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Biological effects of Osteocalcin in human endothelial cells

Saoirse O'Sullivan, Amanda Walker, Susan Anderson. University of Nottingham, Derby, UK

Osteocalcin (OC) or Bone Gla Protein (BGP) is a 5.8kD bone hormone comprising 49 amino acids coded by the BGLAP gene in osteoblasts. Once produced can either be carboxylated (cOC) at 3 glu residues, or remain uncarboxylated (ucOC). A proportion of both cOC and ucOC is released into the circulation. It has been proposed that OC may stimulate the differentiation & mineralisation of vascular smooth muscle cells, but it is unclear whether OC has direct effects of vascular functions other than calcification. The aim of the present study was to establish any effects of either cOC or ucOC on human endothelial cells.

Human aortic endothelial cell lines were grown to confluence in endothelial cell growth medium MV (Promo Cell) and treated with either cOC (Cat. 08060-09C, US Biological), ucOC (Cat. 65307, Anaspec) (both 10ng/ml representing plasma levels of OC) or vehicle for 5 or 30 min (n=6). The cell supernatant was used to screen for changes in cell signalling proteins using multiplex technology (Millipore, Multi-pathway 9-plex magnetic bead panel, Cat. 48-680MAG). Confluent cells were treated with either cOC, ucOC or vehicle (1ng/ml or 10ng/ml) for 72 hours (n=6) and analysis of cellular and secreted levels of Endothelin-1 was carried out by ELISA of cell lysate and medium respectively (Duoset ELISA, Endothelin Pan Specific Cat. DY1160). Data were analysed using one-way analysis of variance and Dunnett's *post hoc* analysis comparing against the vehicle control response (Prism GraphPad 6).

ERK phosphorylation in human endothelial cells was significantly increased by 5 min treatment with ucOC (median↑ 63%, $P<0.05$) or cOC (median↑ 114%, $P<0.001$). The phosphorylation of Akt ($P<0.01$) and its downstream target p70s6k ($P<0.05$), were significantly reduced with cOC, but not with ucOC. No significant changes were seen in phosphorylated levels of CREB, JNK, NFkappaB, p38, STAT3 or STAT5. After 30 min, Akt phosphorylation was still decreased in response to cOC ($P<0.05$). At 30 min, phosphorylated STAT5 was significantly increased after cOC treatment ($P<0.05$).

After 72 h treatment with 1 ng/ml ucOC, but not cOC, the secreted ($P<0.05$) and cellular ($P<0.05$) levels of endothelin-1 were significantly reduced. At 10 ng/ml, both ucOC significantly reduced the cellular ($P<0.01$) levels of endothelin-1. At 10 ng/ml, cOC significantly reduced the secreted ($P<0.001$) and cellular ($P<0.0001$) levels of endothelin-1.

These pilot data demonstrates for the first time that OC has direct effects on endothelial cells at physiologically relevant concentrations. The rapidity of changes in intracellular signalling proteins suggests a receptor target for OC on endothelial cells. One of the biological effects OC is a reduction in endothelin-1. Differences were observed in the actions of the carboxylated and uncarboxylated forms of OC which may have clinical relevance. Further experiments are required to establish other

biological effects of OC on endothelial cells, whether this is coupled to changes in vasomotor activity, and the mechanisms of action.