Lack of cell stress markers in fibrous cap cells in the left main coronary artery

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Summary. Fibrous cap formation is a key aspect of preventing clinical events but animal models to study this are limited and cellular stress plays a fundamental role in fibrous cap formation. Aims: To characterise cellular stress markers in an established animal model to study coronary artery fibrous cap formation. Methods: Male New Zealand White rabbits were fed a diet containing 0.5% cholesterol and 1% methionine for 4 weeks, then 9 weeks of normal diet to induce fibrous cap formation. Immunohistochemistry was used to detect CHOP, GRP78, nitrotyrosine HSP70, HSP90, iNOS and HSP32. Results: The core within the left main coronary artery atherosclerosis contained vast amount of foamy macrophages which readily stained for all markers. However, the smooth muscle cells within the formed fibrous cap were negative for all markers. The endothelium overlying the fibrous cap was positive for CHOP, GRP78, nitrotyrosine, iNOS and HSP32, however it was difficult to detect positive endothelial HSP70 or HSP90 immunoreactivity. Serial sectioning and immunohistochemistry for all factors showed clear dual iNOS+ / HSP32+ / HSP70- / HSP90- single cells within the fibrous cap formed. Conclusion: Smooth muscle cells within fibrous caps appear 'stress free', however isolated single smooth muscle cells within caps and within the core show positive immunoreactivity for stress markers. This model could be used to understand the role of cellular stress in fibrous cap formation in the coronary artery.

Key words: Stress markers, Fibrous cap, Smooth muscle cell, Coronary artery disease

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Introduction

Rupture of the fibrous cap is a major cause of myocardial infarct, however animal models to study fibrous cap formation in the left main coronary artery are limited (Zulli and Hare, 2009; Zulli et al., 2009). Cellular stress is strongly associated with fibrous cap rupture, which includes endoplasmic reticulum stress, nitrosative stress, oxidative stress and inflammatory stress.

Endoplasmic reticulum (ER) stress occurs when improperly folded proteins accumulate in the extracellular space. To restore homeostasis, the unfolded protein response (UPR) is initiated and cell chaperones, such as glucose regulated protein 78 are upregulated (GRP78) (Marciniak and Ron, 2006), however CHOP protein (CCAAT/enhancer binding protein / growth arrest- and DNA damage inducible gene 153 (GADD153), DNA–damage-inducible transcript 3 (DDIT3) and C/EBPz) are also upregulated which partly regulates apoptosis (Oyadomari and Mori, 2004). Therefore, GRP78 is regarded as a marker of ER stress, whereas CHOP is regarded as a marker for ER stress induced apoptosis.

Nitrosative stress occurs due to the excess production of nitric oxide (NO), formed via inducible nitric oxide synthase (iNOS). Excess NO can react with the oxygen radical and form peroxynitrite, which can then nitrate the tyrosine residues of proteins (Tyr-NO2) (Elahi et al., 2007). This can cause protein deactivation as well as stimulate cellular signal transduction (Yeo et al., 2008). To identify nitrosative stress, immunohisto-chemistry to nitrotyrosine is commonly used.

Oxidative stress is strongly implicated in plaque rupture (Bonomini et al., 2008). Enzymes that produce the oxygen radical include NAD(P)H oxidases, xanthine oxidase and lipoxygenases, as well as dysfunctional mitochondria (Madamanchi et al., 2005). Oxidative

stress induces the expression of heat shock protein 70 (HSP70) (Papp et al., 2003) and 90 (HSP90), possibly in an attempt to protect protein from oxidation (Sreedhar et al., 2004). Furthermore, oxidative stress induces the up regulation of HSP32 (heme-oxygenase-1) which produces biliverdin, iron and carbon monoxide, which have potent anti-inflammatory, anti-apoptotic and antioxidant functions (Dulak et al., 2008). Thus, detection of HSP90, HSP70 and HSP32 are indicators of oxidative stress.

Inflammatory stress is believed to be a major cause of acute coronary syndromes, especially in the presence of small, multiple vulnerable plaques throughout the coronary circulation (Spagnoli et al., 2007). Neutrophil myeloperoxidase (MPO) produces the potent oxidant HOCl, which has been implicated in plaque rupture (McCarty, 2004). We have recently shown that high dietary taurine, which absorbs HOCl, can significantly impair atherosclerosis formation and apoptosis of endothelial cells in the left main coronary artery (Zulli et al., 2009). Hence, detection of MPO is a marker of inflammatory stress and the presence of HOCl.

Currently, there are no animal models to study the role of stress systems on coronary artery fibrous cap formation. To this end, we set out to characterise these stress systems in our established model of coronary artery atherosclerosis (Zulli et al., 2004, 2009; Zulli and Hare, 2009).

Materials and methods

Male New Zealand White rabbits (n=5) at three months of age received a normal rabbit chow diet supplemented with 0.5% cholesterol +1% methionine +5% peanut oil for 4 weeks. After this, all animals were fed a normal diet for 9 weeks to induce atherosclerotic regression in the left main coronary artery. Animals were then sacrificed and the heart removed. The left main coronary artery was excised, processed for paraffin, and all coronary arteries were mounted in one paraffin block. Sections of artery were cut at 5 μm and placed on a 45°C water bath to allow the sections to expand to its original size. Sections were then collected onto microscope slides and dried. Immunohistochemistry was performed as previously described. Primary antibody dilutions were as follows: HSP70 (Cat#MAB3516, Millipore, diluted 1:50), HSP90 (Cat# ab-1429-50, Abcam, diluted 1:100), nitrotyrosine (Cat#MAB5404, Millipore, diluted 1:100), iNOS (Cat#sc-7271, Santa Cruz Biotechnology, diluted 1:100), GRP78 (Cat#ab25192-100, Abcam, diluted 1:100), HSP32 (Cat#Ab13248, Abcam, diluted 1:100), myeloperoxidase (Cat#sc-59600, Santa Cruz Biotechnology, diluted 1:50) , CHOP (Cat#MA1-250, ABR, USA), HHF-35, alpha smooth muscle cell actin (1:4, Millipore Corporation, USA, Cat# ICH2020). Antibodies were incubated overnight followed by processing with 'Envision' system (Dakocytomation, Denmark). Immunohistochemistry and image analysis

was performed as previously established in our laboratory (Zulli et al., 2008; Zulli and Hare, 2009). Images were captured by a digital camera (Leica DFC425, Leica Australia) mounted on a biological microscope (Olympus BX53 model, Olympus Australia).

Results

The regression period caused accumulation of spindle shaped cells immunoreactive for smooth muscle alpha actin cells throughout the plaque (Fig. 1). Specifically, such cells accumulated near the lumen and formed a cap (arrows, cropped figure). All stress markers were positively identified in the core of the left main coronary artery (Figs. 2, 3). In particular, the ER stress markers CHOP and GRP78 immunostaining was similar, in that the endothelium overlying the fibrous cap was immunopositive (Fig. 2) as well as cells within the core of the atherosclerotic lesion. However, no spindle shaped cells within the cap (as shown in Fig. 1) showed positive immunoreactivity for these markers.

Nitrosative stress was also present in the plaques (Fig. 2). Nitrotyrosine (NT) positive immunoreactivity showed similar results to CHOP and GRP78 immunoreactivity. NT was identified on the endothelial layer overlying the fibrous cap as well as within the atherosclerotic core. As well, spindle shaped cells within the cap (as shown in Fig. 1) did not stain for NT.

The oxidative stress markers HSP70 and HSP90 were poorly identifiable on the endothelial layer overlying the fibrous cap, however they were more readily detectable within cells of the atherosclerotic core. Likewise, no spindle shaped cells within the cap (as shown in Fig. 1) showed reactivity to HSP70 or HSP90 (Fig. 3).

Interestingly, iNOS and HSP32 were found to co-localise in specific cells that were negative for HSP70 and HSP90 (Fig. 3, arrows-cropped section). Moreover, both proteins were clearly identified in the endothelium overlying the fibrous cap formed as well as co-localised in cells within the core. Similar to other proteins, there were no detectable spindle shaped cells within the cap that also showed positive immunoreactivity for these proteins.

Discussion

The main findings of this investigation are two fold: (a) coronary artery atherosclerosis expresses all markers of stress systems studied, albeit at different localisation and (b) the alpha actin positive spindle shaped cells in the formed fibrous cap do not express any stress marker studied.

Stress systems are believed to play a major role in coronary artery plaque rupture and endothelial erosion, which cause acute coronary syndromes. Endoplasmic reticulum stress and apoptosis of macrophages appears

to be activated by reactive nitrogen species (Gotoh et al., 2002), as well as in endothelial cells in culture (Dickhout et al., 2005). Previous studies in the model presented in this manuscript clearly show a reduction in plaque positive, cap positive and endothelial cell positive cells expressing the endoplasmic reticulum stress marker, GRP78 (Zulli and Hare, 2009), compared to plaques developed in the early stage of atherosclerosis (four week dietary intervention). In the study presented here, we show clear evidence of cells expressing ER stress proteins and nitrotyrosine within the atherosclerotic core (distinct from necrotic core as no necrotic material or cholesterol esters observed) and in the endothelium overlying the fibrous cap formed, indicating that treatments aimed at reducing nitrosative or endoplasmic reticulum stress might be tested on this model to determine if fibrous cap formation can be enhanced. We have recently shown that high dietary taurine can impair CHOP upregulation and apoptosis in coronary artery endothelial layer in a rabbit model (Zulli et al., 2009). As taurine can also absorb the hypochlorite anion, and we provide evidence that the enzyme that produces hypochlorite (MPO, myeloperoxidase) is present in the plaques, whether or not the addition of taurine to the
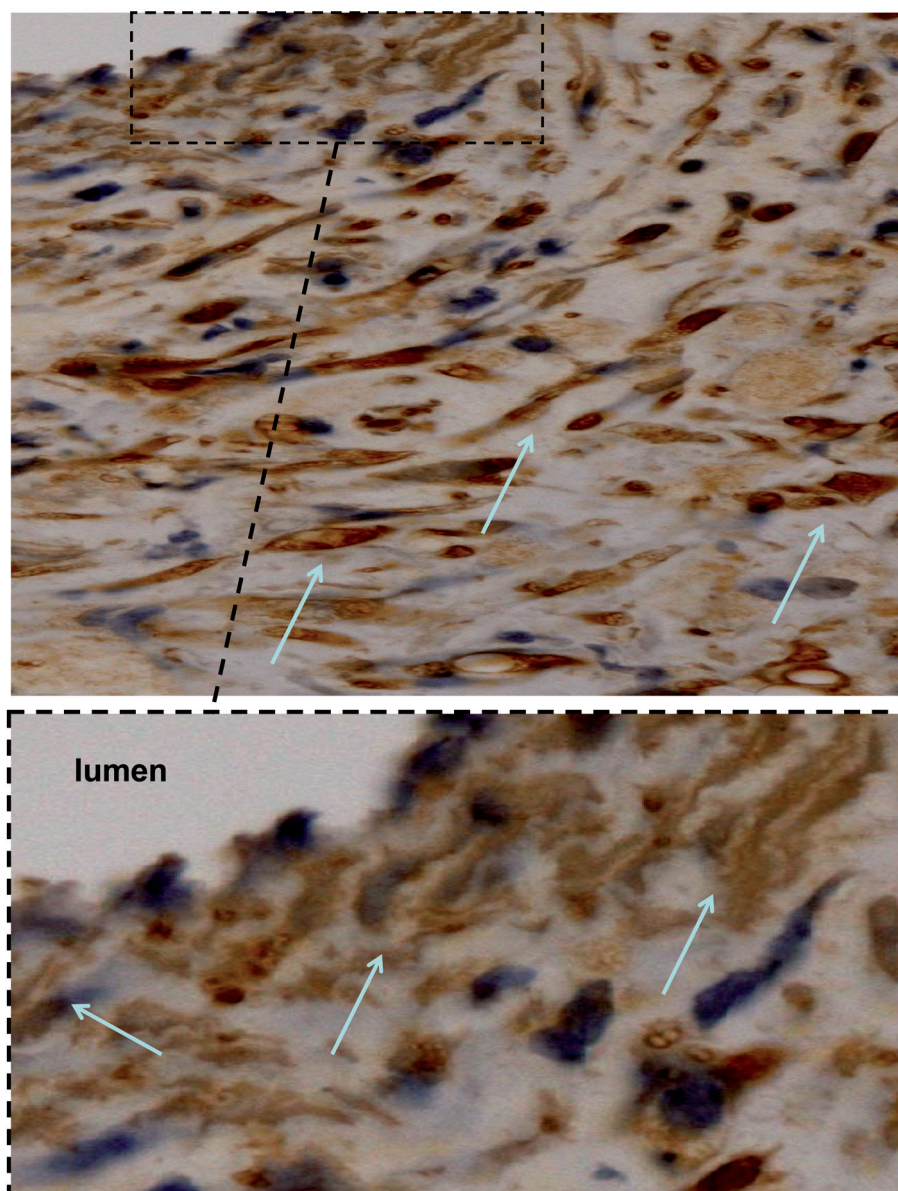

Fig. 1. Figure showing fibrous cap with cells expressing smooth muscle alpha actin. Spindle shaped cells are present throughout the plaque (arrows), but the cells concentrate near the lumen (cropped section). Brown colour represents positive immuno-staining and blue colour indicates nuclear Hematoxylin staining. x 600
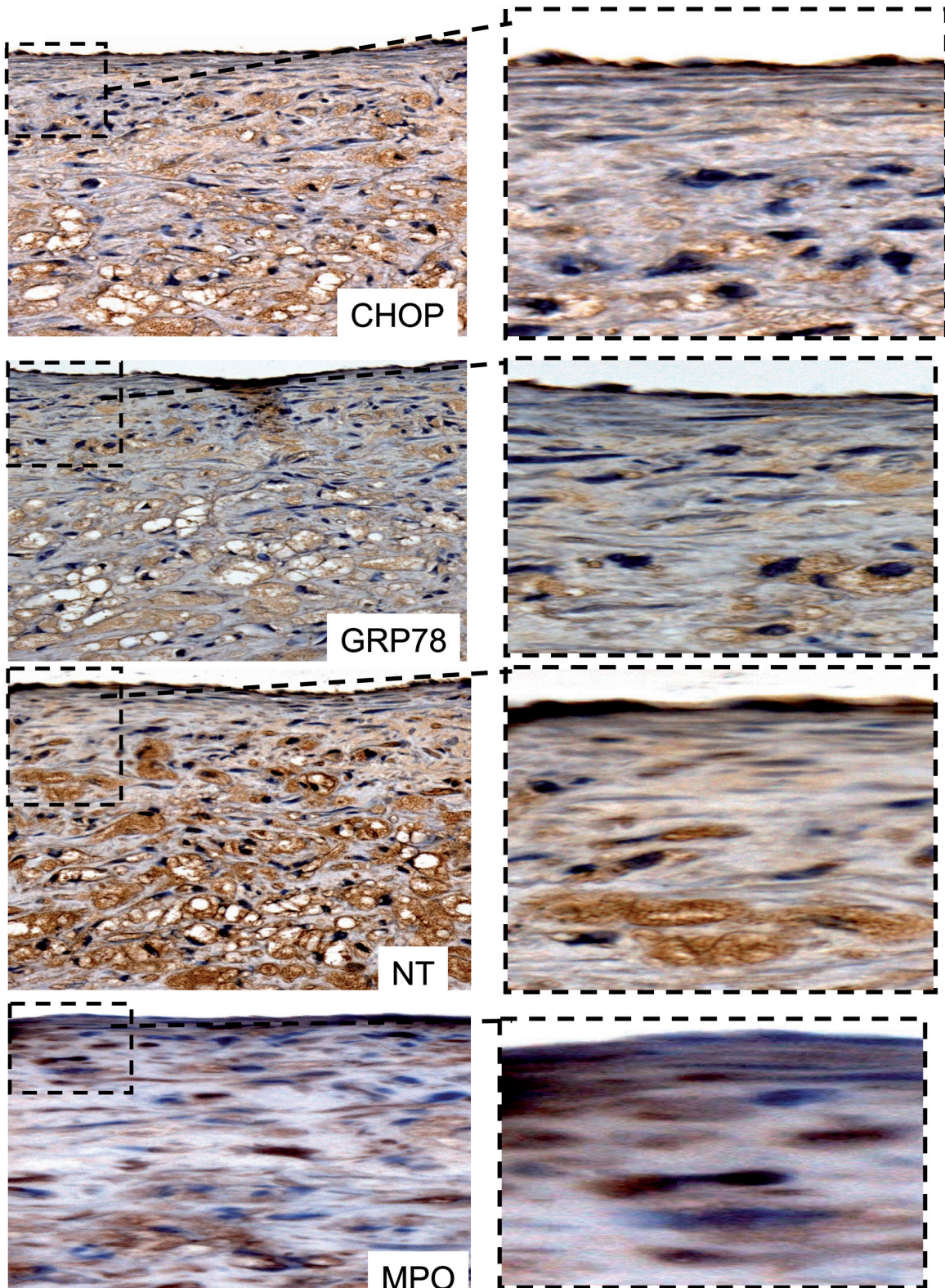
Fig. 2. Figure showing coronary artery atherosclerosis and fibrous cap (cropped section) showing no expression of spindle shaped cells in the cap for stress markers. CHOP and GRP78 (ER stress markers) immunoreactivity is clearly observed in the core of the plaque and the endothelial layer, and this is similar to nitrotyrosine immunoreactivity (NT, nitrative stress). Myeloperoxidase immunoreactivity (MPO, marker for inflammation) was present in plaques but was difficult to identify in the endothelial layer. Brown colour represents positive immunostaining and blue colour indicates nuclear Hematoxylin staining. x 400
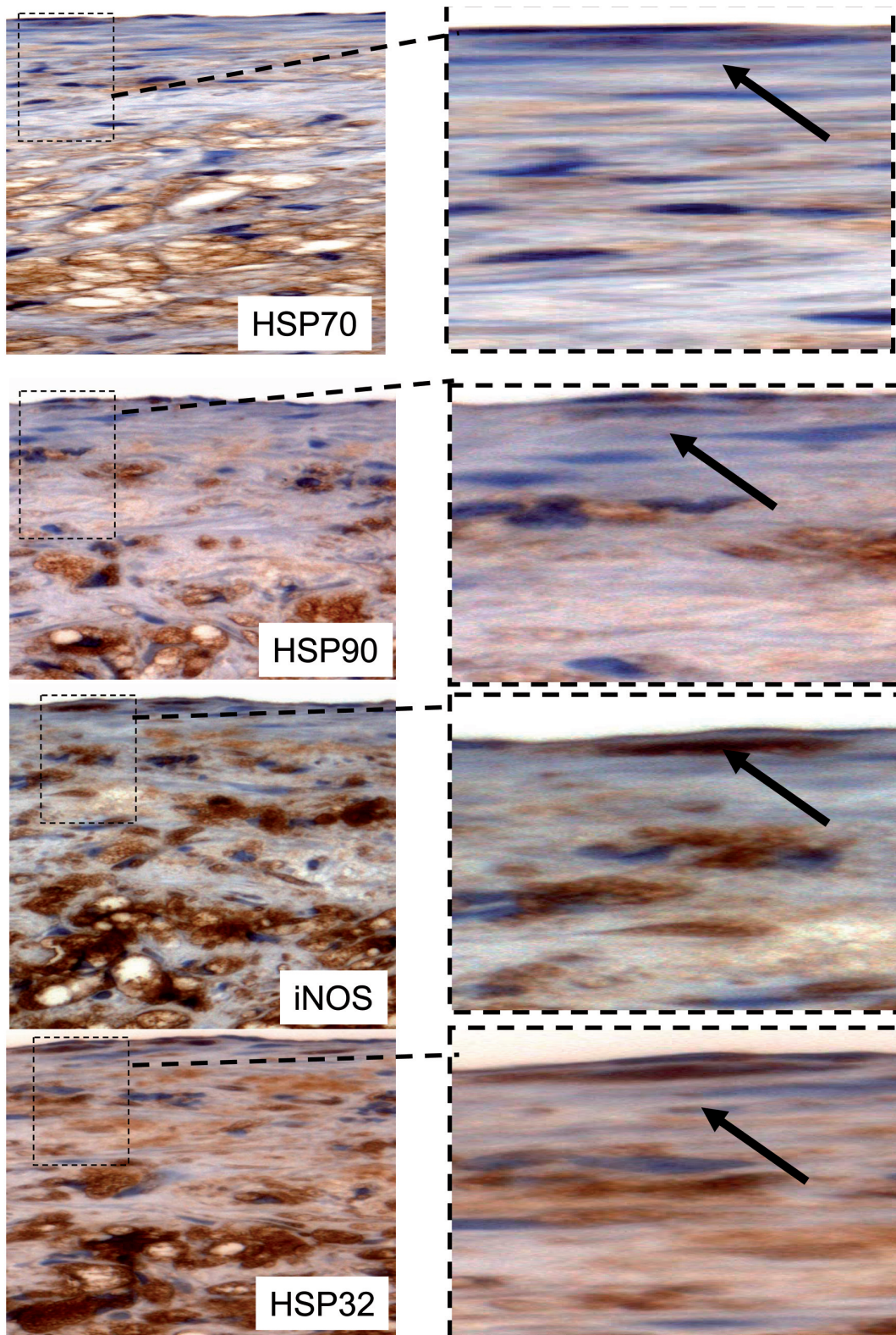
Fig. 3. Figure showing coronary artery atherosclerosis and fibrous cap (cropped section) showing no expression of spindle shaped cells in the cap for stress markers. HSP70 and HSP90 (oxidative stress markers) immunoreactivity is clearly observed in the core of the plaque but not the endothelial layer. iNOS was also present in the plaque and endothelial layer similar to HSP32, but no spindle shaped cells were visible in the cap. Interestingly, a subgroup of cells was clearly positive for iNOS+HSP32 and negative for HSP70/90 (cropped images, arrow). Brown colour represents positive immunostaining and blue colour indicates nuclear Hematoxylin staining. x 400

regression period in these animals promotes a stable plaque phenotype warrants further investigation.

The oxidative stress system is upregulated in human vulnerable coronary plaques (Kobayashi et al., 2003). The upregulation of heat shock proteins in vulnerable plaques (Lepedda et al., 2009; Andrie et al., 2011) could protect against endothelial cell apoptosis and thus prevent thrombosis formation. For example, an early study showed that oxidized LDL induces the expression of HSP32 and that vitamin C reduced this effect (Siow et al., 1999). We found clear evidence of iNOS and HSP32 (heme-oxygenase-1) co-localisation, indicating possible cross-talk between these two systems, as recent studies suggest that HSP32 is a key element in the cytoprotective effect of NO (Chung et al., 2008). For example, NO stimulates HSP32 expression in endothelial cells (Yee et al., 1996), and HSP32 possibly protects cells via carbon monoxide production (Choi et al., 2003). Taken together, this information suggests that co-expression of iNOS and HSP32 could aid in endothelial cell survival. Studies aimed at inhibition of HSP32 would help understand the role of iNOS and HSP32 in plaque remodelling and endothelial layer erosion.

An interesting finding in this investigation is that HSP70 and HSP90 were not readily identifiable on the endothelial layer overlying plaques, however these proteins were clearly indentified within the core of the coronary plaque. The reason for this remains unclear at this stage, however it could be argued that either the endothelial layer overlying the fibrous cap is not undergoing oxidative stress or that the expression of these proteins are inhibited. Indeed, the latter hypothesis is supported by studies in mice, whereby cigarette smoke decreased carotid HSP70 expression (Matsumoto et al., 2008). As well, Zhu and colleagues showed that endothelial cells in culture express HSP70 only when proliferating (Zhu et al., 1996), suggesting that the endothelial cells overlying the fibrous cap formed in this study are not proliferating. Endothelial HSP90 has been identified in human carotid artery atherosclerotic plaques (Businaro et al., 2009), but it is suggested to be a marker of plaque instability (Madrigal-Matute et al., 2010). As the results presented in this manuscript show poor endothelial HSP90 expression, this could suggest that the plaques observed are of stable phenotype. Taken together, these data suggests treatments aimed at increasing endothelial HSP70 or HSP90 in this model could be used to determine the role of these proteins on plaque structure.

In summary, we provide evidence that smooth muscle cells, identified by smooth muscle cell actin + shape (spindle cells) in the atherosclerotic cap do not express markers of cellular stress, however cells expressing stress markers are present in the core. This infers that smooth muscle cells must become 'de-stressed' to migrate to the cap to form a stable plaque. This novel model can be used to study stress systems in coronary artery fibrous cap formation.

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