Activation of the Proapoptotic Unfolded Protein Response in Plaques of the Human Carotid Artery

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WHAT THIS PAPER ADDS
This is the first study examining UPR-activation in human rupture-prone carotid plaques, and the proapoptotic UPR-CHOP signaling pathway was identified as a potential inducer of apoptosis in vascular cells. On the basis of these results, acute cerebrovascular events (stroke and TIA) are now joined with acute coronary syndromes as clinical events that warrant intense research into development and implementation of anti-apoptotic strategies, including targeting activation of the UPR-PERK/CHOP-branch specifically. That, in turn, may relieve ER-stress-induced apoptosis and thereby has the potential to reduce plaque progression/ruptures in cardiovascular disease.

Objective: To analyze expression of keystone markers of apoptosis and the proapoptotic signaling pathway “unfolded protein response” (UPR) in rupture-prone plaques of the human carotid artery.

Methods: Plaque specimens were obtained during endarterectomy for high-grade carotid stenosis, and were formalin-fixed. Ten specimens were identified that exhibited criteria of advanced rupture-prone atherosclerotic plaques, and histological and immunohistological analysis of markers of apoptosis (cleaved Caspase-3, TUNEL) and UPR (KDEL, ATF3, CHOP, CHAC-1) was performed. In addition, co-localization of apoptosis and UPR-activation was assessed by double-immunohistochemistry.

Results: The mean size of the necrotic core was 44 ± 7% and the mean minimum/representative thicknesses of the fibrous cap were 129 ± 39 μm/280 ± 60 μm, respectively. Each specimen fulfilled at least two of the criteria for rupture-prone plaques. Semi-quantitative analysis of immunohistochemistry showed a significant increase in cleaved Caspase-3-positive (1923 ± 93 cells/mm²) and TUNEL-positive cells (1387 ± 66 cells/mm²) when compared with control tissue. Furthermore, expression of UPR-markers KDEL, ATF3 and CHOP was significantly increased (1175 ± 40 cells/mm², 1971 ± 69 cells/mm² and 2173 ± 120 cells/mm², respectively). Co-localization of UPR-activation with apoptosis was confirmed by double-immunohistochemistry, and lesional macrophages were identified as the primary cell-type involved.

Conclusion: For the first time, activation of the proapoptotic signaling pathway UPR has been identified in advanced rupture-prone plaques of the human carotid artery. This provides additional evidence for adding UPR to the potential targets for controlling plaque apoptosis and thereby preventing plaque progression/rupture.

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INTRODUCTION
Plaque rupture is the most common cause of coronary artery thrombosis and serves as the pathomorphological correlate for acute coronary syndrome. In the cerebrovascular circulation, plaque rupture of carotid lesions has been identified as being positively associated with recent TIA (transitory ischemic attack) and stroke. Atherosclerosis as the underlying disease is a progressive inflammatory process triggered by retention of lipids in the arterial wall, leading to multifocal lesion development and formation of vulnerable plaques, the precursor of plaque rupture, being characterized by a large necrotic core (>30% of plaque) and a thin fibrous cap (<65 μm). Increased apoptosis of lesional macrophages is one of the molecular mechanisms that leads to increased size of the necrotic core and thinning of the fibrous cap. Among several triggers of apoptosis, ER (endoplasmic reticulum)-stress was identified as a potent inducer of macrophage apoptosis.
ER-stress occurs as a consequence of a mismatch between the load of unfolded and misfolded proteins in the ER and the capacity of the cellular machinery that copes with that load and sets a compensatory mechanism, the unfolded protein response (UPR), in motion, which reduces protein load and increases folding capacity. If cellular homeostasis cannot be re-established, programmed cell death (apoptosis) is initiated.8

This is achieved by phosphorylation of the ER-membrane bound protein kinase RNA-like ER kinase (PERK), which, in turn, phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2a), which then promotes translation of activating transcription factor 4 (ATF4) and subsequent induction of CHOP (C/EBP-homologous protein).9 CHOP itself causes proapoptotic changes, such as downregulation of BCL-2 and activation of calcium signaling pathways with subsequent induction of FAS death receptor.10

Robust evidence has accumulated from animal models of ApoE-deficient mice that activation of the PERK-CHOP branch of the unfolded protein response occurs in atherosclerotic lesions and promotes necrotic core formation.11–15 Interestingly, only one study is available that has examined activation of the PERK-CHOP branch in a human specimen (coronary artery16), but UPR-activation has not been studied so far in carotid plaques. Therefore, the aim of this study was to analyze activation of the PERK-CHOP branch of the UPR in human advanced rupture-prone carotid lesions.

METHODS

Carotid artery specimens

Carotid artery specimens were obtained from patients undergoing carotid endarterectomy for high-grade stenosis of the internal carotid artery at University Medical Center, Johannes-Gutenberg University, Mainz, Germany. The excised plaques were fixed in 4% formalin (Roth, Karlsruhe, Germany) immediately after retrieval in the operating room, and subsequently embedded in paraffin wax. The plaque cylinder was manually trimmed to an approximately 3 mm specimen containing the segment with maximum lumen reduction, which was subsequently cut into 3 μm transverse sections (perpendicular to the lumen), mounted on Superfrost Plus slides (Menzel, Thermo Scientific, Braunschweig, Germany) and deparaffinized in xylene. Carotid specimens were analyzed by histology and 10 specimens complying with criteria for rupture-prone plaques were chosen for further detailed analysis. Control specimens of common carotid artery and common femoral artery were obtained from patients without clinical evidence of atherosclerotic disease (vascular trauma, organ donor).

Clinical data were recorded for each patient. The use of human tissue for this study was approved by the local ethics committee and consent was obtained from the patients.

Histopathology

For morphological analysis, every 10th section was stained with hematoxylin and eosin (H&E, Gill’s No.3, Polyscience Inc. Eppelheim, Germany) as well as Masson-Goldner (Roth). From those sets of 10 sections each, five sets containing the area of maximum lumen reduction were selected and five representative overviews of H&E staining of total plaque area of each cylinder were taken with a digital camera-equipped microscope (ZEISS Axioplan, Oberkochen, Olympus XC30, Hamburg, Germany). Cap thickness, area of the necrotic core and total area of the plaque were measured using CellSens Imaging Software (Olympus). The necrotic core was defined as amorphous material containing cholesterol crystals. The fibrous cap was identified, and the thinnest part (minimum cap thickness) and the part with a thickness considered to be most representative of the cap as a whole (representative cap thickness) were measured and averaged.17 In addition, plaques were graded according to the AHA-classification.18

Immunohistochemistry

For each antibody, at least five representative sections of each specimen were stained. The sections were selected from the above described sets of 10 slides that allowed staining of the markers of apoptosis and UPR (n = 8). Depending on additional stainings (double-immunohistochemistry) and for technical reasons, additional sets of 10 sections adjacent in both directions to the above described sets were used if needed. Thus, on average, representative sections stained for each plaque specimen covered a cylinder length of 300–450 μm. All antibodies used in this study are listed in Table 1 and were used according to the manufacturer’s instructions and established protocols for detection of UPR-markers.19,20 Prior to blocking of endogenous peroxidase activity with 3% H2O2, antigen retrieval was performed for cleaved Caspase-3 and ATF-3 (activated transcription factor 3) by heating for 20 minutes at 95 °C in citrate buffer (pH 6.0) in a waterbath, for CHAC-1 (cation transport regulator-like protein 1) with pre-digestion in ready-to-use proteinase K solution (DakoCytomation, Glostrup, Denmark) for 10 minutes, and for CHOP using

Table 1. List of antibodies, suppliers and concentration used.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Isotype</th>
<th>Clone</th>
<th>Supplier and Catalog#</th>
<th>Concentration</th>
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<td>CD68</td>
<td>Mouse IgG3</td>
<td>PG-M1</td>
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<td>Mouse IgG2A</td>
<td>1A4</td>
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<td>1:500</td>
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<tr>
<td>KDEL</td>
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<td>10C3</td>
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<td>ATF-3</td>
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<tr>
<td>CHAC-1</td>
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<td>Polyclonal</td>
<td>Abcam, #ab105101</td>
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</tr>
<tr>
<td>cCaspase-3</td>
<td>Rabbit monoclonal</td>
<td>Asp175 (5A1)</td>
<td>Cell Signaling, #9664</td>
<td>1:100</td>
</tr>
<tr>
<td>CHOP</td>
<td>Rabbit IgG</td>
<td>Polyclonal</td>
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</table>
For CHAC1, staining mographics using freely available ImageJ Software (http://rsb.info.nih.gov/ij/disclaimer.html). For staining analysis and semiquantitative analysis, one overview of total cross-sectional area (25× magnification) and three representative high-power fields of the shoulder region (200× magnification) were captured from each of the five sections with a digital camera equipped microscope (ZEISS Axioplan, Olympus XC30). Semiquantitative analysis of immunohistochemical stainings was performed by counting positively stained cells/nuclei in the high-power images by two independent researchers blinded for patient demographics using freely available ImageJ Software (http://rsb.info.nih.gov/ij/disclaimer.html).

Double-immunohistochemistry

For the simultaneous detection of two different antigens within the same specimen the EnVision G2 Doublestain System visualization kit (DakoCytomation) was used. The visualization is based on peroxidase (HRP) using DAB+ as chromogen (brown color deposits, KDEL) and alkaline phosphatase (AP) using Permanent Red as chromogen (red color deposits, cCaspase-3/CD68/Actin) and were used according to the manufacturer’s instructions. All antibodies used in this assay are detailed in Table 1.

TUNEL-assay

For examining apoptosis by DNA fragmentation, the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore) was used according to the manufacturer’s instructions. DNA strand breaks were detected by enzymatically labeling the free 3′-OH termini with modified nucleotides, and methyl green was used to counterstain nuclei.

Statistical analysis

Semiquantitative results were presented as indicated and testing of differences between values was performed using the Mann–Whitney test (two-tailed). A p value of <.05 was considered statistically significant (*), a p value of <.001 was considered highly significant (****).

RESULTS

For this study, 10 specimens were selected and the respective patient characteristics were retrieved from the computerized database of the Division of Vascular Surgery of the University Medical Center (Table 2). Analysis of lesion morphology confirmed that all specimens exhibited features of rupture-prone plaques corresponding to the inclusion criteria of this study. The average values for total representative cap thickness (all specimens) were 280 ± 60 µm and for total minimum cap thickness 129 ± 39 µm (Fig. 1A,B), both being below the cut-off values of 500 µm and 200 µm, respectively. Selective analysis confirmed that each specimen had a representative cap thickness of less than 500 µm (Fig. 1A), and all but one (#5) had a minimum cap thickness of less than 200 µm (Fig. 1B). The size of the necrotic core was determined to be 44 ± 7% averaged for all specimens, and by selective analysis all specimens were again demonstrated to have a necrotic core size of at least 25% (Fig. 1C). When criteria of the Stary-classification were applied, three specimens could be classified into Stary group IV (atheroma with necrotic core) and seven into Stary group VI (lesion with surface defect).

Immunohistochemistry was used to identify markers of apoptosis (cleaved Caspase-3 and TUNEL) and UPR-activation (KDEL, ATF3, CHOP, CHAC1) as well as cellular markers for macrophages (CD68). Fig. 2A shows a panel of representative stainings at low magnification to allow assessment of the whole circumferential cross-sectional area and spatial distribution of the individual stainings. In addition, Fig. 2A shows a representative H&E staining that was used for measurement of lesion morphology. It could be demonstrated that markers of apoptosis and UPR-activation showed increased expression in the shoulder region of the plaque and around the necrotic core. The appropriate sections of control tissues (common femoral artery, CFA, common carotid artery, CCA and internal carotid artery, ICA) as well as the negative and positive

Table 2. Patient characteristics (n = 10).

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>n</th>
<th>%</th>
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<tr>
<td>Age</td>
<td>73 ± 6 years</td>
<td>Range 62–84 years</td>
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<td>Male gender</td>
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<tr>
<td>Risk factors</td>
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<td>100</td>
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<td>30</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
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<td>60</td>
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<tr>
<td>Diabetes mellitus</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>ASS</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Degree of carotid stenosis</td>
<td>88 ± 7%</td>
<td>Range 75–99%</td>
</tr>
</tbody>
</table>
controls for each antibody used for semiquantitative analysis are depicted in Fig. 2B. For semiquantitative analysis, high-power magnifications were obtained from those regions and the individual markers were quantified. A highly significant increase of markers of apoptosis was found, that is TUNEL (1387 ± 66 cells/mm²) and cleaved Caspase-3 (1923 ± 93 cells/mm²) in the shoulder region of each plaque when compared with control sections (Fig. 3A,B).

In addition, markers of UPR-activation were studied and, here, robust expression could be demonstrated for markers of the early UPR-signaling chain like Chaperons GRP 78 and 94 (summarized as KDEL, 1175 ± 40 cells/mm²), ATF3 (1971 ± 69 cells/mm²) as a central signaling molecule of the PERK-CHOP branch, and CHOP as the canonical pro-apoptotic UPR-effector (2173 ± 120 cells/mm², Fig. 3C–E). Again, expression of the above markers was found to be significantly increased when compared with control sections.

To provide additional evidence for the activation of CHOP, CHAC1 was studied, which has been reported to act as a downstream signaling molecule of CHOP. Here, it was found that CHAC1 is expressed in the shoulder region (Fig. 4A) and quantification revealed an expression rate of 27 ± 1%, which was highly significant when compared with control sections (Fig. 4B).

Having shown that both markers of apoptosis and UPR-activation exhibited significantly increased expression in the plaques, a colocalization analysis was performed. When adjacent sections were stained for CHOP and TUNEL, both showed a matching staining pattern (Fig. 5A,B) that was further corroborated by similar cell counts in semiquantitative analysis of positively stained cells (Fig. 5C).

To further corroborate those findings, double-immunohistochemistry was performed using antibodies for KDEL and cleaved Caspase-3. Again, simultaneous staining of cells with KDEL and cleaved Caspase-3 was demonstrated indicating presence of UPR-activation (ER-stress) in apoptotic cells (Fig. 5D,E). To further elucidate the cell type being involved, co-expression of UPR-marker KDEL with markers for smooth muscle cells and macrophages was analyzed. Using double-immunohistochemistry for KDEL and α-Actin/CD68, it was demonstrated that UPR-activation occurs only to a small extent in smooth muscle cell-rich areas (Fig. 5F), but predominantly in macrophage-rich areas (Fig. 5G).

When plaque apoptosis and UPR-activation were correlated with patient demographics, men showed a significant increase in plaque apoptosis (TUNEL, $p = .004$) and UPR-activation (KDEL and CHOP: $p < .0001$, ATF3: $p = .09$) when compared with women. There was no age difference between men and women (median age 74 vs. 72 years, range 62–81 vs. 68–84 years, 95% CI 65–82 vs. 66–81 years, $p = n.s.$). Furthermore, patients on statin medication had a significant higher level of plaque apoptosis (TUNEL and cleaved Caspase-3, $p < .001$) when compared with patients not taking statins. Markers of UPR-activation, however, were not affected by statin medication (KDEL: $p = .85$, ATF3: $p = .85$, CHOP: $p = .25$). For both factors (male gender and statin medication), parameters of plaque morphology (representative plaque thickness, minimum plaque thickness and size of necrotic core) were statistically similar compared with female gender ($p = .42$, $p = .10$ and $p = .42$, respectively) and no statin medication ($p = .55$, $p = .31$ and $p = .31$, respectively). With regards to clinical symptoms, a comparison of parameters for plaque morphology (representative and minimum cap thickness and size of necrotic core), lesional apoptosis (cleaved Caspase-3) and UPR-activation (KDEL, ATF3, CHOP) showed no significant difference between asymptomatic ($n = 3$) and symptomatic ($n = 7$) patients ($p = .38$, $p = .38$, $p = .83$, $p = .27$, $p = .12$, $p = .67$ and $p = .33$, respectively).
Figure 2. Immunohistochemistry. (A) View of total cross-sectional area of a representative specimen showing staining for morphological analysis (H&E) and immunohistochemistry of markers of apoptosis (cleaved Caspase-3, TUNEL) and UPR-activation (KDEL, ATF3, CHOP, CHAC1) (magnification 50×, scale bar indicates 500 μm). (B) Stainings of representative sections of control tissues as well as negative/positive controls for each antibody used for semiquantitative analysis. Control tissues are common femoral artery (CFA), common carotid artery (CCA), and internal carotid artery adjacent to the plaque (ICA). Negative controls (NC, irrelevant isotype-matched antibody) and positive controls (PC, Tonsil for TUNEL and cleaved Caspase-3, Pancreas for UPR-markers) are also shown (all magnifications 400×).
Figure 3. Semiquantitative analysis of apoptosis and UPR-markers. A representative image (magnification 200×), and the results of the quantification (bar graph) are shown for each marker of apoptosis (TUNEL, A, and cleaved Caspase-3, B) and activation of UPR (KDEL, C, ATF3, D, and CHOP, E). Control specimens are common femoral artery (CFA), common carotid artery (CCA), and internal carotid artery adjacent to the plaque (ICA). Values represent mean ± SEM of positively stained cells per mm². p < .001 for plaque versus all controls (***).
DISCUSSION

Although the underlying pathomechanisms that govern plaque disruption in both the coronary and the carotid arteries share similarities, different cut-off values have been described, which define the “rupture-prone” plaque. The Oxford plaque study found that critical cap thickness is greater in carotid plaques than in coronary plaques with a minimum cap thickness of less than 200 μm and a representative cap thickness of less than 500 μm. A lipid core size of less than 25% of cross-sectional area was identified as one of the predominant histologic features in ruptured carotid plaques.

Consequently, for the present study the focus was on advanced carotid plaques and included only study specimen that fulfilled the three parameters of ruptured/rupture-prone carotid plaques (minimum cap thickness <200 μm, representative cap thickness <500 μm, necrotic core >25%). The majority (seven) of the specimens were complicated plaques (Stary VI) with surface fissure/rupture, the other three were advanced plaques (Stary IV), a ratio that has also been found in other studies examining culprit lesions.

In those specimens, a significant increase of apoptosis as a key-stone element of plaque progression towards rupture could be demonstrated by immunohistochemical detection of cleaved Caspase-3 and DNA strand breaks (TUNEL) in comparison with control specimens. For evaluation of activation of the UPR, the focus was on three well-established signaling molecules involved in the proapoptotic PERK-CHOP branch: Chaperones GRP78 and GRP94 (recognized by the c-terminal retention sequence KDEL11,16,19), ATF3 and CHOP. Using semi-quantitative analysis of immunohistochemical detection of KDEL, ATF3 and CHOP, it was shown that expression of all three signaling molecules is robust and significantly increased in plaques when compared with control tissue (common femoral, common carotid, and non-diseased internal carotid artery).

Those findings are in line with results obtained from an analysis of 16 human coronary atherectomy specimens of patients where a significant increase in KDEL- and CHOP-positive cells in patients with unstable angina versus stable angina was documented.16

In addition to the work of Myoishi et al., which so far is the only available study on human tissue, convincing evidence from genetically modified atherogenic mouse models (ApoE−/−) exists wherein a significant increase in the expression of KDEL and CHOP in lesions could be documented.11,15 Vice versa, by using a combined CHOP−/−ApoE−/− deficient mouse model, a significant reduction of UPR-activation (GRP78) and plaque size,12 as well as a significant reduction of lesional apoptosis (TUNEL-positive cells) was shown.13,14

To corroborate the above findings of UPR-activation, additive evidence of increased CHOP expression was evaluated, that is the key element of the proapoptotic UPR-branch, CHAC-1 (cation transport regulator-like protein 1), has been identified to act as a proapoptotic component of the UPR downstream of CHOP.20,23,24 It was shown that CHAC1 expression is significantly increased in plaques when compared with control tissue.

Colocalization analysis confirmed that markers of UPR-activation and apoptosis can be detected in close proximity at the cellular level, and it was shown that UPR-activation can be predominantly localized in lesional macrophages and, to a lesser extent, smooth muscle, which again is in line with findings obtained by other groups.14–16

In addition, analysis of plaque apoptosis revealed a significant effect of statin therapy and supports findings of apoptosis induction in vascular cells by HMG-CoA reductase inhibitors.25 A potential influence of gender was also seen in this study, with increased apoptosis and UPR-activation in male patients that may be attributed to increased susceptibility of men to inflammatory processes. However, the mean age in this study was 73 years and the role of gender differences related to postmenopausal hormone changes (estrogen) remains controversial.26

Taken together, specimens were selected in this study that fulfilled morphologic criteria of rupture-prone carotid plaques and it was shown that activation of the proapoptotic CHOP-branch of the UPR occurs in lesional macrophages, and to a lesser extent smooth muscle cells, and

Figure 4. Semiquantitative analysis of CHAC1. A representative section is shown for quantification of CHAC1 (A, magnification 200×). As CHAC1 staining characteristics comprised cellular (arrow) as well as necrotic core (asterisk) patterns, quantification was achieved by measuring the positively stained area (B). Control specimens are common femoral artery (CFA), common carotid artery (CCA), and internal carotid artery adjacent to the plaque (ICA). Values are given as mean ± SEM of percent of CHAC1-positive stained area of total image area. p < .001 for plaque versus all controls (***)
Figure 5. Colocalization analysis. Staining for CHOP (A) and TUNEL (B) of adjacent sections is shown (magnification 200×). (C) Semi-quantitative analysis of CHOP and TUNEL in five pairs of adjacent sections. To assess colocalization of apoptosis and UPR-activation in the same section, double immunohistochemistry was performed (cleaved Caspase-3, brown pigment and KDEL, red pigment) and shown as high-power magnification (200×, D and 400×, E) of two representative specimens. Representative arrows (black) indicate positively stained cells for cleaved Caspase-3 and KDEL. Colocalization analysis of UPR-activation (KDEL, brown pigment) with smooth muscle cells (F, red pigment) and macrophages (G, red pigment) was performed using double immunohistochemistry. For smooth muscle cells (F) α-actin was used, and for macrophages (G) CD68 was used as antigen (both magnifications 100×).
colocalizes with markers of apoptosis. To the authors' knowledge, this is the first study examining UPR-activation in human carotid plaques and the findings fit well with results obtained from human coronary plaques and data from atherogenic mice models highlighting ER-stress and the UPR as a potential and potent inducer of apoptosis of vascular cells and thereby contributing to plaque progression and rupture.

Potential limitations of this study include the lack of multivariate regression analysis to identify potential influences on apoptosis and UPR-activation related to the number of specimens analyzed. Furthermore, in this pilot study, a correlation was performed between plaque morphology and individual clinical status (symptomatic vs. asymptomatic), which yielded no significant difference between the groups. Of note, the actual number of specimens in the asymptomatic group was low (n = 3) and there are controversial reports on the validity of such a correlation. Furthermore, the current clinical definition of a "symptomatic" carotid stenosis covers a wide time interval of up to 6 months after the onset of neurological symptoms. In the present study, "symptomatic" specimens showed a considerable variation in time from insult to operation, of between 1 and 35 days. However, it is realized that correlation of clinical status with UPR-activation in a study enrolling a larger specimen series of both asymptomatic and symptomatic stenoses with short interval from onset of symptoms to operation (<7 days), may provide additional insight and should be addressed in a future study. Thirdly, owing to the indication for operative endarterectomy (high-grade stenosis), it was not possible to include a control group of early stages of plaque development (moderate stenosis). Therefore, there is the possibility that the activation of apoptosis and UPR observed under the study conditions may also be a response to plaque remodeling rather than an actual cause of plaque rupture.

Putting the results into perspective, acute cerebrovascular events (stroke and TIA) may now be considered as the clinical events together with acute coronary syndromes that warrant intense research into development and implementation of anti-apoptotic strategies. Targeting activation of the UPR-PERK/CHOP-branch specifically may serve as a potential mechanism to relieve ER-induced apoptosis, and thereby reduce acute events/plaque ruptures in cardiovascular disease. However, because of the complexity of the UPR-signaling network and its fundamental role in cellular homeostasis (protein synthesis) and survival, further work is needed to understand the interplay of the UPR effectors and dissect elements with the most promising therapeutic gain.

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CONFLICT OF INTEREST
None.

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