INTRODUCTION

Premature cardiovascular disease (CVD) is a serious long-term complication of chronic systemic inflammatory diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). Despite advances in disease-modifying and biological therapy for these diseases, we lack specific strategies aimed at retarding development of premature CVD and have limited knowledge of whether individual drugs offer vascular protection. Since endothelial dysfunction precedes overt atherosclerosis, investigating the ability of drugs to activate cytoprotective signalling pathways that prevent or reverse endothelial dysfunction is an attractive research strategy. Cytoprotective pathways include those regulated by AMP-activated kinase (AMPK). AMPK, a ubiquitous signalling kinase composed of a heterotrimeric complex of a catalytic α subunit and regulatory β and γ subunits, is generally considered a sensor of cellular metabolic status. In endothelial cells (EC), AMPK activity exerts multiple protective effects including enhanced endothelial nitric oxide synthase phosphorylation and nitric oxide synthesis, mitochondrial biogenesis, and protection against apoptosis and oxidative damage.

Methotrexate (MTX) therapy is central to the current treatment paradigms for RA. There is sound evidence that MTX reduces CVD in RA\(^4\) and improves clinical markers of endothelial dysfunction.\(^7\) These findings led to the recently initiated Cardiovascular Inflammation Reduction Trial, in which MTX or placebo is prescribed to patients with prior myocardial infarction (MI) to test the inflammatory hypothesis of atherothrombosis,\(^10\) an intriguing new direction in the treatment of CVD.

Mechanistic understanding of the vasculoprotective actions of MTX is sparse. However, it is known that long-lasting polyglutamate metabolites of MTX inhibit 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase and adenosine deaminase, leading to a rise in intracellular levels of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase and adenosine deaminase, leading to a rise in intracellular concentrations of AICAR-monophosphate (ZMP) and AMP.\(^11\) The subsequent accumulation and extracellular release of adenine has been proposed as the principal anti-inflammatory mechanism of action of MTX.\(^12\) 13 However, both ZMP and AMP are AMPK activators. We, therefore, hypothesised that the low-dose MTX regimen used in contemporary rheumatology practice activates AMPK, leading to enhanced expression of cytoprotective proteins within vascular EC: a new mechanistic of action to explain its clinical action in reducing CVD associated with inflammation.

MATERIALS AND METHODS

Detailed methods are supplied in the online supplementary file.
MTX phosphorylates AMPK and induces MnSOD and other cytoprotective target genes

To identify potential mechanisms underpinning vascular protection, we investigated the hypothesis that, through its effects on nucleotide metabolism, MTX may activate AMPK, a signaling kinase with significant vascular protective actions, including manganese superoxide dismutase (MnSOD) induction. MnSOD is a mitochondrial antioxidant enzyme that catalyses the conversion of superoxide into hydrogen peroxide. Induction of MnSOD is an important vascular cytoprotective response, capable of protecting against mitochondrial damage and atherogenesis. To establish whether MTX activates human endothelial AMPK, HUVECs were exposed to 100 nM MTX for up to 72 h. The concentration chosen is able to increase intracellular AICAR in vitro and is achievable in patient plasma following conventional low-dose therapeutic dosing. AMPKα1-Thr172 phosphorylation was observed after 48 h treatment, but not earlier, in HUVEC (figure 2A, B; 24 and 72 h data not shown) and in HAECs (see online supplementary figure IA, B). The delay in phosphorylation suggests an indirect action, such as the accumulation of AICAR and AMP following inhibition of AICAR transformylase by MTX polyglutamate metabolites, rather than a direct action of MTX on upstream AMPK-kinases.

In addition to AMPK phosphorylation, induction of MnSOD mRNA and protein was seen after 48 h of MTX treatment. This response was concentration-dependent, first seen with MTX 0.1 nM and maximal at 10 and 100 nM (see figure 2C–E; time course online supplementary figure IC) and reproducible in HAECs (see online supplementary figure ID, E). Increased MnSOD was also detected following AICAR treatment of HUVECs (figure 2F, G), and has previously been linked with AMPK activity. In addition to MnSOD, 48 h exposure to low-dose MTX increased expression of other important endothelial cytoprotective genes. These included the antioxidant, anti-inflammatory, antiapoptotic enzyme haem oxygenase-1 (HO-1; figure 3A and online supplementary figure IF, G); Bcl-2-related protein AI (A1), an antiapoptotic member of the Bcl-2 family (figure 3B), and uncoupling protein-2 (UCP2), important for regulation of mitochondrial-reactive oxygen species generation (figure 3C). After 72 h, induction of the complement-inhibitory protein decay-accelerating factor (DAF, CD55) was also observed (figure 3D). Thus, MTX-mediated AMPK activation in EC is linked to the regulation of a variety of AMPK-dependent protective genes, all of which are antiatherogenic.

MnSOD induction by MTX requires AMPK and CREB activation

To dissect further, the transcriptional pathway involved in MnSOD induction by MTX, cyclic AMP-response element binding protein (CREB) was identified as a potential candidate. CREB is a direct downstream target of AMPK, its activity is positively associated with vascular health, and it has been implicated in MnSOD induction. CREBser133 phosphorylation was observed following treatment of HUVEC with MTX (figure 4A, B). In order to determine the role of AMPK, a loss-of-function approach was adopted. HUVECs were transfected with siRNA directed against the AMPKα1 or α2 subunit (see online supplementary figure IA, B), prior to MTX treatment. MTX-mediated CREBser133 phosphorylation was abrogated by AMPKα1 or α2 siRNA (figure 4A, B), suggesting a linear signalling pathway between AMPK and CREB.
Next, the same approach was used to establish whether MnSOD induction by MTX is dependent upon AMPK activation. Induction of MnSOD mRNA and protein by MTX was significantly attenuated by AMPKα2 siRNA (figure 4A, C, D). Similar results were obtained following depletion of AMPKα1 (figure 4A; quantification data not shown). Likewise, analysis of HO-1 expression showed that depletion of AMPKα1 or α2 inhibited MTX-mediated HO-1 induction (see online supplementary figure IIC–E). Additionally, treatment of HUVECs with Compound C, a pharmacological AMPK antagonist, significantly reduced induction of MnSOD and HO-1 mRNA by MTX (see online supplementary figure IIF, G). Further investigation demonstrated the functional role of CREB, with siRNA depletion (see online supplementary figure IIIA, B) preventing MnSOD and HO-1 induction by MTX (see figure 4E and online supplementary figure IIIC).

Chromatin immunoprecipitation was performed to look for enhanced CREB binding to the MnSOD promoter in HUVECs.
treated with MTX. Analysis of the SOD2 promoter (GenBank accession number AF059197) using MatInspector software identified two strong potential CREB binding sites (see online supplementary figure IV), the more proximal of which has previously been validated using a reporter assay and a series of deletion constructs.\(^26\) Significant enrichment of CREB binding to the MnSOD promoter following MTX treatment was found using primers designed to interrogate the known validated binding site (figure 4F). No enhanced binding was seen with primers designed around a negative control downstream region.

MnSOD and HO-1 induction by MTX is maintained when ECs are treated with folic acid or TNF\(\alpha\)

To determine whether the low-dose MTX-induced changes in gene expression in quiescent ECs are relevant clinically, we investigated responses in cells coadministered with folic acid (FA), and those exposed to tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) to model an activated, dysfunctional endothelium.

FA is routinely prescribed alongside MTX to reduce side effects. When HUVECs were treated with clinically relevant concentrations of FA (50 nM)\(^27\) and MTX in combination, no

Figure 2  MTX treatment leads to AMPK\(\alpha\) phosphorylation and induction of MnSOD mRNA and protein. (A) and (B) HUVEC were treated with MTX 100 nM for 48 h. AMPK\(\alpha\) phosphorylation was demonstrated by immunoblot (A; with densitometry B), using AMPK agonist AICAR 1 mM as positive control. HUVECs were treated with MTX 0–100 nM and (C) MnSOD mRNA, and (D) and (E) MnSOD protein were quantified after 48 h by qRT-PCR and immunoblotting, respectively. (F) and (G) HUVEC were treated with AICAR for up to 48 h and MnSOD quantified by immunoblotting. Each experiment was performed three to five times. A: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical vein endothelial cells; MnSOD, manganese superoxide dismutase; MTX, methotrexate; UT, untreated. *p<0.05; **p<0.01.
change in the magnitude of MnSOD induction was observed (figure 5A).

Patients with RA and also those with primary coronary artery disease develop endothelial dysfunction as an early feature. TNFα is an important mediator and was chosen to model endothelial dysfunction in vitro. ECs were exposed to TNFα 1 ng/mL for 24 h prior to the addition of MTX for 48 h. MTX-induced upregulation of MnSOD and HO-1 mRNA was preserved (figure 5B, C). These findings confirm the ability of MTX to condition ECs in the face of a chronic proinflammatory stimulus.

MTX protects against endothelial apoptosis induced by glucose deprivation

Next, the cytoprotective actions of MTX were investigated. The principal function of AMPK activation is to conserve energy; it is, therefore, critically important in the cellular response to glucose deprivation.1 We hypothesised that survival of ECs exposed to a glucose-deficient medium would be prolonged if they were pretreated with MTX, as AMPK signalling would already be active.

ECs were treated with MTX 100 nM for 48 h, and then maintained for 18 h in Hanks’ balanced salt solution (HBSS) or glucose-deficient HBSS. Early apoptosis was detected by Annexin V staining, and established cell death by permeability to propidium iodide (PI), using flow-cytometric quantification. Glucose deprivation led to a marked increase in Annexin V binding to the EC surface and doubling of PI-positive cells. These responses were significantly reduced by MTX (figure 5D–G). Moreover, MTX did not increase EC apoptosis under normoglycemic conditions, although there was a modest reduction in total cell numbers consistent with impaired proliferation (see online supplementary figure V). These data suggest that the primary effect of MTX treatment on vascular EC is protective and that AMPK activation and subsequent protective gene induction do not represent a stress response to a noxious stimulus.

MTX phosphorylates AMPK and increases MnSOD in murine aortic tissue and reduces endothelial and adventitial ICAM-1 expression

Finally, snap-frozen descending aortae harvested from the MTX-treated and saline-treated animals described above were used to analyse the effect of MTX therapy on the protective pathways in vivo. Immunoblotting of WBF1 aortic lysates revealed increased AMPKαThr172 phosphorylation and MnSOD protein expression in MTX-treated animals (figure 6A–D).

Given that MTX is also a powerful anti-inflammatory agent, it is likely that vascular protection induced by MTX is mediated, at least in part, by anti-inflammatory actions. To assess this, aortic intercellular adhesion molecule (ICAM)-1 expression was quantified. Transverse aortic sections from MTX and saline-treated WBF1 mice were stained with an antimouse ICAM-1 monoclonal antibody and examined by immunofluorescence confocal microscopy. MTX therapy reduced both endothelial and adventitial ICAM-1 staining (figure 6E–H). The reduction in adventitial staining is likely to be of particular relevance in the amelioration of organ damage in this model, given the reduction in the adventitial leucocytic infiltrate also observed in the intramyocardial arteries (figure 1A–C).

**DISCUSSION**

The current study identifies a novel MTX-activated protective pathway which may underpin the ability of MTX to reduce CVD associated with chronic inflammation. However, the historical general perception of MTX is that it is harmful and clinically effective only because it kills pathological tissue before normal cells are irreversibly damaged. Indeed, early studies on MTX and CVD in RA suggested CVD was increased.28

**Figure 3** MTX treatment results in the induction of AMPK target genes. HUVEC were treated with MTX 100 nM for 48 h and mRNA levels of (A) HO-1, (B) A1, and (C) UCP-2 mRNA were analysed by qRT-PCR. (D) Induction of DAF mRNA was measured after 72 h MTX treatment. Each graph represents the results of four experiments. AMPK, AMP-activated protein kinase; A1, Bcl-2-related protein A1; DAF, decay-accelerating factor; HO-1, haem oxygenase-1; HUVEC, human umbilical vein endothelial cells; MTX, methotrexate; UCP-2, uncoupling protein-2; UT, untreated. *p<0.05; **p<0.01.

Subsequent experience is, however, altering perceptions. Several studies have demonstrated that long-term low-dose MTX therapy in RA (15–20 mg/week) is associated with reduced CVD, with Choi et al reporting a 70% reduction in cardiovascular (CV) mortality. Likewise, MTX therapy reduced atheroma in cholesterol-fed rabbits. These results might reflect an antiatherogenic action of adenosine via ligation of its A2A receptor which, in addition to an anti-inflammatory action, may induce reverse cholesterol transport proteins and prevent foam cell formation. However, our study supports an additional mechanism, namely that MTX exerts a direct beneficial effect on vascular endothelium.

Patients with RA and SLE with normal epicardial coronary arteries exhibit coronary microvascular dysfunction which may precede and contribute to accelerated atherosclerosis. Thus, to explore the arterioprotective actions of MTX, we investigated WBF1 mice, which develop an inflammatory vasculopathy of small muscular arteries and arterioles that predispose to thrombosis and tissue infarction. A previous attempt to treat WBF1 mice with MTX failed to show improvement in mortality or...
renal function at 30 weeks. However, the study used threefold higher doses of MTX than stated herein, and this may have contributed to mortality, given that MTX accumulates in renal failure which is universal in older WBF1 mice. Treatment of mice with early disease demonstrated that MTX reduces the severity of the vasculopathy and attenuates organ damage. MTX exerted specific anti-inflammatory actions, reducing aortic endothelial and adventitial ICAM-1 and preventing leucocytic infiltration of the arterial wall, suggesting an additional mechanism by which MTX may retard CV events in RA. However, despite the fact that MTX therapy activates protective pathways and that basic pathogenic mechanisms of arterial injury in the WBF1 mice including immunoglobulin deposition, complement activation, focal leucocyte infiltration and endothelial damage are generally applicable across many systemic inflammatory diseases, they do not necessarily translate to the situation in human RA. Thus, further studies are now required in patients.

AMPK is associated with anti-inflammatory and desirable metabolic changes in many different systems and disease settings. Understanding of the relationship between AMPK, endothelial dysfunction and atherogenesis remains incomplete. However, current thinking suggests that reduced AMPK activity predisposes to endothelial dysfunction, while AMPK activation by laminar shear stress may contribute to vasculoprotection. Thus, our finding that MTX activates AMPK in human ECs may provide an important mechanistic explanation for the clinical observation of reduced CVD in patients with RA, prescribed this drug.

The current study demonstrates AMPK phosphorylation and induction of protective target genes using MTX concentrations in vitro that are achievable in patient sera. Moreover, increased phosphorylated AMPK and upregulation of the downstream target MnSOD were identified in murine aortae following MTX administration using a regimen analogous to the long-term, low-dose therapy used for chronic inflammatory disease. This strongly suggests that MTX-mediated AMPK activation is likely to be a real phenomenon in patients. We have also reported that, in vitro, the anti-inflammatory drugs, celecoxib, can specifically induce EC AMPK phosphorylation, while at supra-therapeutic concentrations, metformin has a similar effect. Further investigation is required to establish the extent to which these observations can be directly translated to patients.

Although we have yet to determine how MTX activates AMPK, we speculate that this is secondary to increased intracellular ZMP and AMP levels. ZMP and AMP bind to the AMPKγ subunit, delaying dephosphorylation of Thr172 in the α

Subunit. Exposure of EC to MTX resulted in delayed AMPK phosphorylation, consistent with inhibition of AICAR transformylase and adenosine deaminase leading to accumulation of ZMP and AMP. These enzymes are most potently inhibited by MTX-polyglutamates, an important fact, given that MTX is rapidly converted to MTX-polyglutamates, the erythrocyte concentrations of which are more closely associated with clinical responses than MTX plasma levels. MTX is known to increase AICAR levels in HUVECs after a 48 h treatment and can enhance activation of AMPK by AICAR in cancer cell lines. While this manuscript was under review, Pirkmajer et al have shown that MTX and AICAR together increase ZMP in cultured myotubes, supporting our hypothesis as to how MTX activates AMPK.

Previous studies have shown that the AMPKα1 isoform is more abundant in ECs and have, therefore, focused on its functional effects. Recent evidence suggests that important vasculoprotective effects are also mediated through AMPKα2. Our study demonstrates that depletion of either α subunit attenuates MTX-mediated MnSOD and HO-1 induction. For the regulation of MnSOD, AMPKα2 activity seemed somewhat more important than α1, a fact supported by the finding that addition of SOD to aortae from AMPKα2−/− mice can rescue endothelial dysfunction. Interestingly, the α2 subunit is thought to be the more sensitive to AMP, and this is consistent with our speculation that MTX activates AMPK by altering levels of AMP.

MTX treatment attenuated apoptosis induced by glucose deprivation, a response replicated by the AMPK agonist AICAR. The underlying mechanism is likely multifactorial. AMPK activation moves cells away from glycolysis as an ATP source, and promotes mitochondrial oxidative phosphorylation, thus prolonging cell survival in low glucose conditions.
Additionally, induction of A1 and HO-1 by MTX may exert antiapoptotic effects. MnSOD, UCP-2 and HO-1 induction will also impart important antioxidant effects which improve cell survival. Although beyond the scope of this initial report, induction of additional AMPK targets A1, UCP-2 and DAF suggest that a whole-genome approach is now indicated to identify the full extent of the cytoprotective profile of MTX and to determine how its actions may be replicated by novel, more specific compounds.

The demonstration that MTX activates an AMPK/CREB-dependent signalling pathway to induce MnSOD and HO-1 reveals significant atheroprotective potential. MnSOD induction protects against atherogenesis, and ApoE−/− mice deficient in MnSOD exhibit accelerated atherosclerosis.21 Recent evidence points towards a central role for CREB in the maintenance of a healthy arterial wall. Loss of aortic CREB is found in rodent models of hypertension, atherosclerosis and insulin resistance,27 while murine cardiac-specific expression of dominant-negative CREB increases oxidative stress, mitochondrial dysfunction and mortality.38 Although CREB39,40 is a direct target of AMPK,24 CREB activation by MTX might also occur via adenosine binding of G protein-coupled receptors, leading to protein kinase A activation via CAM. However, this well-described mechanism of CREB activation41 is thought only to occur on promoters where the CRE site is within 250 bp of the TATA box.40 The SOD2 promoter does not contain a TATA box,41 and the CRE site likely to be responsible for the effects of CREB binding presented here lies 1200 bp distal to the transcription start site. This favours AMPK activation rather than adenosine as the route to CREB activation by MTX.

The lack of evidence demonstrating a requirement for AMPK-CREB signalling for the protective effects of MTX in vivo is a limitation of this study. Their complex genetic background and the need to generate F1 animals to develop vasculo-inflammation in the need to generate F1 animals to develop vasculo-inflammation precludes crossing WBF1 mice with an AMPK-deficient strain. Likewise, data obtained by treating animals daily with the AMPK antagonist, Compound C, would be confounded by the observation that the drug itself induces protective enzymes including HO-1 and MnSOD.42 However, AMPKα activation and MnSOD induction were demonstrated in aortae from MTX-treated animals, suggesting they play a role in vascular protection. Nevertheless, increased adenosine may also contribute to the vascular effects of MTX. Adenosine reduces cytokine-mediated cell adhesion molecule upregulation in EC,43 and MTX treatment promotes vasodilatory responses attributed to ligation of adenosine 2A receptors.44

In conclusion, we have presented evidence for a novel MTX mechanism of action in the vasculature, which identifies specific effects of low-dose MTX and moves the perception of this drug from toxic to protective. We propose that low-dose MTX therapeutically conditions vascular endothelium via activation of AMPK-CREB signalling, so inducing cytoprotective genes which may contribute to the efficacy of MTX in reducing CV complications in patients with RA.

Acknowledgements The authors thank Lorraine Lawrence for technical assistance.

Contributors CCT helped design the study, developed assays, performed the majority of research and data analysis and helped write the paper. FA-R and DC performed some of the immunoblotting experiments. GMB, AMR and JJB helped with bioinformatics analysis and development of confocal and light microscopy techniques. AB and HM also contributed to the experimental data shown. DOH and JJB contributed throughout to the study design, ongoing research and editing of the manuscript. JCM and BIM conceived the study, participated in the research and data analysis and wrote the paper.

Funding This study was funded by the Wellcome Trust (WT088515MA) and the Rosetrees Trust (A379). The authors acknowledge support from the Imperial College, National Institute for Health Research, Biomedical Research Centre.

Competing interests None.

Ethics approval Form Imperial College ethics committee (06/Q046/21) and the UK Home Office (70/6722).

Provenance and peer review Not commissioned; externally peer reviewed.

Open Access This is an Open Access article distributed in accordance with the terms of the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited. See: http://creativecommons.org/licenses/by/4.0/

REFERENCES

Methotrexate-mediated activation of an AMPK-CREB-dependent pathway: a novel mechanism for vascular protection in chronic systemic inflammation

C C Thornton, F Al-Rashed, D Calay, G M Birdsey, A Bauer, H Mylroie, B J Morley, A M Randi, D O Haskard, J J Boyle and J C Mason

*Ann Rheum Dis* published online January 9, 2015

Updated information and services can be found at:
http://ard.bmj.com/content/early/2015/01/09/annrheumdis-2014-206305

**Supplementary Material**
Supplementary material can be found at:
http://ard.bmj.com/content/suppl/2015/01/09/annrheumdis-2014-206305.DC1

**References**
This article cites 43 articles, 24 of which you can access for free at:
http://ard.bmj.com/content/early/2015/01/09/annrheumdis-2014-206305#BIBL

**Open Access**
This is an Open Access article distributed in accordance with the terms of the Creative Commons Attribution (CC BY 4.0) license, which permits others to distribute, remix, adapt and build upon this work, for commercial use, provided the original work is properly cited. See:
http://creativecommons.org/licenses/by/4.0/

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Topic Collections**
Articles on similar topics can be found in the following collections

- Open access (605)
- Connective tissue disease (4253)
- Degenerative joint disease (4641)
- Epidemiology (1367)
- Genetics (968)
- Immunology (including allergy) (5144)
- Inflammation (1251)
- Musculoskeletal syndromes (4951)
- Rheumatoid arthritis (3258)

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/