

NFκB inhibition mitigates serum amyloid A-induced pro-atherogenic responses in endothelial cells and leukocyte adhesion and adverse changes to endothelium function in isolated aorta

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Abstract (198 words)

The acute phase protein serum amyloid A (SAA) is associated with endothelial dysfunction and early-stage atherogenesis. Stimulation of vascular cells with SAA increases gene expression of pro-inflammation cytokines and tissue factor (TF). Activation of the transcription factor, nuclear factor kappa-B (NFκB), may be central to SAA-mediated endothelial cell inflammation, dysfunction and pro-thrombotic responses, while targeting NFκB with a pharmacologic inhibitor, BAY11-7082, may mitigate SAA activity. Human carotid artery endothelial cells (HCtAEC) were pre-incubated (1.5 h) with 10 μM BAY11-7082 or vehicle (control) followed by SAA (10 μg/mL; 4.5 h). Under these conditions gene expression for TF and TNF increased in SAA-treated HCtAEC and pre-treatment with BAY11-7082 significantly (TNF) and marginally (TF) reduced mRNA expression. Intracellular TNF and IL-6 protein also increased in HCtAEC supplemented with SAA and this expression was inhibited by BAY11-7082. Supplemented BAY11-7082 also significantly decreased SAA-mediated leukocyte adhesion to apolipoprotein E-deficient mouse aorta in *ex vivo* vascular flow studies. In vascular function studies, isolated aortic rings pre-treated with BAY11-7082 prior to incubation with SAA showed improved endothelium-dependent vasorelaxation and increased vascular cGMP content. Together these data suggest that inhibition of NFκB activation may protect endothelial function by inhibiting the pro-inflammatory and pro-thrombotic activities of SAA.

Key words: Nuclear, Transcription, endothelium, atherosclerosis, serum amyloid A, Aorta

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Introduction

The endothelium plays a critical role in regulating immune processes in the vasculature by providing a structural barrier, a non-thrombogenic surface and maintaining vascular tone [1]. However, pathophysiological activation of the endothelium facilitates vascular inflammation and endothelium dysfunction is implicated in the progression of atherosclerosis and other chronic inflammatory diseases [2]. Endothelial cell activation occurs in the earliest stages of cardiovascular disease and is linked to loss of barrier function, focal inflammation, lipid accumulation and pro-coagulation [3]. Endothelium activation and dysfunction also impact on cardiovascular risk factors such as hypertension [4], type 1 diabetes [5] and rheumatic disorders [6] all diseases characterized by a sustained increase in circulatory levels of several inflammatory biomarkers including serum amyloid A (SAA).

SAA is an acute phase protein that is significantly up-regulated by infection or injury [7]. Under these conditions (cardiovascular diseases, type 1 diabetes, rheumatic disorders) SAA is produced primarily in the liver [8] although other source include coronary artery endothelial cells [9]. Whilst SAA is an acute phase protein, circulating levels are evident in various inflammatory disorders such as non-insulin dependent diabetes, and can exceed 2 mg/L in chronic inflammatory conditions [10].

In addition to functioning as an inflammatory biomarker, SAA is proposed to stimulate several cellular activities linked to atherogenesis. Plasma SAA predicts adverse effects in patients with vascular disease and SAA is found at sites of thrombus and plaque rupture in atheroma [11, 12]. SAA is also known to induce pro-inflammatory and pro-thrombotic activities in endothelial cells, including up-regulation of adhesion molecules, decreased nitric oxide (NO) production and bioactivity, and accumulation of reactive oxygen species (ROS) [13], all effects linked to endothelial dysfunction, which precedes atherogenesis [2, 14]. Furthermore, SAA induces production of tissue factor (TF) and tumour necrosis factor (TNF) in peripheral blood mononuclear cells (PBMC) and immortalised macrophage cells [15, 16] as well as other pro-inflammatory cytokines such as IL-1B, monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-8 and macrophage inflammatory protein-1 alpha (MIP-1 α) in both monocytes and local stromal cells [15, 17].

In vivo studies also indicate a pro-atherogenic role for SAA, specifically, the acceleration of atherosclerosis in apolipoprotein E-deficient mice via promotion of recruitment and adhesion of macrophages at the inflammatory sites [18, 19]. The pro-inflammatory mediators (MCP-1, MMP-9, TF) and adhesion molecules (ICAM-1, VCAM-1) are increased upon SAA stimulation in

atherosclerotic lesions [18]. Moreover, SAA up regulates the LDL scavenger receptor that drives foam cell and lesion formation [20].

The pro-atherogenic effects of SAA may be mediated via the transcription factor NF κ B – a key gene expression regulator of various inflammatory cytokines, chemokines and adhesion molecules [21]. NF κ B can be induced by SAA in endothelial cells [22, 23] and is implicated in SAA-mediated TF and/or cytokine expression in monocytes [15, 17, 24], macrophages [16] and endothelial cells [25]. Activation of cell surface receptors for SAA such as receptor for advanced glycation end products (RAGE), toll-like receptor (TLR) and formyl peptide receptor-like 1 (FPR1) also stimulate NF κ B activation [22] and inhibition of these receptors, or SAA activity, can ameliorate SAA-mediated endothelial dysfunction [26]. The cytokine TNF is up-regulated by SAA in many cell types and stimulates adhesion molecule expression in endothelial cells, also induces nuclear factor kappa-light-chain-enhancer of activated B cells-1 (NF κ B1) a member of the NF κ B family of transcription factors [27]. Overall, these data suggest that NF κ B may play a pivotal role in mediating the pro-atherogenic effects of SAA in the endothelium under pathophysiological conditions. The present work employs ex vivo and in vivo studies aimed to determine whether pharmacologic inhibition of NF κ B activation and down-regulation of its transcriptionally activated target genes protects the endothelium from SAA induced adverse effects.

Results

HCtAEC supplemented with 10 $\mu\text{g/mL}$ SAA showed ~2-fold increase in secretory levels of VEGF relative to control cells (Figure 1), albeit this did not reach statistical significance. BAY11-7082 (1-100 μM) dose-dependently inhibited the level of secretory VEGF induced by SAA (Figure 1). At 10 μM (BAY11-7082), secretory VEGF was reduced by ~40% compared to SAA alone and this concentration of the NF κ B inhibitor (BAY11-7082) was selected for all further studies on gene and protein analysis. This selected (active) dose of BAY11-7082 (10 μM) is lower than that used by others [28, 29] and provides an advantage of minimising potential non-specific inhibitory activity in cultured HCtAEC.

Gene expression

HCtAEC supplemented with SAA at normo-glycemic conditions (media containing 5 mM D-Glucose), showed significantly higher TNF mRNA (>30 x; $P<0.05$ vs control cells) and discernibly higher but non-significant TF mRNA expression (Figure 2). Pre-treatment of HCtAEC with BAY11-7082 (10 μM) prior to SAA supplementation significantly inhibited the TNF mRNA expression and showed marked but non-significant inhibition of TF mRNA expression in the same cultured endothelial cells (Figure 2) suggesting that the selected dose of inhibitor was able to inhibit SAA pro-inflammatory actions on HCtAEC.

Inflammatory Protein expression

The secretory levels of IL-6 were assessed as an independent marker of inflammation. The concentration of cell confluency normalised secretory IL-6 was significantly up-regulated (~10-fold; $P<0.05$) in HCtAEC exposed to 10 $\mu\text{g/mL}$ of SAA. By contrast, pre-treatment of cells with BAY11-7082 markedly abated SAA-mediated secretion of IL-6 yielding levels below that detected in the corresponding vehicle-treated control cells (Figure 3). Taken together, these data suggest that pharmacological inhibition of NF κ B can diminish downstream inflammatory markers.

Immunocytochemistry analysis

Next, immunocytochemistry experiments were performed to assess the level and distribution of IL-6 and TNF (Figure 4) in order to determine whether gene regulation resulted in corresponding transcriptional regulation of the inflammatory cytokines. HCtAEC treated with 10 $\mu\text{g/mL}$ SAA displayed more IL-6 positive (red) staining in the cell cytoplasm than the control group in the absence of SAA stimulation, (compare Figures 4A and B). IL-6 immuno-positive staining markedly diminished in cells pre-treated with 10 μM BAY11-7082 prior to SAA insult (compare Figures 4B & C and corresponding insets highlighting IL-6⁺ immune-fluorescence within single cells). As predicted based on SAA-mediated increases in TNF mRNA (see Figure 2), immuno-positive TNF

increased in the cytoplasm of HCtAEC following incubation with 10 $\mu\text{g/mL}$ of SAA (compare Figures 4D & E) while a decreased cytoplasmic accumulation of TNF was noted in cells pre-incubated with BAY11-7082 (Figure 4F). These results confirm that BAY11-7082-mediated inhibition of NF κ B concomitantly decreases secretory IL-6 protein (consistent with data shown in Figure 3) while also demonstrating that TNF protein expression was also sensitive to inhibition of NF κ B in HCtAEC in the presence of SAA.

Leukocyte endothelial adhesion

Aortae isolated from ApoE-deficient mice administered with SAA showed a significant increase in adherent leukocytes (Figure 5A, C), which was almost completely inhibited by pre-treatment with BAY11-7082 prior to administering SAA (Figure 5B, C). Under these experimental conditions, control mice that were injected with sterile saline (as a control) showed minimal adherent cells that did not differ markedly from the aortae from mice treated with BAY11-7082 (data not shown). Together this suggests that SAA stimulates leukocyte endothelial adhesion to vascular endothelium via NF κ B activation pathways, which can be significantly inhibited by pre-treatment with the NF κ B inhibitor BAY11-7082.

Vascular function test

Addition of SAA to isolated rat aortic rings inhibited endothelium-dependent relaxation in the presence of ACh (Figure 6A); this loss in vaso-relaxation has been ascribed previously to SAA stimulating increased production of superoxide radical anion that deactivates vaso-dilating nitric oxide [13]. Pre-incubation of aortic segments with BAY11-7082 significantly improved endothelium-dependent relaxation in the presence of SAA compared to rings without BAY11-7082 pre-treatment. However, the level of vascular relaxation did not reach that of the control (in the absence of SAA and BAY11-7082) and was also markedly less than endothelium-independent relaxation stimulated by the endothelium-independent vaso-dilator, SNP (Figure 6A).

Owing to increased TNF expression after SAA insult and its impact on reducing the bioavailability of NO implicated in the vascular relaxation, we measured cGMP levels as an indicator of endothelium function. The levels of aortic cGMP, that are related to the presence and bioactivity of NO were consistent with the altered relaxation profiles exhibited in the same aortic tissue segments (Figure 6B). Thus, aortic segments stimulated with SAA showed significantly reduced tissue cGMP compared to controls in the absence of SAA activation. Furthermore, pre-treatment with BAY11-7082 prior to incubation with SAA significantly increased aortic cGMP compared to vessel segments incubated with SAA alone, although the extent of cGMP recovery did not reach control or SNP-elicited levels (Figure 6B).

Discussion

Previous studies have shown SAA to be a significant promotor of endothelial dysfunction, a precursor to the development of vascular disease [7, 26]. Attempts to use pharmacological approaches to block SAA receptor activation have proven to be less effective in ameliorating SAA bioactivity compared with athero-protective HDL (a potential *in vivo* regulator of SAA activity) [23]. Herein, the data demonstrate that inhibition of the transcription factor NF κ B, concomitantly inhibits the pro-inflammatory and pro-thrombotic activity of SAA on cultured vascular endothelial cells. Thus, HCtAEC stimulated by SAA induced the expression of pro-inflammatory mediators including IL-6 and TNF while prothrombotic TF and growth factor VEGF also increased in the presence of SAA. Pre-treatment of the endothelial cells with the NF κ B inhibitor BAY11-7082 blocked SAA-stimulated mRNA expression of TNF and TF in the endothelium. Likewise, IL-6 and TNF proteins decreased in the presence of the NF κ B inhibitor post-stimulation with SAA. In aortae isolated from apolipoprotein E-deficient mice, supplementation of the mice with BAY11-7082 also significantly decreased SAA-mediated leukocyte adhesion. Further, freshly isolated rat aortic rings bathed in media containing BAY11-7082 both reversed SAA-mediated inhibition of vessel relaxation stimulated by acetylcholine and increased aortic cGMP levels in parallel indicating markedly improved endothelium-dependent vasodilatation. Overall, these data suggest that blockade of this transcription factor may effectively inhibit SAA bioactivity and ameliorate vascular inflammation that may be central to many developing vascular pathologies.

In studies with cultured human carotid artery endothelial cells performed here, addition of recombinant SAA up-regulated inflammation through promoting cellular production of several mediators including TNF and IL-6 as well as pro-thrombotic TF, consistent with prior work [7, 22]. These pro-inflammatory and pro-thrombotic elements are implicated in atherosclerosis and other chronic inflammatory diseases. Cell-derived mediators such as TNF are critical to immune regulatory and pro-inflammatory responses that are central to combating acute and chronic events. The data herein indicate that TNF mRNA increases after exposure of endothelial cells to SAA, which is in agreement with previous studies that observed a similar TNF response in other primary endothelial cells [22] as well as human monocyte cell line THP-1 [7]. Indeed, increased production of TNF has been linked to a number of pathologies where SAA is also increased [7]. In particular, TNF levels have been found to be significantly up regulated in mice prone to developing atherosclerosis, and levels of TNF were directly linked to SAA increase SAA plasma [18].

Furthermore, the chemokine TNF activates cell surface expression of important adhesion molecules (ICAM-1, VCAM-1) that are central to leukocyte recruitment [30]. In addition, TNF acts as an

instigator of the expression and biosynthesis of other mediators of the innate immune system such as TF and IL-6 [31]. This is particularly relevant in the model of SAA-mediated endothelial dysfunction used here where increased TF and IL-6 were detected, consistent with TNF amplifying the local pro-inflammatory environment and promoting endothelial dysfunction, a characteristic of the SAA-stimulated vascular endothelium [13].

Closely related to pro-inflammation is TF and its cascade of pro-coagulatory events through interaction with thrombin [32]. Thus, TF is clinically significant in thrombus formation and is implicated in atherogenesis [33], and can be a predictor of acute coronary syndromes [34]. Discernibly higher TF mRNA was detected in HCtAEC stimulated with SAA consistent with other cell models of SAA induced endothelial dysfunction [22, 35]. Interleukins and particularly, IL-6 may play a multi-functional role in SAA-mediated endothelial dysfunction [7]. Increased accumulation of IL-6 has been shown by others to promote a local pro-inflammatory environment [36] as well as increase vascular permeability to blood borne components [37]. In this study, we observed a marked increase in IL-6 protein secretion by SAA-stimulated HCtAEC. Interestingly, secretion of IL-6 is not only implicated in downstream activities of SAA but may also act upstream of the acute phase protein. Thus, IL-6 is known to be a potent inducer of cellular synthesis of SAA during inflammation [38] by various cell types including those of the liver such as hepatocytes, Kupffer cells [8] and in human monocytic leukaemia cell lines (THP-1) [39]. If SAA-stimulation leads to production of IL-6 by a dysfunctional endothelium, then this interleukin may induce the production of additional SAA, thereby establishing an autocrine loop and effectively exacerbating endothelial dysfunction. Evidence for this IL-6-driven autocrine loop has been reported previously in thrombin-activated endothelial cells [40].

It should be noted that for SAA-induced IL-6 production by HCtAEC, the secretory form of IL-6 was more prevalent than intracellular accumulation of this interleukin, suggesting that a majority of IL-6 produced is trafficked to the extracellular domain. Similar cytokine trafficking trends have been found to occur in studies of rheumatoid arthritis patients [41]. The observation that much of the interleukin was exocytosed 24 h after SAA insult supports the notion that trafficking of this cytokine favours its release into the extracellular compartment and this is likely to involve a mechanism of exocytosis of vesicles enriched with IL-6 as detected by immune-fluorescent microscopy in this study (refer to Figure 4).

Of the observed mediators in our system, IL-6 displayed the strongest response to BAY11-7082 suggesting that the NF κ B transduction pathway is central in its induction by SAA. Following the premise that IL-6 production is intensified by autocrine regulation, the same may hold true for its inhibition or depletion. In related studies involving rheumatoid arthritic synovium, SAA showed a

similar ability to up-regulate IL-6 and this bioactivity was markedly abrogated by NFκB inhibition using BAY11-7082 [42].

In addition, previous studies in endothelial cells and synoviocytes have reported a role for SAA in the promotion of angiogenesis through its ability to induce VEGF [22] and this pro-angiogenic activity can be partially inhibited by pharmacological blockade of a range of SAA receptors at the cell surface [22]. The present results showing that BAY11-7082 dose-dependently decreased VEGF production in endothelial cells stimulated with SAA also indicate that inhibition of the canonical NFκB pathway is an effective alternative to pharmacological approaches aimed at blocking SAA receptors on the endothelial cell surface. Whilst, blockade of NFκB resulting in a corresponding decline in angiogenic VEGF has not been well documented in endothelial cell models, related studies with breast cancer cell lines such as those in MDA-MB-231 have displayed successful VEGF suppression with an NFκB inhibitor [43].

Owing to high rate of atherosclerosis and associated cardiac complications resulting from plaque formation and rupture; we aimed to investigate the potential role of BAY 11-7082 on endothelial adhesion and vascular functions. Our results showed significant reduction of the endothelial adhesion in mice treated with NFκB inhibitor compared to SAA alone group. These findings suggest that BAY11-7082 prevents the activation of NFκB thus inhibiting the expression of adhesion molecules and minimising the risk of vascular plaque formation. Further to endothelial adhesion implicated in plaque formation is the lost vascular elasticity occurring under inflammation mediated oxidative stress leading to depletion of nitroxides essential to vascular dilatation and hemodynamics. Endothelial cell dysfunction can lead to increased permeability to lipoproteins, leukocytes adhesion and cytokines generation [44]. To assess the role of BAY11-7082 on vasoconstriction and vasodilatation we treated the rat aortae with SAA alone and in supplementation with BAY11-7082. The vasodilatation was significantly improved in the aortas pre-treated with BAY11-7082 compared to SAA alone. These findings were further supported by assessments of aortic cGMP levels, which were significantly higher in vessel segments treated with BAY 11-7082 suggesting its potential to limit SAA-mediated decrease in nitric oxide production/bioactivity on the vascular endothelium.

In summary, our results confirm SAA induced over expression of TNF and IL-6; increased leukocytes endothelial adhesion and compromised vascular activity. Pre-treatment with BAY11-7082 effectively reversed these changes supporting a role of NFκB in SAA bioactivity. Moreover, NFκB inhibition by BAY11-7082 may be a potential mechanism to modulate thrombus formation in the endothelium, a risk factor of cardiac disease particularly in diabetic patients.

Materials and Methods

Materials

Chemicals, cell culture reagents and proteins were of the highest quality grade available and sourced from Sigma-Aldrich, Australia unless specified otherwise.

Cell culture

Commercial human carotid artery endothelial cells (HCtAEC) (Cell applications, California, USA) were cultured in MesoEndo Cell Growth Medium (Cell applications, California, USA) containing endothelial cell growth serum (ECGS) (Millipore, Sydney, Australia). Cells were grown to ~90% confluence at 37°C in a humidified atmosphere with 5% (v/v) CO_{2(g)} as described previously [23]

Dose selection for *in vitro* cell culture experiments with BAY11-7082

In our previous reports [17, 22] 10 µg/mL recombinant SAA (PeproTech, Lonza Australia, Mount Waverly, Australia) has induced significant expression of TF, TNF and VEGF in cultured HCtAEC exposed to SAA and was therefore used (10 µg/mL) in all current *in vitro* experiments conducted in this study. This SAA concentration represents a median value between normal, low circulating and acute phase SAA levels (5-25 µg /mL; [17]). All reagents and media were rigorously tested for endotoxin levels using the Limulus Amebocyte Lysate (LAL) buffer and endotoxin standards, visualised with Spectrozyme LAL (American Diagnostica, Stamford, CA). Reagents were discarded if endotoxin levels were >5 pg/mL. We exhaustively tested preparations of recombinant SAA for LPS contamination and routinely found <2 pg LPS/µg SAA/mL. This low-level contamination was unable to induce pro-inflammatory/pro-coagulant responses in human peripheral blood monocytes that are highly sensitive to LPS and denaturing the SAA protein by heating to 100 °C ablates its biological activity while similar treatment of LSP has no impact on its biological action [35].

Owing to positive association of VEGF-A with atherosclerosis particularly in ApoE^{-/-} mice [45] optimal dose of the NFκB inhibitor BAY11-7082 was selected based on its inhibitory effects on VEGF measured by an enzyme-linked immunosorbent assay (ELISA) as described previously [23]. Briefly, cells (density 1x10⁷ cells/well) were designated to four different treatment groups and pre-treated with vehicle (dimethyl sulfoxide; DMSO as a control) or 1, 10 or 100 µM BAY11-7082 in DMSO. Control and drug-treated cells were then incubated in HEPES Physiological Saline Solution (HPSS) (containing: 22 mM HEPES, 124 mM NaCl, 5.6 mM D-Glucose, 5 mM NaHCO₃, 5mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 0.16 mM Na₂HPO₄, 0.4 mM NaH₂PO₄, adjusted to pH 7.4) at 37 °C and 5% CO_{2(g)} for 1.5 h. Cells were then treated with 10 µg/mL recombinant human

Apo-Serum Amyloid A (SAA1) (Peprotech, USA) and incubated at 37 °C and 5% (v/v) CO_{2(g)} for 4.5 h.

Immediately prior to harvest, cell confluency (%) was measured using an IncuCyte Zoom® live cell imaging system (Essen BioScience, Sydney Australia). For this, 4 x 4 field HD phase-contrast images of each well were acquired using a 10x objective lens (Nikon, Australia) and analyses were completed by the IncuCyte Zoom® system software with user input for segmentation adjustment (differentiation of background from cells). After SAA treatment of cells, the HPSS overlay was collected and stored at -80 °C for further analyses. Treated cells were harvested in buffer (50 mM phosphate buffered saline (PBS) containing: 1 mM EDTA, 10 µM butylated hydroxytoluene, and 1X Complete™ protease cocktail inhibitor tablet (Roche, Bern, Switzerland) and then exposed to 3 freeze-thaw cycles. The lysate was collected and stored at -80 °C for subsequent analyses. For all biochemical assessments, total cell protein was determined using the bicinchoninic acid assay with bovine serum albumin used as standard.

Gene analysis

TF and TNFα were chosen for gene analysis as SAA is known to stimulate expression of these genes in a range of cell types. TF expression is induced by inflammatory cytokines in atherosclerotic plaques and being potent physiological trigger of the downstream coagulation cascade leads to thrombus formation at the site of plaque rupture. TNFα targets vascular endothelium and increases the expression of several other pro-inflammatory, pro-coagulant and pro-apoptotic genes [46]. This also reduces NO bio-availability [47, 48] secondary to NO scavenging by reactive oxygen species (ROS) or inhibition/decline in NO synthesis [49] which further aggravates vascular endothelial dysfunction.

Where required, total mRNA was extracted from cell lysates using a commercial Isolate II RNA Mini Kit (Bioline, Sydney, Australia) and cDNA thereof was synthesised immediately using a Tetro cDNA Synthesis Kit (Bioline, Sydney, Australia) with an Eppendorf MasterCycler gradient System (Eppendorf, Sydney, Australia). Reverse-transcriptive polymerase chain reaction (RT-PCR) was performed with 0.5-6 µL cDNA (final volume was optimised by normalising the corresponding house-keeping gene). A PCR master mix was prepared by adding 1 µL of specific forward primer and 1 µL of reverse primer (Table 1) and 12.5 µL 2x MyTaq Red (Bioline, Sydney, Australia) and 14.5 µL of the PCR master mix was added to each PCR tube followed by cDNA and finally diluting to 20 µL with DEPC-treated water (Bioline, Sydney, Australia).

PCR reactions were cycled at 95 °C for 1 min to activate DNA polymerase, heated to denature step at 95 °C for 15 s, allowed to anneal at 60 °C for 15 s followed by elongation at 72 °C

for 10 s. Amplified products were visualised using a 2% (w/v) agarose gel and SYBR®Safe (Life Technologies, USA). PCR reaction samples were loaded into wells with 6 µL of Hyperladder I (Bioline, Sydney, Australia) and PCR products separated using electrophoresis (Bio-Rad, Sydney, Australia) for 45 min at 90V. The gel was imaged on a GelDoc™ MP system (Bio-Rad, Sydney, Australia) using ImageLab™ Software V5.2 (Bio-Rad, Sydney, Australia). Gene expression was quantified using gel densitometry with ImageLab™ Software in conjunction with ImageJ (National Institutes of Health, USA).

Sandwich enzyme-linked immunosorbent assay (ELISA)

Cells were normalized for cell density/confluency and interleukin-6 protein was quantified in media samples and cell lysates (after normalising for total cell protein) using a commercial sandwich ELISA (Elisakit.com, Australia). Monitoring of IL-6 in relevance to atherosclerosis were several fold (i) it plays primary role in hepatic C-reactive protein synthesis which leads to generation of pro-inflammatory cytokines [50]; ii) possess significant pro-coagulant activity [51]; iii) activates endothelial cells which in turn express cellular adhesion molecules (ICAM, VCAM) leading to plaque formation [19, 36] and iv) its potential to develop atherosclerosis in mice when administered (IL-6) exogenously [52]. As per manufacturers guidelines absorbance readings were recorded at 450 nm using an Infinite® M200 PRO Plate reader (Tecan, Männedorf, Germany) and analysed using Microsoft Excel (2013, v7). Standard curves generated on the same plate were employed to express protein units in pg/mL. All data was normalised to the corresponding level of cell confluence (expressed as a percentage %) determined using an IncuCyte system immediately prior to cell harvest for ELISA (See Supplemental Figure S1 and Table S1).

Immunofluorescence

Immunofluorescence imaging of HCtAEC was performed as described previously [23] for TNF and IL-6. Briefly, cells were grown to ~80% confluence on Nunc™ Lab-Tek™ II Chamber Slides (Thermo Scientific, Melbourne, Australia) before treatment with SAA (10 µg/uL) in the absence and presence of BAY-11 (10 µM). Slides were then washed 3 x with HPSS and cells fixed with ice-cold acetone for 20 min before addition of rabbit anti-human primary antibody (Abcam, Sydney, Australia), (1:50 v/v dilution in antibody diluent (0.1% (v/v), Triton-X 100, 1% (w/v) BSA in TBS) and incubation at 4 °C overnight. The chambers were washed gently with TBS-T three times for 3 min and treated with a secondary IgG- Alexa Fluor® 594 conjugated antibody (Life Technologies, Sydney, Australia) at 1:200 v/v final dilution for 45 min at 20 °C followed by 3 wash cycles (TBS-T).

A glass cover slide was mounted onto chamber slides using SlowFade Gold Antifade Reagent with DAPI (Life Technologies, USA). The slides were allowed to dry overnight in the dark at 4°C and then sealed with clear nail varnish. Slides were imaged on a Zeiss Axio Scope A.1 (Carl Zeiss, Melbourne, Australia) using a 20x objective lens and Zen2 Lite software (Carl Zeiss, Melbourne, Australia) was utilised to obtain images. All images were converted to TIF and were manually edited by adjusting the brightness and contrast uniformly to ensure balanced contrast and to remove background distortions using Microsoft PowerPoint (Office v7, 2013).

Animal studies

Leukocyte adhesion

After gaining ethical approval from the local Animal Ethics Committee (E/1573/2015/B) (Melbourne, Australia), 8 weeks old male atherosclerosis-prone Apolipoprotein E^{-/-} mice on a C57BL/6 background were allowed to acclimatise for one week prior to experimentation. Intraperitoneal (*i.p.*) administration of BAY 11-7082 (5 mg/kg) or sterile PBS (as vehicle control) commenced on day 0 and was repeated on days 4, 7, 10 and 13. In parallel, recombinant SAA (120 µg/kg; Lonza Australia, Mount Waverly, Australia) was administered via *i.p.* injection on days 8, 11 and 13, while the same vehicle control mice (above) were administered sterile PBS (100 µL, 0.01M; via *i.p.* injection) on days 0, 4, 7, 8, 10, 11 and 13 as a further control measure. All mice were anaesthetised and culled via cervical dislocation on day 14 for use in *ex vivo* vascular adhesion studies as described previously [53].

Leukocyte-endothelial interactions were observed using an *ex vivo* dynamic flow adhesion assay [54]. Briefly, abdominal aortae were excised and aortic surfaces thoroughly cleaned of excess fat to facilitate clear imaging. Each end of the abdominal aorta was then carefully mounted on the cannula in a vessel chamber. Whole human blood was freshly isolated on the day of the experiment and labelled with VybrantDil dye (Invitrogen, USA) (final dilution 1:1000 v/v) for 10 min and then perfused at a rate of 7.5 mL/h through the isolated aorta and leukocyte adhesion was visualized by fluorescence microscope. Images were acquired at 160x magnification with a 1.4 mm field and readings were taken at 1 field along the vessel at 5 separate time points in 2.5 min intervals for 30 s.

Vascular function studies

Next, we extended our analysis to assess the vascular functionality (Srage-Dawley) using freshly isolated rat aortic rendered surplus and made available to other researchers in line with animal ethics guidelines vessels (Sydney South West Area Health Services ethics protocols 2014/020 and 2014/030), thereby minimising the usage of new animals. Briefly, isolated aortae were excised and flushed with HBSS and vascular activity study was performed within 12-16 hours of harvest.

Aortae were then placed in modified Krebs-Henseleit solution (containing in mM: 11 D-glucose, 1.2 MgSO₄, 12 KH₂PO₄, 4.7 KCl, 120 NaCl, 25 NaHCO₃, and 2.5 CaCl₂·2H₂O) and cut into 3 mm ring segments as described previously [2].

Aortic rings were then mounted in a Myobath system (World Precision Instruments, Sarasota, FL, USA) containing 15 mL of modified Krebs–Henseleit solution aerated at 37°C with carbogen gas (5% v/v CO₂/oxygen). Next, individual rings were contracted with phenylephrine (10⁻⁷–10⁻⁵ mol/L), and the dose that elicited half-maximal contraction force in each mounted aortic segment was selected for further studies. Mounted rings were then treated with vehicle (control) or SAA (final concentration 1 µg/mL a dose known to activate the endothelium and impair vaso-relaxation [13] for 4 h followed by thorough washing prior to relaxation assessment. In some experiments, the aortic ring segments were pre-treated with BAY11-7082 (10 µM) for 1 h prior to addition of SAA. Concentration–response curves (10⁻⁶–10⁻² mol/L) were then generated for endothelium-dependent (acetylcholine; ACh) or endothelium-independent (sodium nitroprusside; SNP) relaxation in the presence of added indomethacin (25 µM). Vessel relaxation was expressed as a percentage of initial phenylephrine-mediated contraction force. At the completion of the experiments each vessel was immediately frozen in liquid nitrogen and stored at -80 °C for further biochemical assessment.

Measurement of aortic cGMP

Aortic levels of the vaso-active effector molecule cGMP were determined using a commercial ELISA kit (Sapphire Biosciences, Redfern, Sydney, Australia). Where required, aorta samples were thawed, homogenized and centrifuged (16,000 g for 10 min at 4 °C) to obtain cytosolic protein from the supernatant fraction, as previously described (Cho et al., 2011). Aortic cGMP levels were subsequently determined. Standard curves generated on the same plate were employed to express protein units in pg/mL the data was normalized to total homogenate protein.

Statistical Analysis

Statistical analysis was performed using the GraphPad® Prism software Version 6.0 (GraphPad Software Inc., USA). Data was analysed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test to compare differences between groups. Statistical significance was reached at 95% confidence interval ($p < 0.05$).

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Author Contributions

Conceptualization: PKW, JD

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Formal Analysis: AV, BC, MS, AS, AA

Investigation: AV, BC, MS, WAS, AS, NM

Data Curation: AV, BC, GA

Writing – Original Draft Preparation: AV and GA.

Writing – Review & Editing: PKW, JD, JdeH, JPD

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Conflicts of interest

All authors have no conflicts to declare.

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Table 1. Primer sequences employed in this study^a

Primer	Forward sequence (5'- 3')	Reverse sequence (5'- 3')	Accession
β-actin	AGCACTGTGTTGGCGTACAG	GGACTTCGAGCAAGAGATGG	XM_006715764.1
TNF-α	CAGAGGGCCTGTACCTCATC	GGAAGACCCCTCCCAGATAG	NM_000594.3
TF	GTGACCTCACCGACGAGATT	CCGAGGTTTGTCTCCAGGTA	NM_001178096.1
NFκB	TGGAAGCACGAATGACAGA	TGAGGTCCATCTCCTTGGTC	NM_001319226.1

^aPrimer sequences used in RT-PCR studies with annealing temperature set to 60 °C for all experiments. Primers were synthesised by Sigma-Aldrich, Australia and Geneworks, Australia. β-actin used as a housekeeping gene to normalise results. All primer sequences were verified as targeting the gene of interest in the species nominated using Blast Search (an on-line database screening freely available at the NIH site: https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch); identifying accessions numbers are as reported above.

Table S1. HCtAE cell confluency as determined by imaging with an IncuCyte system.

Well	Treatment group	Confluency (%)
A1	1.1 Control	56.85298
B1	1.2 Control	56.13226
A2	1.1 SAA	59.34018
B2	1.2 SAA	64.7347
A3	1.1 BAY11	33.76793
B3	1.2 BAY11	35.37162

^a High-definition D phase-contrast images were acquired for each well in a 6-well plate using the The IncuCyte Zoom® live cell imaging system (Essen BioScience, Australia). The system software calculated the average confluency of each individual field imaged to obtain the overall mean (n=4) level of confluency for the well expressed as a percentage of the total area imaged (%). Levels of confluency were then used to normalise total secretory IL-6 as determined by ELISA.

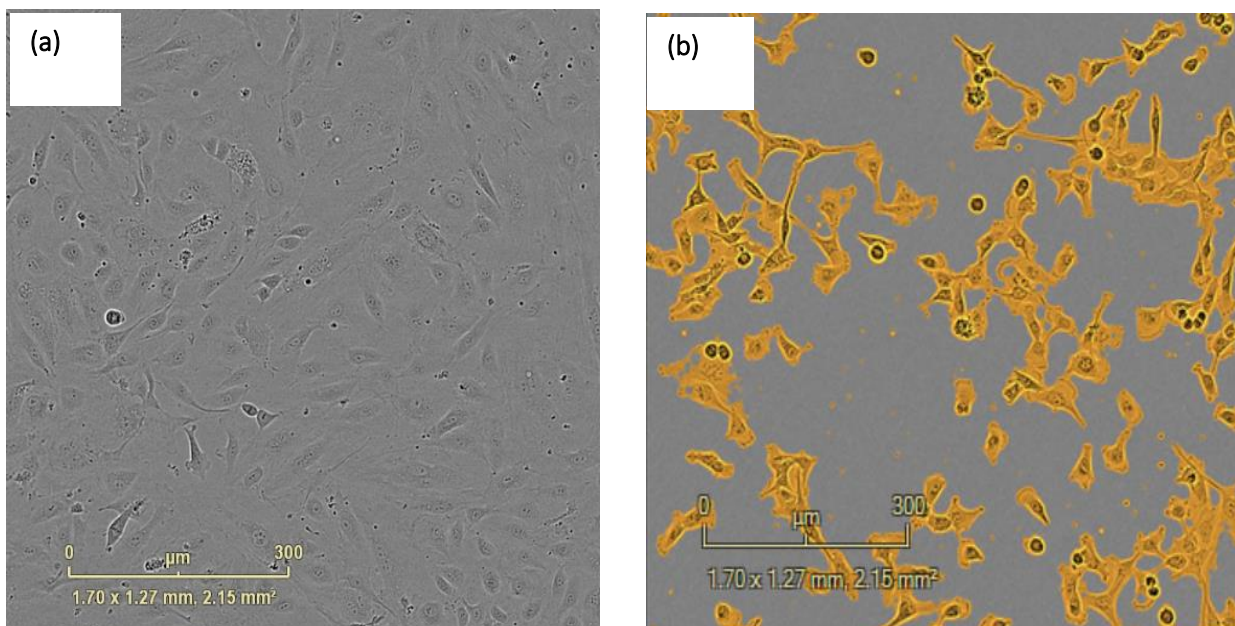
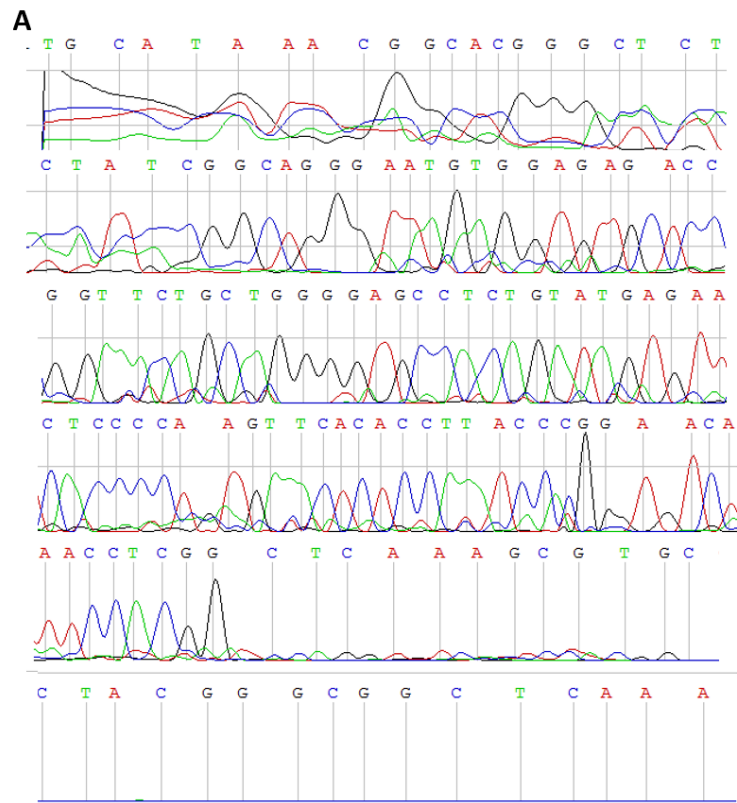
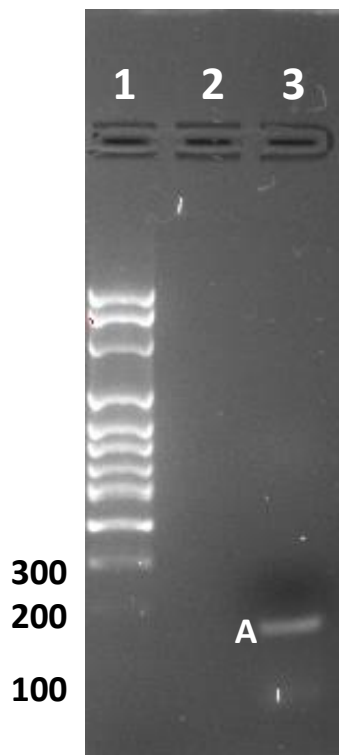


Figure S1. Cultured HCtAE cell confluence as assessed with an IncuCyte imaging system.

The IncuCyte Zoom® live cell imaging system (Essen BioScience, Australia) was used to measure the level of cell confluency (expressed as a percentage of the total area imaged) immediately prior to the time of harvest. (a) Representative field image of well A1 (*Table S1*); showing areal coverage at the 56% confluency level. (b) Representative field image of well B3 (*Table S1*) showing fewer cells in the same areal field (35%) – orange highlight shows confluence mask employed by the software to identify cells). Images were taken using a fixed 10x objective lens (Nikon, Australia).

Figure S2. Gene sequencing**Figure S2(i). RT-PCR gel for TF expression**

Lane 1. Hyperladder 100 Bp Plus

Lane 2. No sample

Lane 3. HCtAE cDNA

Note Band A corresponds to an amplification product of 157bp

Figure S2(ii). Sequencing chromatogram for purified bands (Gentle Software)

DNA from bands noted as A in Figure 1 were extracted from agarose gel, purified and sequenced. Figure shows good quality chromatography data for reliable sequence data.

Experimentally determined Sequence

TGCATAAACGGCACGGGCTCTCTATCGGCAGGGAATGTGGAGAGACCGGTTCTGCTGGGGAGCCTCTGTATGAGAACTCCCCAAGTTCACACCTTACCCGGAACAAACCTCGGCTCAAAGCGTGCCTACGGGCGGCTCAAATTATTGCATCTTTGCTGGGGGTCCCCCGGCGGGGGGGGGGGGAGGGCCAAAAGGAAATATTGCACG

Figure S2(iii). Tissue factor sequencing result

Homo sapiens coagulation factor III (thromboplastin, tissue factor) mRNA, complete cds
Sequence ID: [BT019808.1](#) Length: 888 Number of Matches: 1
[▶ See 1 more title\(s\)](#)

Range 1: 312 to 422 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
148 bits(80)	3e-32	102/111(92%)	7/111(6%)	Plus/Plus
Query 10	GGCACGGG-C-TCT-CTA-TCGGCAGGGAATGTGGAGAG-ACCGGTTCTGCTGGGGAGCC	64		
Sbjct 312	GGCACGGGTCTTCTCTACCCGGCAGGGAATGTGGAGAGCACCAGTTCTGCTGGGGAGCC	371		
Query 65	TCTGTATGAGAACTCCCCA-AGTTCACACCTTACCCGGA-ACAAACCTCGG	113		
Sbjct 372	TCTGTATGAGAACTCCCCAGAGTTCACACCTTACCTGGAGACAAACCTCGG	422		

Figure S2(iv). Band A NCBI Nucleotide Blast result

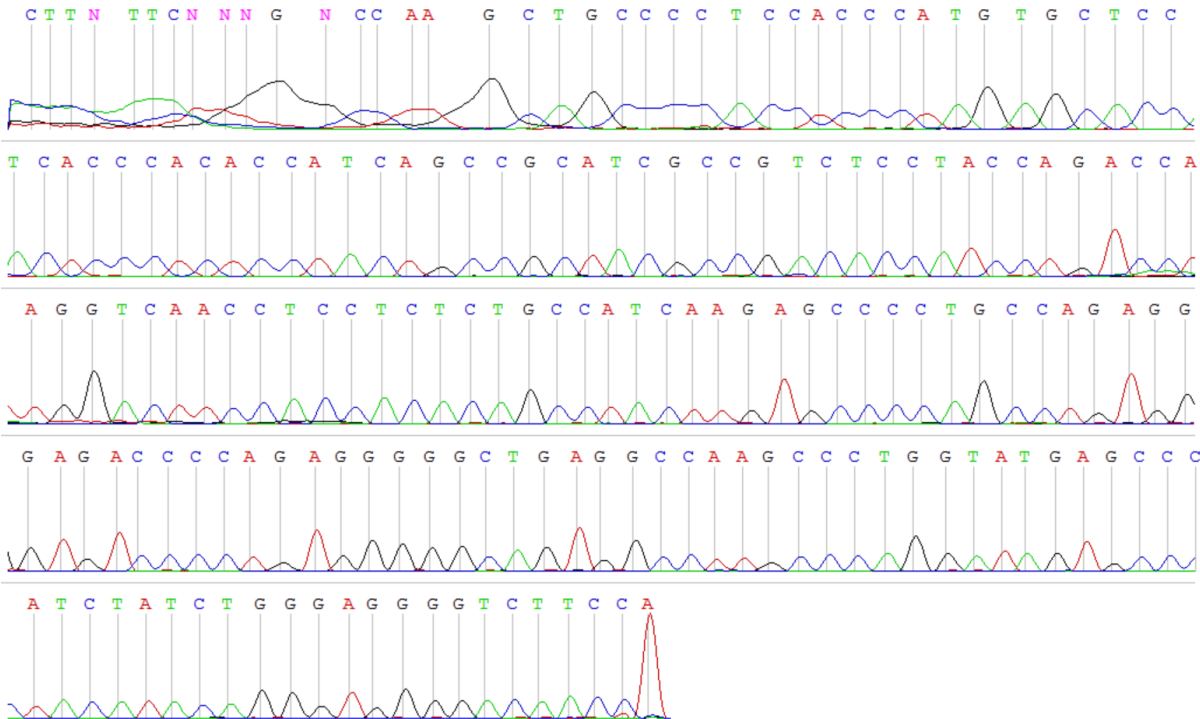


Figure S2(i-iv). Sequencing Chromatogram for purified bands (Gentle Software)

DNA from bands noted A Figure 1 were extracted from agarose gel, purified and sequenced. Figure shows good quality chromatography data for reliable sequence data. Percentage match obtained was 92% identity.

Figure S3(i) NFkB sequencing result

Experimentally determined Sequence

AGCGCATATCTGGATCTTGGTGCACCCTGACCTTGCCTATTTGCAAGCAGAAGGTGG
AGGGGACCGGCAGCTGGGAGATCGGGAAAAAGAGCTAATCCGCCAAGCAGCTCTGC
AGCAGACCAAGGAGATGGACCTCAAGGGCCGCGCCTAGGGGATTTCGGAGGCGCCC
GTGGGCGGCGCGGGGGCCGGGGAGCAGAGCCATACTAGCGGGGGGGGGGGGGGGG
GGGGGGGGGGGGGGGGGGAGAC

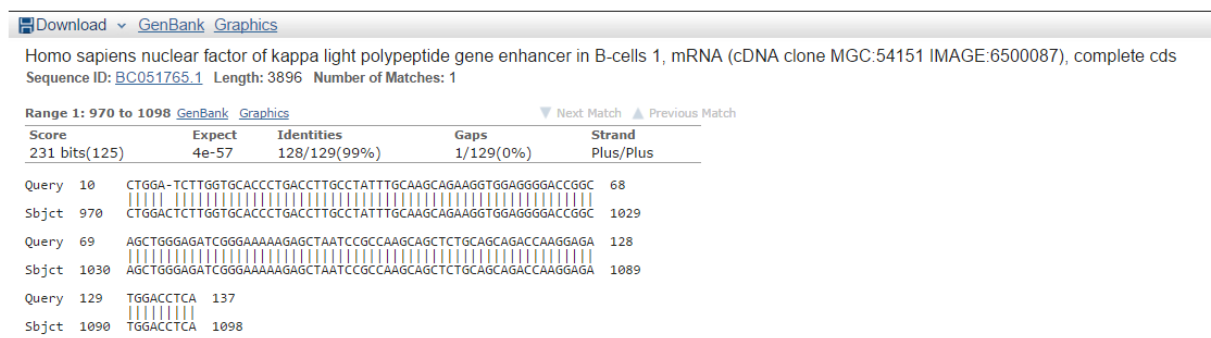


Figure S3(ii). NFkB Nucleotide Blast result

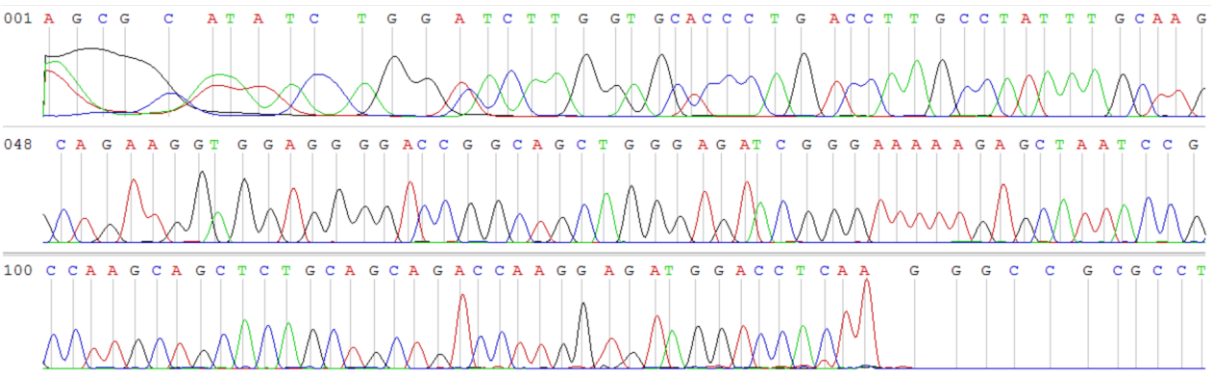


Figure S3(iii). NFkB Sequencing Chromatogram

DNA from a single band was extracted from agarose gel (as per figure S2(i) above), purified and sequenced. Figure shows good quality chromatography data for reliable sequence data. Percentage match obtained was 99% identity.

Figure S3(i). Beta actin house keeping gene

Experimentally determined Sequence

TGGTCTCATATTCATTGAAGGAGCGAAGGTGTCTCGTGGATGCCCAGGACCCTGCCA
GGAAGGAAGGCTGGAAAGTGCCTAGGACACCGGACCGCTATTGCCAATGGTGA
CCTGGCCGTCAGGCAGCTCGTACTCTTCTCCAGAGAAGAGCAGGAAGCAGCCGTGG
CCATTTGGCTCGAATCAGGTGT

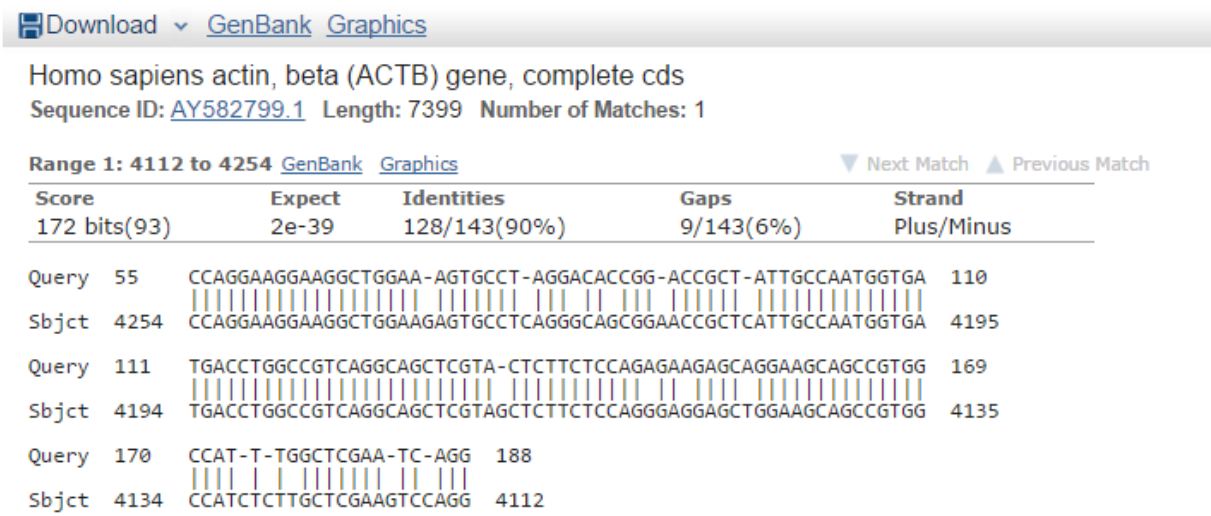


Figure S3(ii). Beta actin Nucleotide Blast

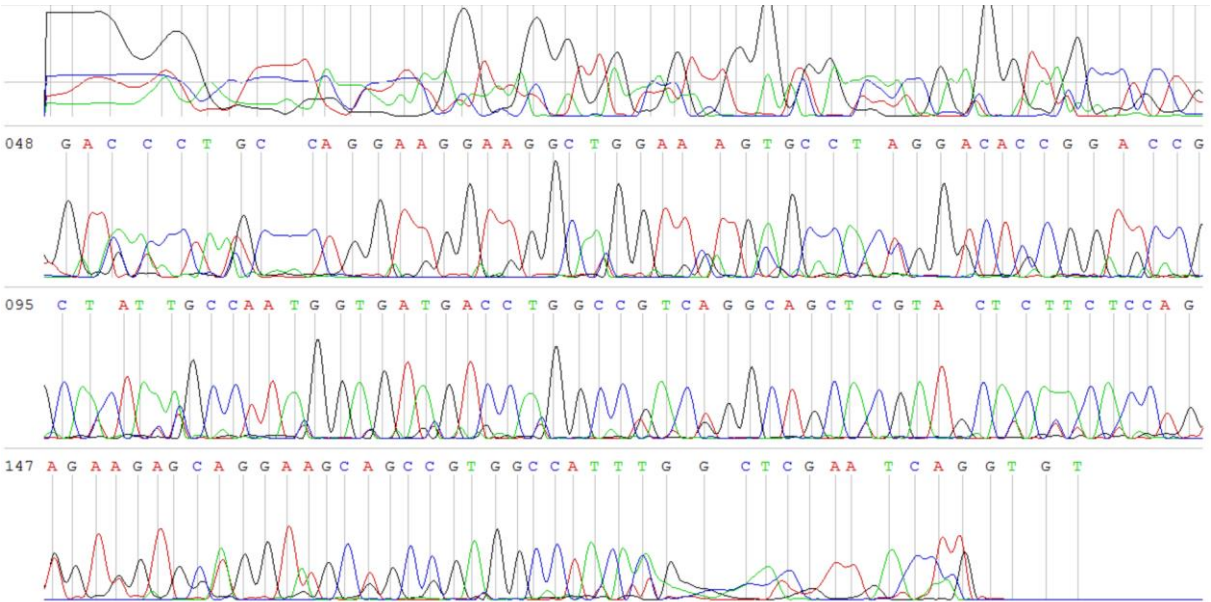


Figure S3(iii). Beta actin Sequencing Chromatogram

DNA from a single band was extracted from agarose gel (as per figure S2(i) above), purified and sequenced. Figure shows good quality chromatography data for reliable sequence data. Percentage match obtained was 90% identity.