1, cold stress caused no statistically significant increase in any serum lipid profiles, including cholesterol, triglyceride, and LDL (P >0.05). However, the serum levels of hs-CRP, IL-8, and ox-LDL were significantly higher in the cold stress group than in the control group (P<0.05) (Table 2).

Effects of cold stress on plaque morphological and histopathological characteristics in atherosclerotic lesions

In ultrasonographic images of the 17 surviving rabbits in the cold stress group, 31 atherosclerotic plaques were detected; in the 18 surviving rabbits in the control group, 25 were detected. Hematoxylin and eosin staining suggested that plaques in the cold stress-exposed animals had larger volumes (7.96±0.51 mm² vs. 4.52±0.45 mm²) and lipid cores, a generally irregular eccentric appearance, and thinner fibrous caps compared to plaques in the control group (Fig. 1A). In the cold stress group, 21 plaques had a lipid core:plaque ratio >40%, and 12 had a thin fibrous cap measuring <7 mm, while in the control group, only six had a lipid core:plaque ratio >40% and three had a thin fibrous cap measuring <7 mm (Table 3). The mean fibrous cap thickness of the aortic plaque was smaller in the cold stress group.
than in the control group (112 ± 53 μm vs. 249 ± 66 μm, P<0.05), and the mean abdominal aortic IMT was also decreased (599 ± 139 μm vs. 923 ± 150 μm, P<0.01). Furthermore, increased lipid accumulation and reduced collagen fibers were observed in the abdominal aortic segments of cold stress–exposed rabbits compared to the control group, as suggested by the areas that stained positive with Oil-red O, Masson, and Sirius red, respectively (P<0.05 for each comparison) (Fig. 1B).
Cold stress promoted angiogenesis and infiltration of monocytes in atherosclerotic plaques

Of the 31 plaques found in the 17 surviving rabbits in the cold stress group, 23 exhibited neovascularization as visualized by contrast agents; among the 25 plaques found in the 18 surviving rabbits in the control group, only four with enhanced contrast were identified (Fig. 2A). The plaque angiogenesis rate was 74.1% (23/31) in the cold stress group, a rate significantly higher than that of 16% (4/25) in the control group (P<0.05) (Table 3). The rate of positive staining for CD31, a marker of angiogenesis, in the aortic sections was much higher in the cold stress group than in the control group (126.4 ± 33.1% vs. 38.9 ± 14.2%) (P<0.05) (Fig. 2B).

Furthermore, we evaluated the distribution of macrophages in the atherosclerotic plaques and found that 18 ± 3.5% of the area of the arterial walls in the control group was positively stained with Mac-1, a common antibody to macrophages, while a significantly higher proportion was stained in the cold stress group (33 ± 2.2%) (P<0.01) (Fig. 2C).

Cold stress–enhanced cell apoptosis via ERS

We measured the proportion of apoptotic cells in the abdominal aortic lesions using a TUNEL assay and found that the proportion of TUNEL-positive cells was significantly higher following cold stress exposure (P<0.05) (Fig. 3A). To further investigate the roles of ERS in response to cold stress, we determined the expression levels of two ERS-associated markers, GRP78 and CHOP, using RT-PCR, and found that both were significantly greater in the cold stress group than in the control group (P<0.05) (Fig. 3B). Simultaneously, the cold stress-exposed animals exhibited higher phosphorylated c-Jun than control animals did, as shown in Fig. 3C (P<0.05).
Moreover, we further investigated the correlation between ERS and the ox-LDL–mediated macrophage foam cell apoptosis in RAW264.7 cells. After being pretreated with Tunicamycin (1 mg/L) and JNK inhibitor sp600125 (10 μmol/L) for 30 min before the application of ox-LDL, the mean OD of lipid droplets and apoptotic cells were quantified. Western blotting analysis and quantification of CHOP, GPR78, and p-JNK after various treatments in RAW264.7 cells. (C) RT-PCR analysis of the expression of three main ER stress sensors before and after ox-LDL treatment. Each experiment was performed in triplicate. *P < 0.05 vs control, **P < 0.01 vs control; *P < 0.05 vs ox-LDL treatment; **P < 0.01 vs ox-LDL treatment.

Moreover, we further investigated the correlation between ERS and the ox-LDL-mediated macrophage foam cell apoptosis in RAW264.7 cells. After being pretreated with...
ox-LDL (100 mg/L) for 24 hours, the cells exhibited higher levels of lipid-drop accumulation and apoptosis as suggested by the Oil-red O and TUNEL staining, and tunicamycin treatment further triggered cell apoptosis by enhancing ERS. However, sp600125, a commonly used JNK inhibitor that dose dependently inhibited the phosphorylation of c-Jun[14], prevented apoptosis in foam cells compared with the ox-LDL–treated cells (Fig. 4A). Additionally, cellular CHOP, GPR78, and p-JNK significantly increased after ox-LDL and tunicamycin treatments, alone and in combination. However, sp600125 did not significantly change the levels of CHOP and GPR78 in spite of p-JNK (Fig.4B). Subsequently, we determined the three main ERS sensors on the ER membrane, including PERK, ATF6, and IRE1. We found that the mRNA levels of ATF4 (indicator of PERK activation), ATF6, and spliced X-box binding protein-1 (spliced XBP1, indicator of IRE1 activation) were significantly increased in response to ox-LDL treatment 4 (Fig. 4C). Collectively, these results indicate that ox-LDL activates ER-associated apoptosis signaling pathways.

Discussion

The current study revealed a significant increase in aortic plaque instability, characterized by increased IMT, macrophage accumulation, cell apoptosis, angiogenesis, and thinner fibrous caps, in a cold stress–exposed atherosclerotic rabbit model. Moreover, we demonstrated that ERS was associated with the progression of cold stress–induced plaque vulnerability.

Many factors may affect the incidence of cardiovascular diseases, including war, natural disasters, psychological stress, and exposure to extreme environmental conditions [15, 16]. Even cold winter weather can increase hospital admission rates, as well as ACS morbidity and mortality [17]. When humans are exposed to cold, peripheral blood vessels contract, resulting in increased vascular resistance, blood pressure, and cardiac load, which make it easier for a thrombus to form and thereby increase ACS occurrence [18]. Epidemiological evidence suggests that ACS incidence is generally higher in winter than in the summer [7]. Animal studies have also indicated adverse impacts of cold stress on cardiovascular disease, beginning with the first successful creation of a cold stress–induced rat hypertension model by Van [19].

In our study, we subjected animals with atherosclerotic lesions to intermittent, repetitive cold stress to mimic the progress of human atherosclerosis in naturally cold environments. We observed the development of plaques in these animals, thus combining the methods of the cold stress–induced hypertension model in rats and the atherosclerotic plaque model in rabbits. Pathological studies in humans have also identified certain features that are common to most unstable atherosclerotic plaques, such as increased lipid core size and macrophage infiltration as well as decreased collagen content. Although an animal atherosclerotic model with cold-induced plaque instability may not fully mimic conditions in human patients with ACS and the plaque-destabilizing effects of naturally occurring cold stress, it still provides a useful tool for assessing the effects of cold stress on plaque stability and merits use in further investigations.

Over the last decade, studies have suggested that ERS is an important factor in the initiation and clinical progression of atherosclerosis [20]. Recent reports have shown that advanced atherosclerotic plaques exhibit significantly more apoptotic macrophages and smooth muscle cells compared with earlier or more stable lesions, which exhibit higher expression levels of CHOP, a key mediator of ERS-induced cell apoptosis [21]. Therefore, excessive or sustained activation of ERS can be responsible for the vulnerability of atherosclerotic plaques. In the current animal study, we also found that the expression of CHOP was markedly increased in cold conditions together with the elevated apoptotic cells in the atherosclerotic plaques. Moreover, we found that serum ox-LDL levels in cold stress–exposed animals were significantly higher than in the controls, indicating that ox-LDL participated in the induction of plaques vulnerability. It has been suggested that ox-
LDL or oxidized lipids are responsible for triggering ERS apoptosis in human aortic smooth muscle cells and causing the death of macrophages via a CHOP-dependent pathway, with this process being inhibited by antioxidants [22]. In addition, a compelling study revealed that ox-LDL can induce excessive generation of reactive oxygen species (ROS), which was well established as a strong inducer of ERS [23, 24]. With regard to the establishment of the cold-stressed animal model, rabbits with atherosclerotic lesions were exposed to a cold condition at 4°C for 1 hour per day and then returned to 37°C. The return to normothermia after a cold shock can induce a heat shock–like response, with a burst of ROS [25, 26]. Therefore, in the current study, ox-LDL and oxidative stress induced by ox-LDL or rewarming may contribute to the activation of UPR and ER apoptosis. However, the role of the heat shock–like response after cold stress in inducing ERS requires more investigation.

With regard to the in vivo study, we did not evaluate the effects of intermittent and repetitive cold stress exposition on the cultured macrophage cells just as the in vitro works, mainly considering the discrepancy between cell cultures and animal physiological conditions based on the fact that neuro-humoral regulation plays critical roles in homothermal animals, and hypothermia may not directly induce the reduction of body temperature and atherosclerotic plaque instability while it induces the increased production of inflammatory cytokines animals. Therefore, exposing cultured cells to cold conditions could not fully mimic the in vivo works. Additionally, we found that ox-LDL was significantly higher in cold-stressed animals, which is an well-established inducer of atherosclerosis. Thus, we used ox-LDL to induce the formation of macrophage foam cells to further investigate the roles of ERS on cell apoptosis. After enhancing the ERS by tunicamycin, apoptotic cells significantly increased. Moreover, we found that the ERS sensors PERK, ATF6, and IRE1 increased after ox-LDL treatment. PERK and ATF6 were previously demonstrated to be responsible for enhancing CHOP levels and initiating apoptosis [27]. Meanwhile, IRE1 initiates apoptosis through the IRE1-XBP1-JNK pathway [28]. In our study, the JNK pathway was activated through cold stress, and the JNK inhibitor alleviated ox-LDL–induced cell apoptosis, indicating that the IRE1-XBP1-JNK pathway contributed to the cold stress–induced cell apoptosis. Thus, we concluded that ERS was involved in the progression of plaque instability induced by daily exposure to cold stress over a long period.

Furthermore, growing evidence has demonstrated that ERS may be both a trigger and a consequence of chronic inflammation. Chronic inflammation is often associated with misfolding mutations and ERS. Likewise, ERS and activation of the UPR are features of many chronic inflammatory and autoimmune diseases [12]. Meanwhile, the ER and related signaling networks are emerging as potential sites for the intersection of inflammation and metabolic disease. It is reported that the three branches of the canonical UPR interact with a variety of inflammatory and stress signaling systems including the Nuclear factor-κB(NF-κB)-IκB kinase (IKK) and JNK-AP1 pathways, as well as networks activated by oxidative stress [10]. In our study, both the inflammatory markers and ERS markers were augmented in cold-stressed animals, indicating that inflammation was associated with the activation of ERS. However, the underlying mechanisms need further investigation.

In summary, we have demonstrated in the present study that cold stress can increase plaque instability in an atherosclerotic rabbit model. The major possible mechanism underlying these effects involves the triggering of ERS by up-regulated CHOP levels mediated by PERK and ATF6 and the activated IRE1-XBP1-JNK pathway, eventually leading to increased apoptosis and plaque instability. These data will provide new insights into the mechanisms responsible for the greater incidence of ACS in cold weather conditions.

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Disclosure Statement

The authors declared no conflicts of interest.

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