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Endothelial endoplasmic reticulum and nitrative stress in endothelial dysfunction in the atherogenic rabbit model

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ABSTRACT

Oxidative stress causes endothelial dysfunction which ultimately leads to atherogenesis, yet anti-oxidant therapy has failed to reduce human clinical outcomes. We hypothesise that endoplasmic reticulum stress and oxidative stress are both present in the endothelial layer of aorta with atherosclerosis. Rabbits were fed for 4 weeks a diet supplemented with 1% methionine +0.5% cholesterol (MC). Control animals received a normal diet. The endothelial function of the abdominal aorta was examined using organ bath techniques. Semi-quantitative immunohistochemistry was used to determine endothelial nitrotyrosine (for nitrative/oxidative stress) and glucose regulated protein 78 (GRP 78) and CHOP to determine endoplasmic reticulum stress. Endothelium dependent relaxation in response to acetylcholine significantly decreased in MC. Stress markers were significantly elevated in endothelia in MC compared to control. The total endothelial area examined for GRP78 increased by $8.4 \pm 0.25\%$ in MC vs control ($p = 0.026$) and C/EBP homologous protein (CHOP) increased by $21.9 \pm 0.05\%$ in MC vs control ($p = 0.014$). Nitrotyrosine increased by $13.3 \pm 0.03\%$ in MC vs control ($p = 0.012$).

Conclusions: Both endoplasmic reticulum stress and nitrative stress are present during endothelial dysfunction. Treatment directed at both stresses might be beneficial in the prevention of atherosclerosis.

1. Introduction

Intervention that improves endothelial function improves patient morbidity and mortality. Overwhelming evidence suggests that oxidative stress is involved in endothelial dysfunction (Alsaadon et al., 2015) and the oxidation of low density lipoprotein (LDL) leading to the development of atherosclerosis (Kucera et al., 2015; Hertelyova et al., 2015; Sabaka et al., 2013; Kang et al., 2014), and in this regard, several antioxidant therapies have been used in clinical studies to help prevent the burden of cardiovascular disease. Clinical trials using antioxidant therapies have extensively shown that they are ineffective (especially vitamin E) at preventing morbidity and mortality associated with atherosclerotic disease, and indeed in some cases could increase mortality (Bjelakovic et al., 2007; Wagner et al., 2014). However, with abundant evidence suggesting the opposite should hold true, it is important to determine whether alternative approaches to 'oxidative stress therapy' should be considered.

In this regard, accumulating evidence suggests that endoplasmic reticulum stress is also important in atherogenesis (Erbay et al., 2009). The accumulation of misfolded proteins in the endoplasmic reticulum has been shown to cause endoplasmic reticulum stress and activation of a protective response known as the unfolded protein response (UPR), which is believed to be one of the key initiating events in disease. Homocysteine (Outinen et al., 1999) and LDL (Sorensen et al., 2006) all cause ER stress and thus UPR and this can be detected via an increase in the proteins, glucose-regulated protein 78 (GRP78) and CHOP (Zulli et al., 2009).

Thus, this study was aimed at determining whether endothelial dysfunction caused by an atherogenic diet caused both oxidative and endoplasmic reticulum stress. We used the pan stress marker nitrotyrosine to detect both oxidative and nitrative stress, as well as GRP78 and CHOP to detect endoplasmic reticulum stress.

2. Methods

Male New Zealand White rabbits at three months of age received a normal rabbit chow diet supplemented with 0.5% cholesterol

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plus 1% methionine. The animals were housed in individual cages and maintained at a constant temperature of approximately 21 °C. Food and water were supplied *ad libidum*. The animals were fed their respective diet for 4 weeks ($n = 4$) to induce atherosclerosis as established in our laboratory (Zulli and Hare, 2009; Zulli et al., 2008a). Age matched controls were used in this experiment ($n = 6$). The experiments were carried out according to the National Health and Medical Research Council "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (6th Edition, 1997). The animals were then sacrificed by an overdose intra venous injection of ketamine and xylazine via the main ear vein as previously described in our laboratory (Zulli et al., 2009). The abdominal aorta proximal to the diaphragm was then excised and cleaned of connective tissue and fat. The project was approved by Victoria University Animal Ethics Committee (AEC03/11).

2.1. Isometric tension studies

The abdominal aortae was isolated and cut into 6 mm × 3 mm rings and every second ring mounted in organ baths filled with Krebs solution at 37 °C bubbled with carbogen (95% O_2/5% CO_2) (Zultek Engineering, OB8, Australia). Rings were gently stretched after 1 h to 2 g, and then after 1 h, the maximum constriction was determined by a high potassium Krebs solution (124 mM K^+). Once maximum contraction was achieved (approximately 6 min), the rings were rinsed with Krebs and then allowed to rest for 1 h. After this, rings were precontracted with phenylephrine to approximately 30–40% of maximal contraction. After the contraction stabilised, an acetylcholine concentration response curve (10^{-8} to 10^{-6}M Ach, half log units) was performed.

2.2. Semi-quantitative immunohistochemistry

Excess rings and the rings used in the organ baths were added to 4% paraformaldehyde in PBS pH 7.3 and left overnight. Then, rings were placed into PBS and all blood vessels were processed for paraffin embedding in one batch, as to maintain equal shrinking of vessels during processing. Paraffin infused rings were mounted vertically in two paraffin blocks, cut on a microtome at 5 μm, mounted on microscope slides and immunohistochemistry performed as previously published in our laboratory (Zulli et al., 2009, 2003a). Nitrotyrosine (Cat# MAB5404, mouse monoclonal (Rai and Zulli, 2013)) were purchased from Chemicon International and GRP78 from Santa Cruz (Cat# sc-1050, goat polyclonal (Zulli and Hare, 2009)), and CHOP from ABR (Cat#MA1-250, mouse monoclonal, USA (Zulli et al., 2009)). For mouse monoclonals, the 'Envision system' (Dakocytomation, USA) secondary polymer/peroxide was used and the chromagen was Diaminobenzideine (DAB). For goat polyclonal, the secondary donkey anti-goat at a dilution of 1:100 was used for 1 h (Abcam, Cat#ab6884) followed by steptavidin peroxidise (Sigma Aldrich, Cat# S5512) for 1 h. DAB was also used as the chromagen. Briefly, four images of endothelia overlying normal wall (ENW) or plaque (EP) of each aorta were obtained by an imaging camera (DFC 480, Leica Microsystems, USA). Then, three blinded, independent observers quantified the endothelium using image analysis software (MCID Elite 6.0, Imaging Research Inc. UK), by selecting the ribbon tool and selecting the hue, saturation and intensity to detect the brown pigment immunostain. Then, intensity and proportional area acquired and multiplied as established in our laboratory (Zulli et al., 2006, 2008b, 2009). All data pertaining to ENW or EP were then averaged, as well as ENW + EP for the MC group. Thus, data points shown are the mean of each animal ($n = 4$ (MC) or $n = 6$ (control)) and is the average of the entire section.

2.3. Data analysis

All data points were compared by unpaired Students' two tailed *t*-test. A $p < 0.05$ was accepted in all cases as significant. Endothelial function graphs were normalised to 100% according maximum relaxation observed in the control group and semiquantitative immunohistochemistry was normalised to control as 1. All data is expressed as mean ± SEM.

3. Results

After 4 weeks of dietary intervention, atherosclerosis was heterogeneously present throughout the aorta, however some areas were not affected and other areas only had intimal thickening. Endothelial dysfunction was present in the MC group compared to control (82 ± 6% vs 32 ± 13%, $p < 0.05$, Fig. 1A). Semi-quantification of stress markers revealed over expression of both nitrative and endoplasmic reticulum stress markers. The mean endothelial area detected for GRP78 increased by 8.4 ± 0.25% in MC vs control (Fig. 1B, $p = 0.026$) whereas CHOP increased by 21.9 ± 0.05% in MC vs control (Fig. 1C, $p = 0.014$). Nitrotyrosine increased by 13.3 ± 0.03% in MC vs control (Fig. 1D, $p = 0.012$). Fig. 2 shows the images used for endothelial quantification. For all stress markers, we found no difference between endothelium overlying normal wall between control and MC, but stress markers all increased in endothelium overlying plaque. Thus, in MC group, total mean area scanned was used for statistical analysis, that is all endothelia were averaged in this group. GRP78 (Fig. 2A–C) showed no difference between endothelial overlying normal wall area in control (Fig. 2A) and MC group (Fig. 2B, intimal thickened area shown for comparison in this instance), and this was also shown for CHOP (Fig. 2D, control vs Fig. 2E, normal endothelia in MC and Fig. 2F, endothelia overlying plaque). Similarly for nitrotyrosine (Fig. 2G, control vs Fig. 2H, normal endothelia in MC and Fig. 2I, endothelia overlying plaque). Plasma lipids and homocysteine were increased in the MC group vs control (Fig. 3).

4. Discussion

The major findings in this study is that both nitrative/oxidative stress and endoplasmic reticulum stress are present in endothelia overlying plaques in early atherogenesis, but not in endothelial overlying normal walls.

The disappointment of clinical trials with classic vitamin antioxidants has led to a rethink of the established theory. As common techniques used to detect oxidative stress have limitations (Dikalov et al., 2007), we chose to detect oxidative stress via a pan oxidative stress marker, nitrotyrosine. Peroxynitrite (ONOO—) is a 'reactive nitrogen species' that is formed by reaction of superoxide with nitric oxide, which reacts with the tyrosine residue of proteins to form nitrotyrosine. However, nitrotyrosine can also be formed by tyrosyl radicals, $NO_2^{\bullet}$, NO_3^-, NO_2Cl and HOCl (Halliwell, 1997). We show that nitrotyrosine was increased only in endothelia overlying atherosclerotic plaque, suggesting excess nitrated endothelial proteins which can cause endothelial dysfunction.

Recently, endoplasmic reticulum stress (ERS) has gained attention as a possible primary event involved in cardiovascular disease (Yoshida, 2007), and the association of nitric oxide with ERS has been recently reviewed (Gotoh and Mori, 2006). ER stress is mainly caused by the accumulation of misfolded proteins, leading to the unfolded protein response (UPR). Homocysteine (Outinen et al., 1999) and LDL (Sorensen et al., 2006) can cause ER stress and thus UPR. To determine UPR, an increase in both glucose-regulated protein (GRP) 78 and CHOP can be assessed. As determined in this study, both markers were increased only in endothelia overlying