alpha(1)-Antitrypsin, old dog, new tricks - alpha 1-Antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevatin cAMP

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α1-ANTITRYPSIN-OLD DOG NEW TRICKS:

α1-ANTITRYPSIN EXERTS *IN VITRO* ANTI-INFLAMMATORY ACTIVITY IN HUMAN MONOCYTES BY ELEVATING cAMP

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Running title: α1-antitrypsin induces cAMP in monocytes

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Regulation of serine protease activity is considered to be the sole mechanism for the function of α1-antitrypsin (AAT). Recent reports, however, of the anti inflammatory effects of AAT are hard to reconcile with this classical mechanism. We discovered that two key activities of AAT *in vitro*, namely inhibition of endotoxin-stimulated TNFα and enhancement of IL-10 in human monocyte, are mediated by an elevation of cAMP and activation of cAMP dependent protein kinase A. As expected with this type of mechanism, the AAT-mediated rise in cAMP and impact on endotoxin-stimulated TNFα and IL-10 was enhanced when catabolism of cAMP was blocked by the phosphodiesterase inhibitor rolipram. These effects were still observed with modified forms of AAT lacking protease inhibitor activity.

Regulation of proteolytic activity by endogenous anti-proteases represents a major mechanism limiting host tissue destruction at sites of inflammation. α1-antitrypsin (AAT), the major circulating serine protease inhibitor was first isolated in 1955, and was so named because of its ability to inhibit trypsin (1,2). It is now recognized that AAT, also known as α1-protease inhibitor, is a potent inhibitor of multiple serine proteases with particularly high activity toward the neutrophil serine proteases, neutrophil elastase and proteinase-3 (3,4). Most of the circulating AAT is synthesized by the liver and is released rapidly during the acute phase response to inflammation or infection (5,6). Alterations of the AAT molecule that compromise its structure and/or secretion and thereby lead to the AAT deficiency, are known to predispose the individual to diseases (7). Clinical expressions of AAT deficiency can be seen in the lung, liver, and the skin, with considerable variability in the severity of disease (8, 9). In fact AAT deficiency is the only known genetic risk factor for the development of chronic obstructive pulmonary disease, a chronic inflammatory lung disease characterised by progressive proteolytic destruction of the lung (10).

Although it is generally assumed that the anti-inflammatory effects of AAT are mediated by its anti-protease activity, recent data suggests that other mechanisms may be involved (11-16). Our own *in vitro* studies, using monocytes stimulated with lipopolyscharide (LPS), have demonstrated inhibition of TNFα production by native, AAT and by AAT chemically modified to abolish its protease inhibitor activity. Furthermore we have shown that both native and modified forms of AAT enhance LPS stimulated IL-10 generation (14). The data generated with IL-10 was important since it inferred a specific mechanism for the effects of AAT

rather than a general depressive effect of AAT on cell function.

The aforementioned studies raised the important question as to how AAT might exhibit anti-inflammatory activity independent of protease inhibitor activity. Since the effects of AAT on LPS-stimulated monocyte TNFα and IL-10 were similar to those reported for PDE4 inhibitors (17) and receptor agonists such as PGE2 (18,19), we hypothesized that AAT may mediate its anti-inflammatory activity through elevation of 3'5'-cyclic adenosine monophosphate (cAMP). cAMP is ubiquitously found in all mammalian cells and plays a key role in the regulation many cellular functions (20). Classically, cAMP is thought to exert the majority of its intracellular effects by binding and activating cAMP-dependent PKA, thereby controlling the phosphorylation status and activity of multiple intracellular substrates (21,22). In inflammatory cells, elevation of cellular cAMP either through activation of a multiple membrane receptors or inhibition of cAMP catabolism results in inhibition of LPS stimulated cytokine and chemokine release and leukocyte recruitment (23-26) and of T cell activation and proliferation (27). This activity forms the mechanistic basis for the action of a number of new generation anti-inflammatory drugs (28-30)

Here we report that AAT, independently of its proteinase inhibitor activity, increases cAMP levels in monocytes and thereby exerts its anti-inflammatory effects in a model of LPS mediated inflammation *in vitro*.

EXPERIMENTAL PROCEDURES

α1-antitrypsin (AAT) preparations

The α1-antitrypsin (Human) Prolastin® (Lot 26N3PT2) was donated by Bayer (Bayer Corporation, Elkhart, IN, USA). The vial of Prolastin® contained 1059 mg of functionally active AAT, as determined by its capacity to inhibit porcine pancreatic elastase. Prolastin® was dissolved in sterile water provided by the manufacturer for injections and stored at +4°C. Purified human AAT were obtained from Sigma-Aldrich and Calbiochem (USA). The purity of the AAT preparations was >97% and the inhibitory activity >75%. AAT was diluted in phosphate-buffered saline (PBS), pH 7.4. To ensure the removal of endotoxins, AAT was decontaminated using Detoxi-Gel AffinityPak columns according to instructions from the manufacturer (Pierce, IL, USA). Purified batches of AAT were then tested for endotoxin contamination with the limulus amebocyte lysate endochrome kit (Charles River Endosafe, SC, USA). Endotoxin levels were less than 0.1 enzyme units/mg protein in all preparations used. The protein concentrations of the prepared AAT in the endotoxin-purified batches were determined according to the Bradford method (31). Various AAT preparations were tested in all the experimental models, with the objective of demonstrating that our results are not dependent on the specific properties of one AAT preparation.

Non-inhibitory forms of AAT

Temperature-inactivated AAT was produced by incubation at 60°C for 10h. The AAT was oxidised by N-chlorosuccinimide (Sigma-Aldrich, St Louis, USA) in a 25 molar excess in a 0.1 M Tris-HCl buffer, pH 8. The buffer was changed to PBS using a centrifugal micro-concentrator (Centricon YM30, Millipore, MA, USA). The temperature-inactivated and oxidised AATs were tested for their ability to form complexes with pancreatic elastase (EC 3.4.21.36) (Sigma, USA) and to inhibit elastase activity. Samples of inactivated or native AAT were incubated with pancreatic elastase at a 1.2:1 molar ratio for 15 and 30 min, respectively. The reaction was stopped by adding SDS sample buffer, mixtures were analysed using 7.5% SDS-PAGE and stained with Coomassie Blue. Temperature-inactivated and oxidised AAT did not form any complexes with elastase.

Elastase inhibitory activity was assessed spectrophotometrically (spectrophotometer

DU 600, Beckman). In brief, native, oxidised or polymerised AAT was incubated with pancreatic elastase at a molar ratio 1.2:1 for 5 min at room temperature in 0.1M Tris buffer, pH8. After addition of 25µl of chromogenic elastase substrate (SAPNA, succinyl-(Ala)3-P-nitroanilide, stock solution 1 mg/ml) to give a total sample volume 300µl, absorbance was measured at 405 nm, for 280 s. The absorbance values used for the calculation of elastase inhibition by AATs were corrected with blanks for buffer plus substrate. Temperature-inactivated and oxidised AAT had no inhibitory activity.

Monocyte isolation
Human blood monocytes were isolated from buffy coats (total blood was obtained from 65 donors in this study) using Ficoll-Paque PLUS (Pharmacia, Sweden). Briefly, buffy coats were diluted 1:2 in PBS with addition of 10 mM EDTA and layered on Ficoll. After centrifugation at 400 *g* for 35 min at room temperature, the cells in the interface were collected and washed 3 times in PBS-EDTA. Cell purity and amounts were determined in an Autocounter AC900EO cell counter (Swelabs Instruments AB, Sweden). The granulocyte fractions were less than 5 %. Cells were seeded at a concentration of 5 x 10^6 cells/ml in RPMI 1640 medium supplemented with penicillin 100 U/ml; streptomycin 100 µg/ml; non-essential amino acids 1x; sodium pyruvate 2 mM and HEPES 20 mM (Gibco, UK). After 1h 15 min, non-adherent cells were removed by washing 3 times with PBS supplemented with calcium and magnesium. Fresh medium was added and cells were stimulated with lipopolysaccharide (LPS, 10 ng/ml, J5 Rc mutant), 10-50µM rolipram, 10-50µM forskolin (Sigma-Aldrich, Inc, USA) or predetermined concentrations of AAT, separately and in combination for 30 min, 1h and 18h at 37°C, 5 % CO_2.

Neutrophil isolation
Human neutrophils were isolated from the peripheral blood of healthy volunteers using Polymorphprep TM (Axis-Shield PoC AS, Oslo, Norway) as recommended by the manufacture. In brief, 25 ml of heparin anti-coagulated blood was gently layered over the 12.5 ml of Polymorphprep TM and centrifuged at 1600 rpm for 35 min. Neutrophils were harvested as a low band of the sample/medium interface, washed with PBS, and residual erythrocytes were subjected to hypotonic lysis. The neutrophil purity was more than 95% as determined on an AutoCounter AC900EO.

Cytokine assays
Cell culture supernatants from monocytes treated with LPS alone or in combination with AAT were analysed to determine TNFα and IL-10 levels by using DuoSet ELISA sets (R&D Systems, MN, USA; detection levels 15.6 and 31.2 pg/ml, respectively).

Quantitative real-time RT-PCR analysis
500 ng of total RNA was used for cDNA synthesis with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on cDNA corresponding to 25 nanograms of total RNA in a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a total volume of 25 µl. Samples were diluted 25 times for 18S rRNA analysis. IL-10 primers were designed to span an intron and melting curve analysis was performed on the 7900 HT instrument after the run to make sure that the signals were generated from cDNA and not genomic DNA. IL-10 and 18S rRNA transcript levels were analysed using the SYBR® GREEN PCR Master Mix (Applied Biosystems) with primers; IL-10 F: GGGAGAACCTGAAGACCCTCA IL-10 R: TGCTCTTGTTTTCACAGGGAAG 18S rRNA F: CGGCTACCACATCCAAGGAA and 18S rRNA R:GCTGGAATTACCGCGGCT.

Taqman® Gene Expression Assays Hs00174128_m1, was used to quantify TNFα mRNA levels according to the standard protocol (Applied Biosystems, Foster City, CA, USA). Fold expression values were calculated versus the sample with the lowest levels of each transcript using the ΔΔCt-method after normalisation to the internal control 18S rRNA.

Total cellular cAMP assay

Total cAMP levels in monocytes ($1x10^7$ cells/ml) alone, treated with various concentrations of rolipram, forskolin, AAT (0.1-4 mg/ml) or LPS (10 ng/ml) alone, or pre-treated with LPS, 10μM rolipram or 30μM forskolin for 1 h following exposure to AAT were determined by the cAMP Direct Biotrak scintillation proximity assay (SPA) system according to the manufacturer's recommendations (Amersham Biosciences, USA). Briefly, cells alone or with stimulating agents were incubated for the *pre*-determined time periods and lysed for 5 min. The antiserum, tracer and SPA were reconstituted with lysis reagent and mixed with the analysed sample. The antibody-bound cAMP reacts with the SPA reagent which contains anti-rabbit second antibody bound to fluomicrospheres. Any ^{125}I cAMP that is bound to the primary rabbit antibody will be immobilised on the fluomicrosphere, which will produce light. Measurement in a β-scintillation counter enables the amount of fluomicrosphere-bound labelled cAMP to be calculated. The concentration of unlabelled cAMP in a sample is then determined from a standard curve.

Electrophoresis and Western blot analysis

Monocytes were incubated alone, with 30μM forskolin or 0.5 mg/ml AAT separately or in combination with 10μM H89, for different time periods, lysed and protein-determined using the Bradford method. Equal amounts of analysed protein were subjected to 10% SDS-PAGE gel. Proteins were transferred to a polivinylidene fluoride (PVDF) membrane (Millipore, Millipore Corporation, Bedford, MA 01730) using a semi-dry blot electrophoretic transfer system. Western blot analysis was performed using rabbit polyclonal anti-PKA catalytic α/β [pT197] phosphospecific antibodies (BioSource International, Inc., USA) and polyclonal goat anti-actin (I-19) antibodies (Santa Cruz Biotechnology, Inc, USA). The immunocomplexes were visualised with secondary horseradish peroxidase-conjugated rabbit anti-mouse antibodies (1:10 000) (DAKO, A/S, Denmark) and developed using the ECL Western Blot analysis system (Amersham, UK).

Phosphodiesterase type 4 (PDE4) specific enzymatic activity determination

PDE4 activity in the absence and in the presence of 10 μM rolipram (positive control) or 0.5, 1 and 2mg/ml of AAT was determined using the PDE4 enzymatic assay kit (FabGennix Inc, USA). Ten μl (7.5 μg protein) of PDE4 enzyme supplied with the kit was incubated with rolipram or various concentrations of AAT at 30°C for 5 min. The enzyme was incubated in buffer only for total PDE4 activity measure. Freshly prepared PDE-buffered substrate containing 15μl of 2,8 ^3H-cAMP ammonium salt (25-40 Ci/mmol, PerkinElmer Las, Inc, Boston, MA, USA) was added to the reaction tube and incubated for 10 min at 30°C with shaking. The reaction was terminated by transfer of the tubes to a 100°C water bath for 3 min. According to the manufacturer's protocol the cAMP is hydrolysed by PDE activity into the non-cyclic form. The PDE4 enzymatic activity is directly proportional to the adenosine formed. Separation of adenosine from AMP and cAMP was performed according to kit recommendations. The PDE4 activity was calculated by subtracting non-PDE4 activity from total PDE activity.

PKA activity assay

Monocytes ($1x10^7$ cells/treatment) alone and treated for 1h with 0.5 mg/ml AAT or 50μM forskolin, an activator of PKA (32), were harvested, washed with PBS and

suspended in 0.5 ml of cold extraction buffer (25 mM Tris-HCl pH7.4, 0.5mN EDTA, 0.5mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1μg/ml leupeptide and 1μg/ml aprotinin). Cells were homogenised using cold homogeniser, centrifuged for 5 min at 14 000 x *g* at 4°C and assayed for cyclic AMP-dependent protein kinase A (PKA) using the Protein Kinase A assay kit (Calbiochem, USA) according to the manufacturer's recommendations. P^{32} P-ATP (specific activity 25-40 Ci/mmol) was obtained from PerkinElmer LAS, Inc, Boston, MA, USA). The PKA reaction mixture contained 0.2μCi/μl of P^{32} P-ATP. One unit of activity is defined as the amount of PKA that catalysed the incorporation of 1 pmol of phosphate from ATP into Kempinde/min/mg protein at 30°C and pH 7.2.

In order to confirm our findings we also used a non-radioactive PKA assay kit based on ELISA that utilizes a synthetic pseudosubstrate and a monoclonal antibody that recognizes the phosphorylated form of the peptide (Calbiochem, USA).

Statistical analysis

The Statistical Package (SPSS for Windows, release 13.0) was used for the statistical calculations. The differences in the means of the experimental results were analysed for their statistical significance with the one-way ANOVA with an overall significance level of α=0.05. The independent two-sample t-test was also used.

RESULTS

Time-dependent effects of AAT and AAT/LPS- on TNFα and IL-10 release

TNFα and IL-10 protein and mRNA were measured in monocytes 2, 6 and 18 after exposure to LPS and AAT, either alone or in combination. At 2h, both LPS and AAT induced TNFα protein, albeit at very modest levels, whereas AAT alone induced IL-10. Exposure of monocytes to LPS (10 ng/ml) and AAT (0.5 mg/ml) in combination however, resulted in a more than additive increase in TNFα compared to either agent alone. No significant difference was found between effects of AAT alone and AAT and LPS in combination on IL-10 at 2 h (Fig. 1A). Thus, the effects of combinations of LPS and AAT at 2 h on TNFα protein appeared to be synergistic whereas those on IL-10 reflected substantially an effect of AAT alone. At 6 h combination of AAT with LPS resulted in significant inhibition of TNFα protein whereas IL-10 levels appeared to be enhanced in an additive manner. At 18 h inhibition of LPS-stimulated TNFα protein by AAT was further enhanced whereas dramatic increase of IL-10 release was observed.

In order to explain further these complex changes in protein levels we also performed mRNA analysis. At 2 h we observed a remarkable increase in TNFα mRNA in response to combinations of AAT and LPS with moderate effects of both mediators alone. At 6 and 18 h there was clear inhibition of LPS stimulated TNFα RNA by AAT. For IL-10 we observed an increase in mRNA for combinations of AAT and LPS at 2 and 6 h. However the changes in IL-10 mRNA did not mirror those in protein changes since significant IL-10 protein was observed at 2 h with AAT alone in the absence of detectable mRNA synthesis. The effects of AAT and LPS on IL-10 mRNA levels were transient with lower IL-10 mRNA levels in cells treated with LPS and AAT at 18 h than with LPS alone at this time point (Fig. 1B).

AAT elevates total cellular cAMP levels in LPS-treated monocytes

Monocytes stimulated with LPS alone for up to 1 h showed no detectable rise in cAMP levels [2.6 ± 0.4 pmol/10^7 cells in untreated monocytes ($n = 10$), and

2.45±0.19 pmol/10^7 in cells pre-treated with LPS for 1 h (n = 8)]. However, when monocytes were pre-treated with LPS for 1 h followed by addition of AAT (0.1-4 mg/ml) for 2 minutes cAMP levels increased significantly (82% to 190%, $p<0.001$) compared to controls (Fig. 2A). Furthermore, when monocytes were pre-treated with LPS (10 ng/ml, 1 h), the addition of constant concentration of AAT (0.5 mg/ml) at various time points yielded a rapid up-regulation of total cAMP that peaked after 2 min (Fig. 2B).

AAT elevates cAMP levels independently of its inhibitory activity

We next examined the effects of AAT and chemically modified forms of AAT, which lack protease inhibitor activity, on monocyte cAMP levels. Monocytes exposed to AAT for 2 min markedly increased their total cAMP levels as compared with control. Maximal cAMP responses to AAT occurred following incubation of cells with 1mg/ml AAT for 2 min [2.6 ±0.4 pmol/10^7 cells in untreated monocytes (n=10 experiments), and 6.45±1.2 pmol/10^7 in cells treated with AAT for 2 min (n=8 experiments), $p<0.001$]. AAT was then rendered inactive as an proteinase (verified with neutrophil elastase inhibitor) by either oxidation with *N*-chlorosuccimide or heating to 60°C for 10 h and tested for its ability to elevate cAMP. As shown in Figure 3, both oxidised and polymerized forms of AAT triggered cAMP elevation in monocytes with a similar magnitude of response. When human serum albumin used as a negative control in this experimental model no effect on cAMP levels were observed (data not shown). Moreover, when secretory leukocyte protease inhibitor (SLPI), another serine protease inhibitor with anti-inflammatory activities was included for comparisson, No effect on cAMP levels were observed [3.2±0.54 pmol/10^7 cells in untreated monocytes and 3.8±0.6 pmol/10^7 cells in cells treated with 1 mg/ml SLPI for 2 min, (n=3)]. SLPI was further tested at a range of concentrations with cAMP measurements at various time points up to 30 minutes with no significant effect (data not shown). In addition, to examine whether the effects of AAT on cAMP were cell specific, the influence of AAT on neutophil cAMP were determined. Under the same experimental conditions AAT had no effect on neutrophil cAMP levels (data not shown)

The effects of AAT on cellular cAMP levels are mediated by activation of adenylate cyclase

An important question regarding the AAT stimulated rise in monocyte cAMP was whether it was due to effects on cAMP synthesis or catabolism. Forskolin and rolipram are substances that increase the concentrations of cellular cAMP by either direct activation of adenylate cyclase or by inhibition of PDE 4, a major cAMP catabolising enzyme (33,34). We therefore examined the effects of AAT on cAMP levels in combination with forskolin or rolipram reasoning that a PDE4 inhibitor would enhance the cAMP response to an agent which induces its synthesis but not an agent which prevents its catabolism while a maximum response to forskolin might be enhanced by inhibition of cAMP catabolism but not by an inducer of adenylate cyclase activity. Monocytes were treated with either 30μM forskolin or 10μM rolipram either alone or in combination with AAT before measuring intracellular cAMP levels. As illustrated in Figure 4A, AAT (0.5 mg/ml) alone induced an approximately threefold increase in total cAMP levels at 2 min whereas pre-treatment of monocytes with forskolin resulted in an approximately twofold increase in total cAMP levels. In combination, the effects of forskolin and AAT on cAMP were not additive. This suggests that maximum activation of adenylate cyclase had occurred with AAT. By contrast, pre-treatment of the monocytes with rolipram alone resulted in a 87% ($p<0.01$) increase in cAMP level elevation, and caused a significant augmentation in cAMP levels in response

to AAT (52% increase, $p<0.01$, n=3 experiments) compared to rolipram alone (Fig 4B). Similarly, rolipram increased the forskolin effects on cAMP elevation (from 6.4. ±0.8 pmol/10^7 cells in forskolin treated monocytes to 9.45±1.2 pmol/10^7 in cells treated with forskolin and rolipram in combination (n=3 experiments), $p<0.01$) (data not shown)

To further support the contention that the effects of AAT were due activation of adenylate cyclase rather than inhibition of cAMP catabolism, we evaluated the effects of SQ22536, an inhibitor of adenylate cyclase, on AAT stimulated cAMP. Pre-incubation of monocytes with 25 μM SQ22536 for 45 min almost totally inhibited the ability of AAT to induce a rise in monocyte cAMP (Fig 5). This finding together with the data obtained with rolipram and the failure of AAT to directly inhibit purified PDE4 in vitro (in contrast to 10μM rolipram which inhibited activity 86%, $p<0.001$, (n=3 experiments) strongly suggested that the effects of AAT on monocyte cAMP levels were primarily due to activation of adenylate cyclase.

AAT stimulates the downstream activation of cAMP dependent Protein kinase A.

To confirm that the inhibitory effects of AAT occurred through the classical cAMP/ protein kinase A (PKA) pathway, the effect of AAT on PKA phosphorylation was evaluated. Both AAT (0.5 mg/ml) and forskolin (as positive control) increased PKA activity 213% and 256% ($p<0.001$), respectively compared to control (Fig. 6A). The effects of AAT and forskolin on PKA activity were confirmed by inhibition with 20 μM H-89. Western blot analysis, using a specific anti-PKA catalytic α/β [pT197] phosphospecific antibody, also confirmed downstream activation of PKA in a manner similar to forskolin (Figs. 6B and C). As observed earlier when measuring cAMP levels exposure of monocytes to AAT and forskolin combination had no additive effect on PKA activity (Fig 6 A) and protein levels (Fig 6 C). However of note was that both AAT and forskolin alone induced a similar level of activation of PKA over 1 h (Fig 5) in spite of very different temporal increases in cAMP.

The in vitro anti-inflammatory activity of AAT on LPS-stimulated monocytes are mediated by an elevation in cAMP

In order to link the rise in cellular cAMP to the anti-inflammatory effects of AAT on TNFα and IL-10 we evaluated the effects of AAT, rolipram and forskolin, either alone or in combination, on LPS stimulated monocyte activation after 18 h. In accordance with the data obtained for cAMP, the inhibitory effects of AAT on LPS induced TNFα release were significantly enhanced when added in combination with rolipram. Furthermore, rolipram also enhanced the release of LPS-induced IL-10 release by AAT (73% $p<0.001$) (Fig. 7 A and B). No significant changes in effects of AAT on LPS's induced TNFα release was observed in combination with forskolin (Fig. 8) and forskolin did not enhance the effects of AAT on LPS-stimulated IL-10 release (data not shown)

DISCUSSION

LPS (endotoxin) from gram-negative bacteria induces monocyte/macrophage production of both pro-inflammatory cytokines, in particular TNFα, and anti-inflammatory cytokines, including IL-10. However, the overwhelming balance of LPS activity favours a pro-inflammatory response (35). The result in a clinical setting can be systemic inflammatory response often accompanied by severe tissue injury (36, 37). We and other investigators have reported that AAT, an endogenous inhibitor of serine proteases, may inhibit LPS-induced pro-inflammatory responses *in vitro* and *in vivo* by mechanisms which appear to be independent of inhibition of serine proteases (14, 38-40).

In this paper we have explored the mechanism by which AAT modulates

monocyte responses to LPS *in vitro*. We initially analysed short-term monocyte responses to LPS and AAT separately or in combination. As soon as 2 hr after treatment, AAT induced IL-10 protein release. The discrepancy between mRNA and protein levels at this early time point appear to exclude effects on *de novo* protein synthesis and perhaps reflect release of pre-stored IL-10. However, at 2 and 6 hrs a combination of AAT and LPS clearly increased IL-10 mRNA and this may have been responsible for the large increase in IL-10 protein at 18 hrs if it were sustained. Paradoxically perhaps at 18 hr IL-10 mRNA levels returned to baseline suggesting that a fairly dramatic fall in mRNA synthesis had occurred between 6 and 18 hrs. The mechanism behind this is unknown but may include autocrine modulation of IL-10 mRNA synthesis by IL-10 protein (41) and/or an increase in mRNA instability (42). At 2, 6 and 18 hrs there appeared to be a good correlation between TNFα mRNA and protein levels suggestive of effects on de novo TNFα synthesis. We are unable to explain the relevance of the short term increases in TNFα protein at 2 h by combinations of LPS and AAT although they reflect a magnitude of response only 10% of the 18 hr response to LPS alone. In the long term, the effects of AAT on the LPS-stimulated monocyte activation are predominantly anti-inflammatory with reduction of TNFα and enhancement of IL-10.

It has long been recognized that modulation of cellular cAMP can have a profound effect on leukocyte function (43). Cellular cAMP levels may be tightly regulated by synthesis through activation of adenylate cyclase or hydrolysis by cAMP phosphodiesterases (e.g. PDE4) (44).

Elevation of leukocyte cAMP is generally inhibitory in terms of pro-inflammatory cellular signalling. For example, exogenous cell permeable cAMP analogues (e.g. dbcAMP), inhibitors of PDE4 (e.g. rolipram), receptor agonists mediating the activation of adenylate cyclase (e.g. prostaglandin E2) and direct activator of adenylate cyclase (forskolin), are able to reduce the release of TNFα and enhance the release of the anti-inflammatory cytokine IL-10, in response to LPS (45-49). Since we had observed similar functional activity by AAT on LPS stimulated TNFα and IL-10 (14) we sought to determine whether AAT was operating by a similar mechanism, namely elevation of cellular cAMP.

Initial studies confirmed up regulation of cAMP by AAT and to our surprise indicated that AAT was more efficacious than forskolin a direct activator of adenylate cyclase. We then showed that modulation of the cAMP response with rolipram and the adenylate cyclase inhibitor SQ23356 resulted in the expected enhancement and inhibition respectively of the cAMP response. In functional terms we demonstrated that the effects of AAT effects on cAMP and LPS stimulated TNFα and IL-10 release were enhanced by rolipram also consistent with a mechanism involving the activation of adenylate cyclase by AAT rather than inhibition of PDE4.

Finally we confirmed that the classical downstream signalling pathway for cAMP, activation of PKA, was responsible for mediating the effects of AAT. We found that in spite of very different time courses of cAMP accumulation, i.e. rapid for AAT but delayed for forskolin (50) the effects of both AAT and forskolin on PKA activation, the likely downstream effector of elevated cellular cAMP were similar at one hour. In this study we have not been able to identify the mechanism by which AAT actually activates adenylate cyclase. Although high affinity binding of AAT to the surface of cells has been reported (51), the receptor responsible remains elusive as do the immediate receptor proximal pathways. Interestingly, however, the ability of AAT to elevate cAMP was found to be cell specific for monocytes, since AAT has no effect on neutrophil cAMP. A significant finding in this study was that AAT was able to elevate cAMP levels independently on its protease-inhibitory

activity. Thus both oxidized and heat inactivated forms of AAT, which lack inhibitory activity, caused elevation of cAMP levels similar to that of native AAT. We have shown previously that both forms are also able to modulate LPS stimulated TNFα and IL-10 (14). This finding is exciting in that it confirms a mechanism by which physiologically modified forms of AAT, which are inactive as serine protease inhibitors and presumed to have no anti-inflammatory activity, may still play an important and protective role at sites of inflammation. It is intriguing that a single endogenous protein can express complimentary anti-inflammatory activity by two mechanisms, namely elevation of IL-10 and inhibition of TNFα. Although IL-10 has been reported to inhibit TNFα directly, previous data from this laboratory data suggest that autocrine inhibition of TNFα by IL-10 in is not the mechanism responsible for the inhibitory effects of AAT (14). Thus, although IL-10 antibody alone can enhance LPS stimulated TNF α, production by monocytes, suggestive of an autocrine modulation by IL-10, the inhibitory effects of AAT are similar whether IL-10 antibody is present or not (data not shown) Our findings are in accord with Seldon et al who showed that agents which elevate cAMP and inhibit TNFα in monocytes do so by a mechanism independent of induction of IL-10 (52) .
Thus, as a serine protease inhibitor AAT can block much of the destructive proteolytic activity from activated neutrophils whereas as an elevator of cAMP, AAT may also block the accumulation and activation of monocytes by modulating the production of pro and anti-inflammatory cytokines. It is worthwhile noting that the pluripotential anti-inflammatory effects of AAT are not unique to this serine protease inhibitor. Secretory leukocyte protease inhibitor (SLPI), for example, has also been reported to exert anti- inflammatory effects independent of inhibition of serine proteases (53), and antithrombin III has been shown to inhibit TNFα stimulation of E-selectin expression in endothelial cells. Like AAT, the effects of antithrombin III were due to elevation of cAMP (54). In this study we have shown that in contrast to AAT, SLPI increases neutrophil but not monocyte cAMP suggesting that the proximal signaling mechanisms for SLPI and AAT differ and thereby confer cellular specificity of anti-inflammatory activity.

In vivo, it is unclear which activity of AAT is most significant in suppressing inflammation. The protective role of AAT in smoke-induced emphysema is classically associated with a maintenance of a protease anti-protease balance (39,55). However, AAT also reduces bacterial endotoxin and TNFα-induced lethality *in vivo* (11). In man, Prolastin® therapy has been shown to reduce LTB4 levels in patients with AAT deficient emphysema (56,57). Comparative studies *in vivo* looking at the efficacy of native and modified forms of AAT and low molecular weight serine protease inhibitors may help address the question regarding the dominant anti-inflammatory mechanism of AAT *in vivo*. An important question regarding our findings in isolated blood monocytes is whether they can be extended to effects on blood monocytes or resident and/or newly recruited tissue monocytes/macrophages i.e. are the effects we see *in vitro* relevant to the *in vivo* situation or just an artefact of the way we have prepared the cells *in vitro*.
We have not performed such studies because of the notorious difficulty in working with a cell with the relevant phenotype. Therefore, studies on macrophages isolated from patients with inflammatory disease are necessary. However an intriguing finding by Osawa and co-workers that macrophages stimulated with LPS undergo a sensitization of their cAMP response to exogenous agonists (58) suggests that macrophage-like cells may be potentially more sensitive to the inhibitory effects of AAT. In summary, our studies suggest a mechanism by which AAT may express anti-inflammatory activity *in vitro*, namely via elevation of cellular cAMP. These

novel findings suggest that the effects of AAT *in vivo* are not simply related to modulation of serine protease activity but that more complex cAMP regulated inflammatory mechanisms may be involved.

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FIGURE LEGENDS

Fig. 1A and B. Release (A) and mRNA expression (B) of TNFα and IL-10 by human monocytes in response to exposure for 2, 6 and 18 hrs to LPS (10ng/ml) in the presence or absence of AAT (0.5 mg/ml). Each bar represents a mean of four independent experiments ± SE. Each curve is the mean value of two independent experiments.

Fig. 2. A and B. AAT induces a total cAMP rise in monocytes pre-treated with LPS

(A) The concentration-dependent effects of AAT on total cAMP rise. Monocytes were pre-treated with LPS for 1 h and various concentrations of AAT (0.1-2 mg/ml) were added for the fixed time of 2 min. The maximum cAMP rise was observed at AAT concentrations of 0.5-1 mg/ml. The figure is the result of 4 separate experiments ± SE.
(B) Time-dependent effects of AAT on cAMP elevation. In monocytes pre-treated with LPS (10 ng/ml, for 1 h), addition of AAT (0.5 mg/ml) yielded a rapid rise of total cAMP levels. The AAT effect was time-dependent, with maximum values after 2 min, whereas after 10 min they returned to baseline. This experiment was repeated twice with a similar result, and the mean values are shown in the graph.

Fig. 3. Comparisons of the effects of native (nAAT), temperature-inactivated (pAAT) and oxidised (oxAAT) (0.5 mg/ml) on cAMP rise at 2 min. Each bar represents the mean ± S.E. from three independent experiments, *** $p<0.001$

Fig. 4. AAT effects on induced cAMP rise in monocytes pre-treated with forskolin (A) and rolipram (B)
(A) Monocytes were pre-treated with 30 μM forskolin for 1 h and AAT (0.5 mg/ml) was added for the fixed time of 2 min.
(B) Monocytes were pre-treated with 10 μM rolipram for 1 h and AAT (0.5 mg/ml) was added for the fixed time of 2 min.
The figure is the result of 3 separate experiments ± SE, ***$p<0.001$

Fig. 5. Inhibitory effect of SQ22536 on AAT's ability to raise cAMP.
Monocytes were pre-incubated with 25 μM SQ22536 for 45 min and 0.5 mg/ml AAT was added for the fixed time 2 min. Under these experimental conditions AAT almost totally lost an ability to induce cAMP. Each bar represents the mean ± S.E. from four independent experiments

Fig. 6. AAT and forskolin-mediated PKA activation in monocytes

A, Monocytes were stimulated with AAT (0.5 mg/ml) and forskolin (50μM) alone or in combination with and without adding H89 (20μM) for 1 h. PKA activity was assayed as described in Materials and Methods. Bars represent mean ± SE from three separate experiments. ***p<0.001
B, Western blot shows representative experiment out of 3 performed. Blot with anti-actin antibodies was used as a protein loading control.
C, Western blot shows representative experiment out of 2 performed. Blot with anti-actin antibodies was used as a protein loading control.

Fig. 7. Additive effects of AAT and rolipram on LPS-induced TNFα (A) and IL-10 release (B).
Monocytes were exposed to LPS alone or in the presence of 0.5 mg/ml AAT, 10μM rolipram or their combination for 18 h. An inhibition of LPS-induced TNFα release and enhancement of LPS-induced IL-10 release were significantly improved when AAT and rolipram were added in a combination. Each bar represents a mean of four independent experiments ± SE.

Fig. 8. Effects of AAT and forskolin on LPS-induced TNFα release.
Monocytes were exposed to LPS (10 ng/ml) alone or in the presence of 0.5 mg/ml AAT or various concentrations of forskolin (10 to 50 μM) and AAT combination for 18 h. An inhibition of LPS-induced TNFα release was not improved when AAT and forskolin were added in a combination. Each bar represents a mean of three independent experiments ± SE. Forskolin effects on LPS-stimulated cells are shown as grey bars. AAT and AAT+forskolin effects on LPS-stimulated cells are shown as black bars. ***p<0.001

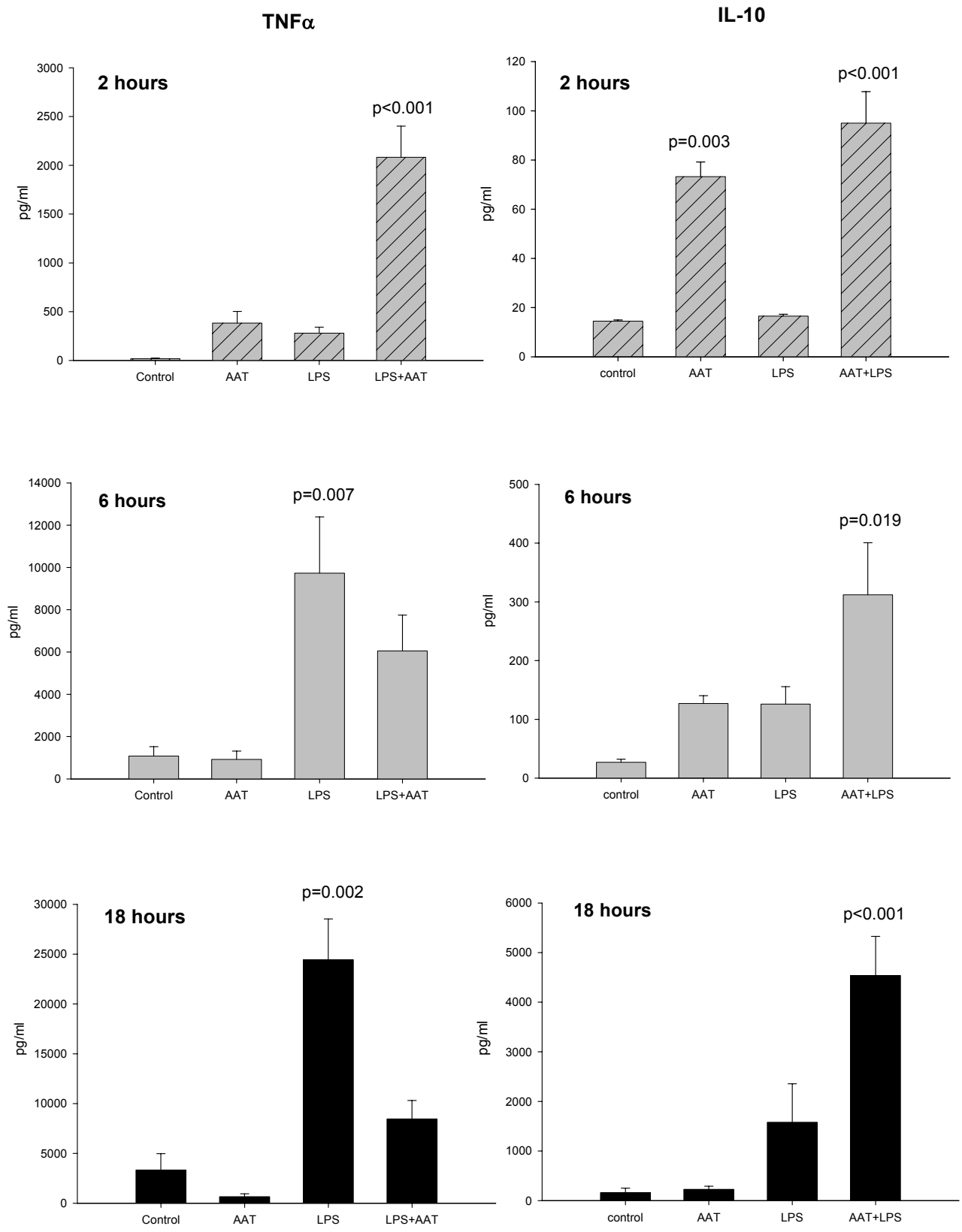
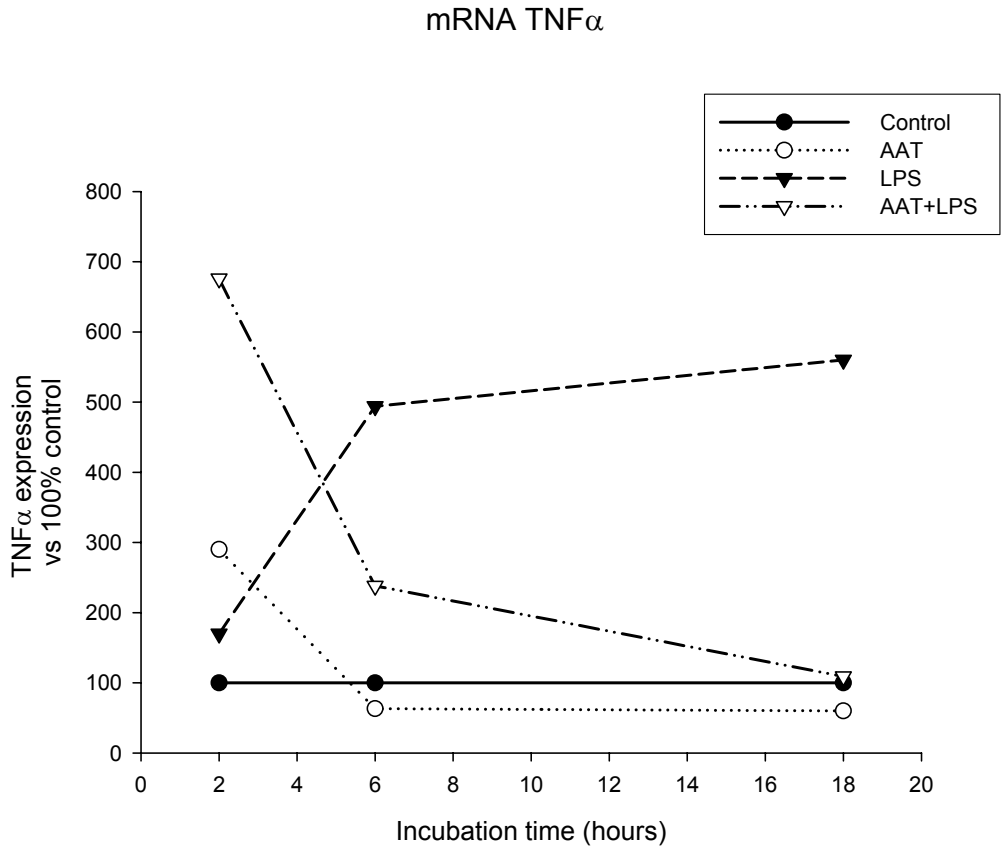

TNFα
IL-10
2 hours
p<0.001
pg/ml
3000
2500
2000
1500
1000
500
0
Control
AAT
LPS
LPS+AAT
2 hours
p=0.003
p<0.001
120
100
80
60
40
20
0
control
AAT
LPS
AAT+LPS
6 hours
p=0.007
14000
12000
10000
8000
6000
4000
2000
0
6 hours
p=0.019
500
400
300
200
100
0
18 hours
p=0.002
30000
25000
20000
15000
10000
5000
0
18 hours
p<0.001
6000
5000
4000
3000
2000
1000
0

FIGURE 1A


mRNA TNFα
Control
AAT
LPS
AAT+LPS
TNFα expression
vs 100% control
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16
18
20
Incubation time (hours)

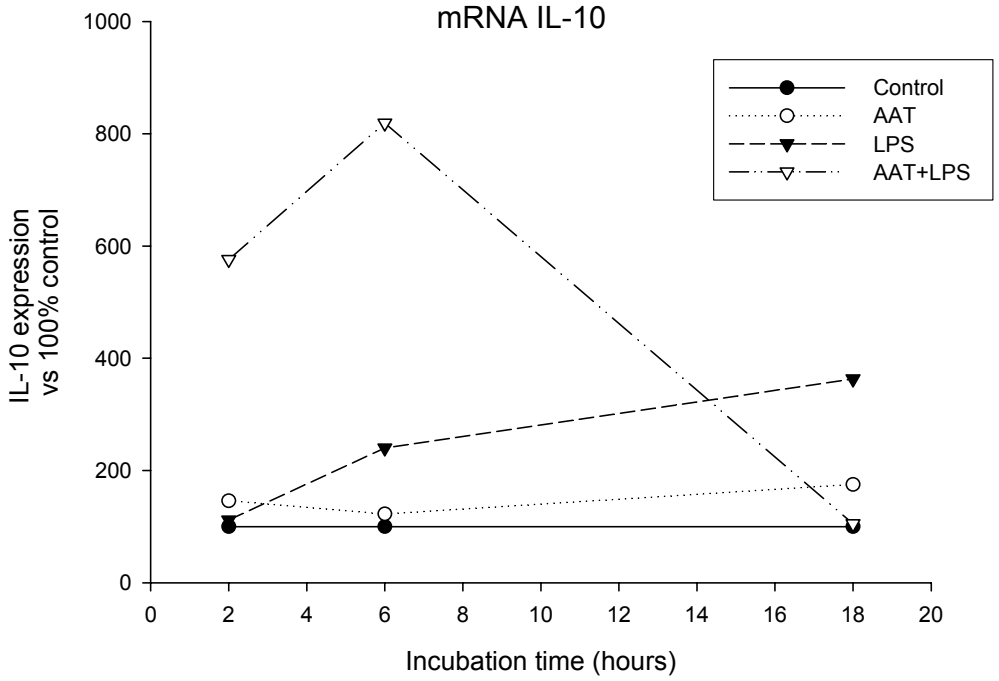

mRNA IL-10
Control
AAT
LPS
AAT+LPS
IL-10 expression
vs 100% control
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
Incubation time (hours)

FIGURE 1B

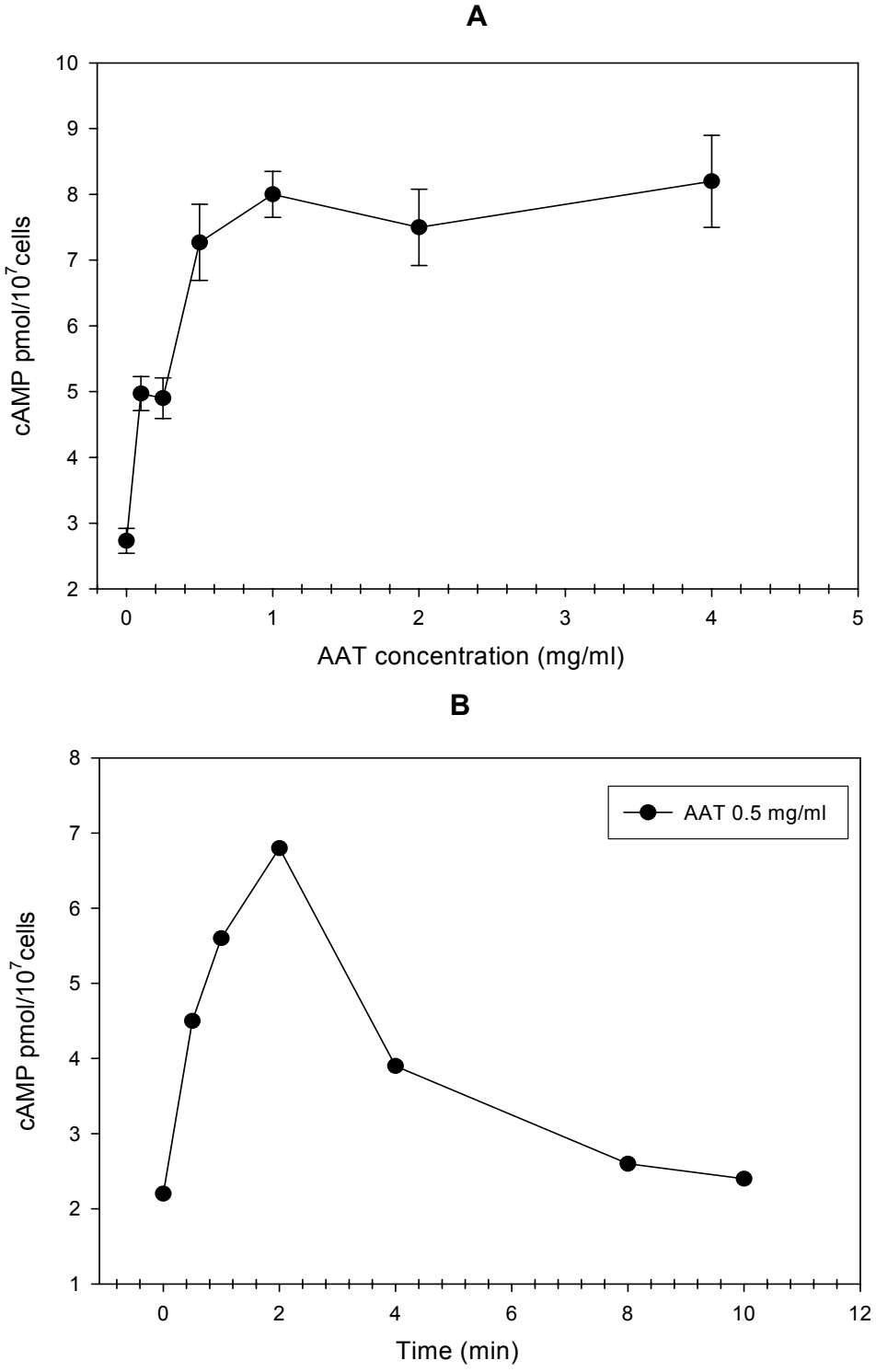

A
cAMP pmol/10^7cells
AAT concentration (mg/ml)
B
AAT 0.5 mg/ml
cAMP pmol/10^7cells
Time (min)

FIGURE 2

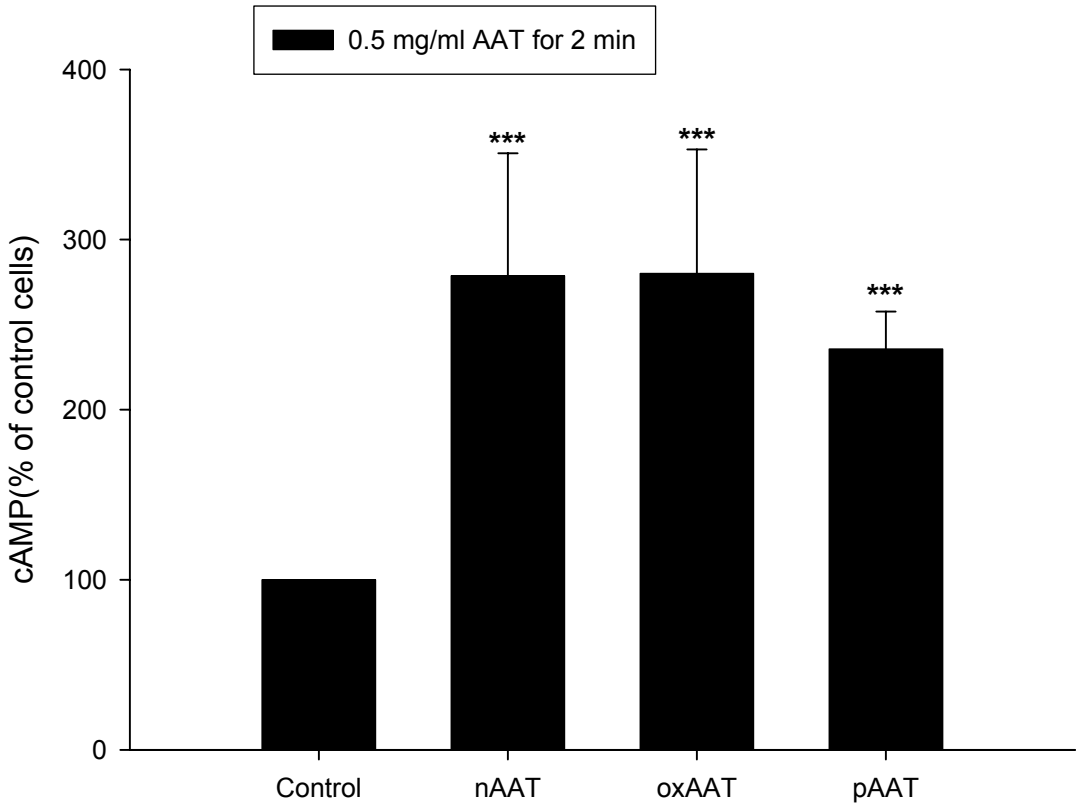

0.5 mg/ml AAT for 2 min
400
300
200
100
0
cAMP(% of control cells)
***
***
***
Control
nAAT
oxAAT
pAAT

FIGURE 3

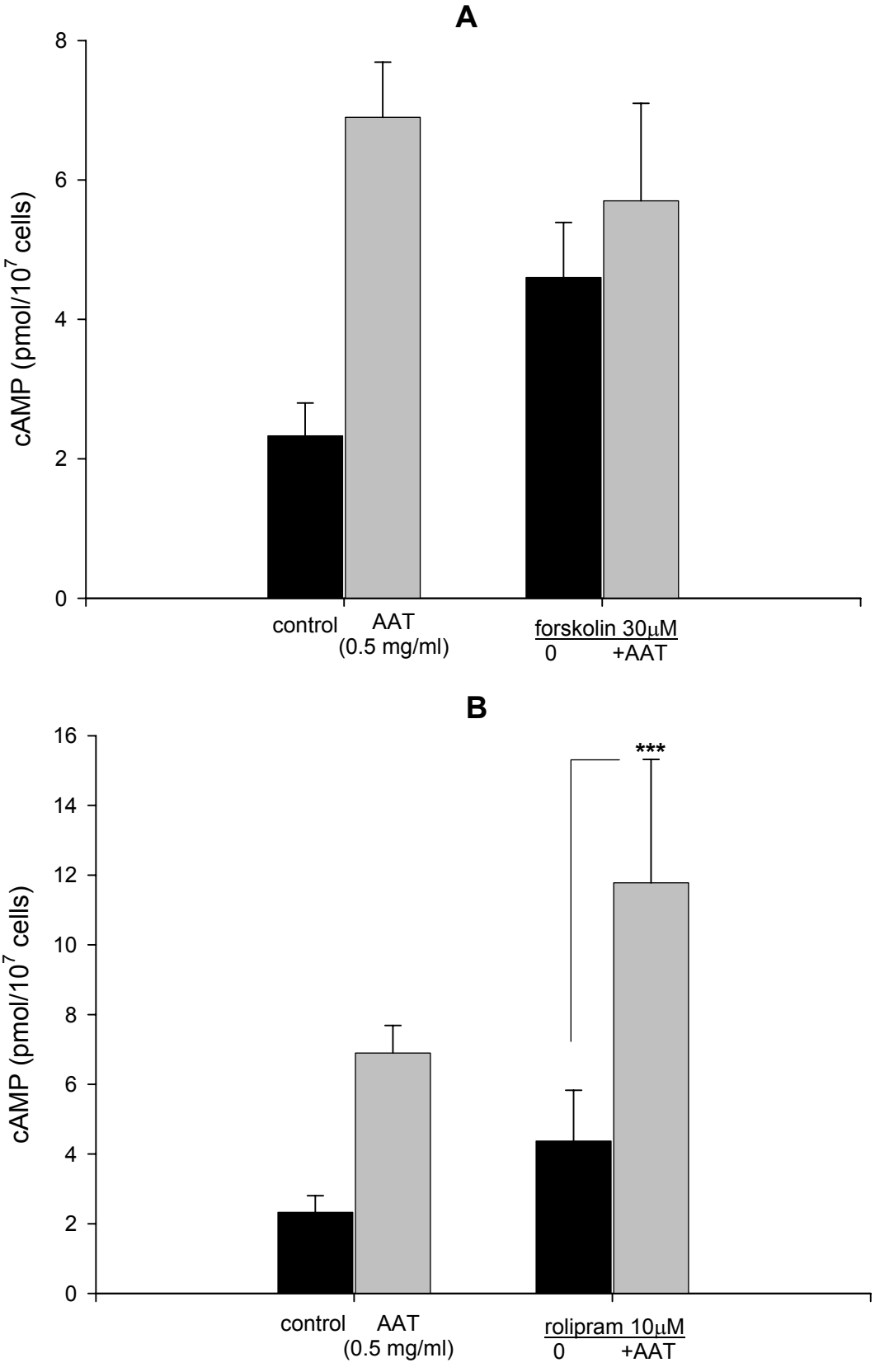


FIGURE 4 A and B

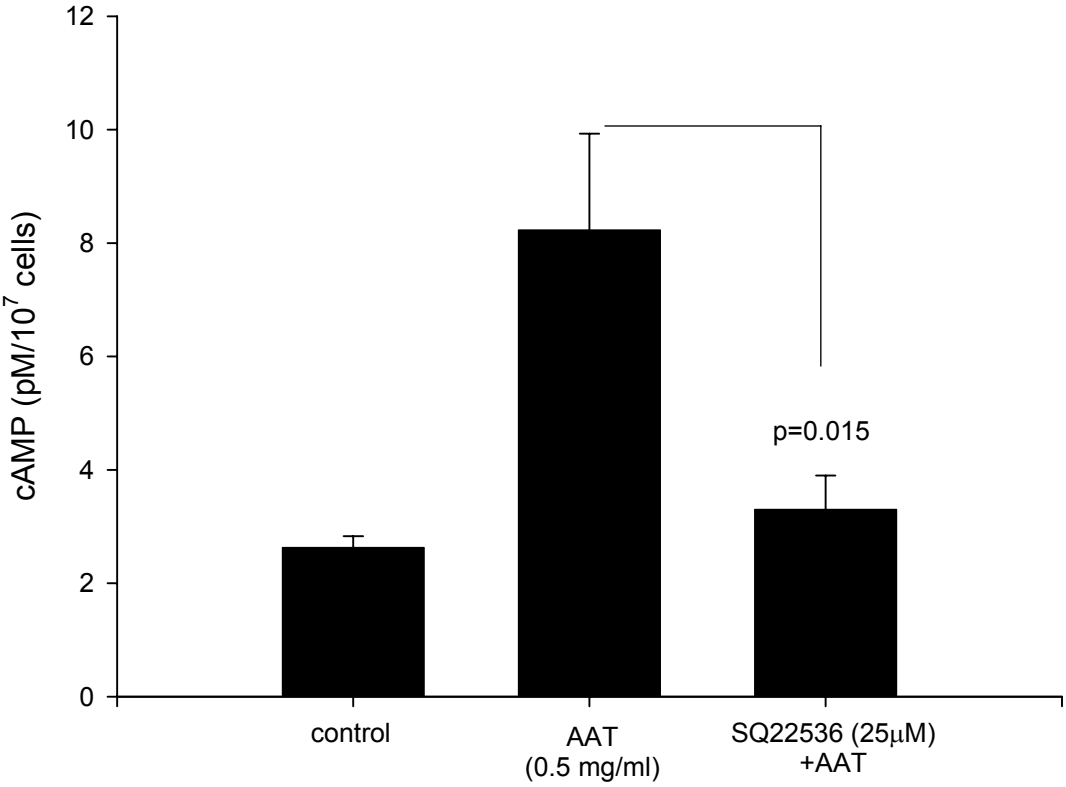
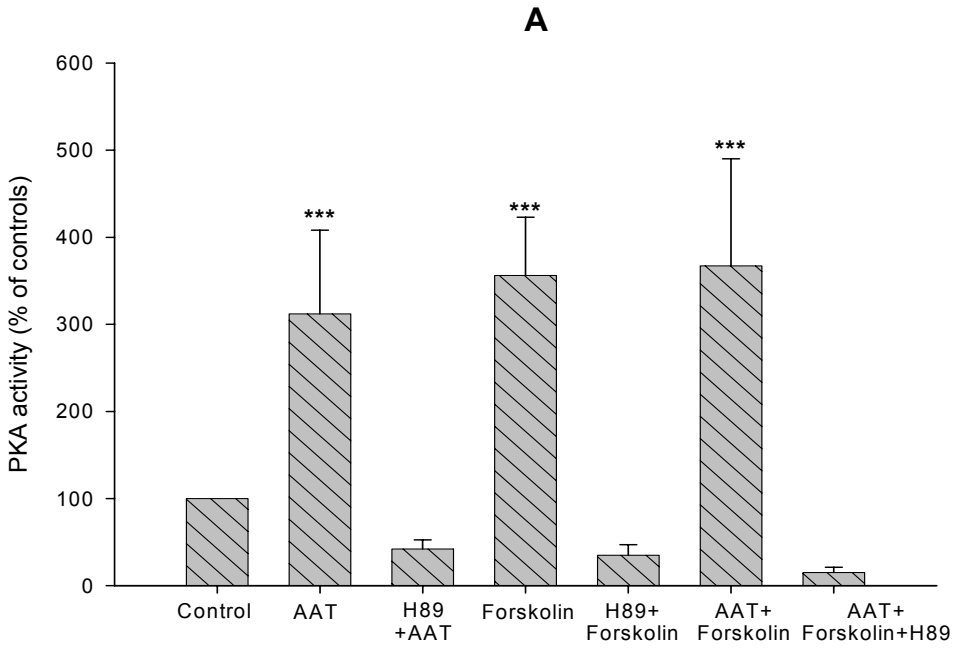
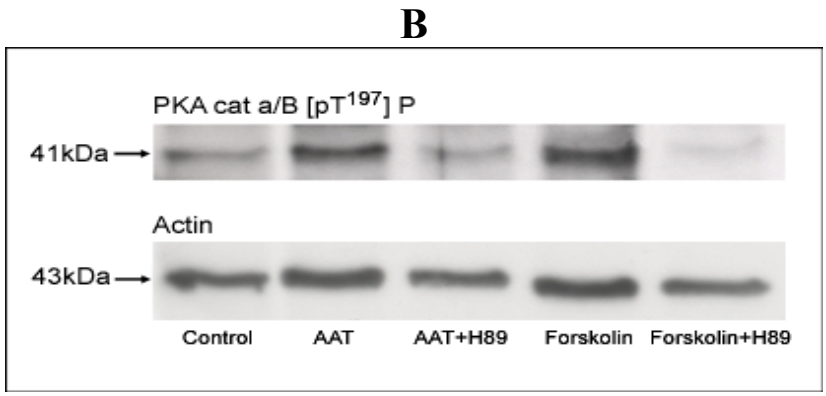

12
10
8
6
4
2
0
cAMP (pM/10^7 cells)
p=0.015
control
AAT
(0.5 mg/ml)
SQ22536 (25μM)
+AAT

FIGURE 5





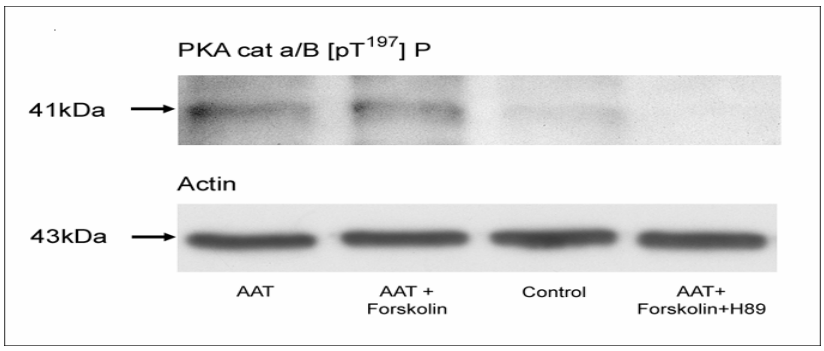


FIGURE 6 A, B and C

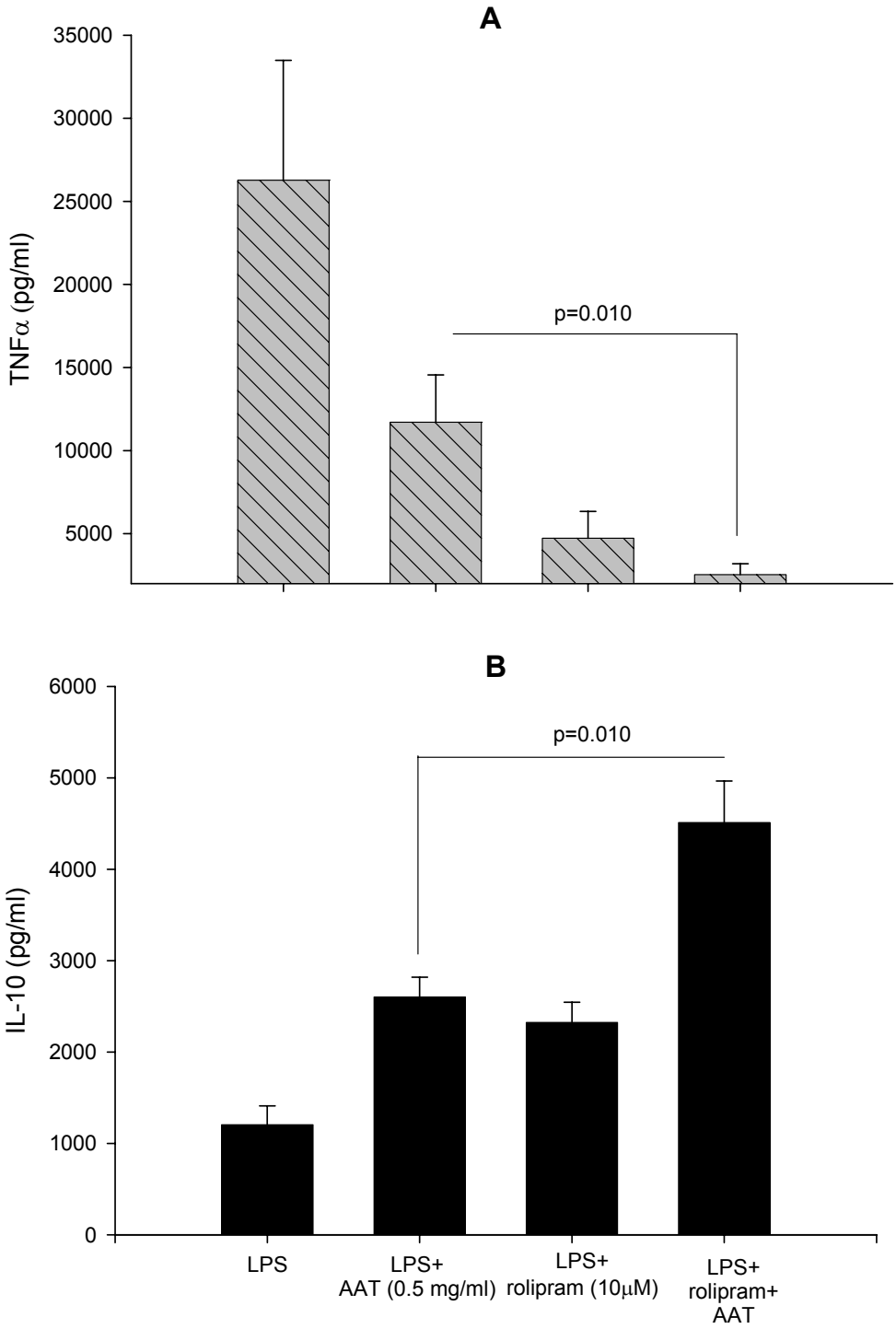


FIGURE 7 A and B

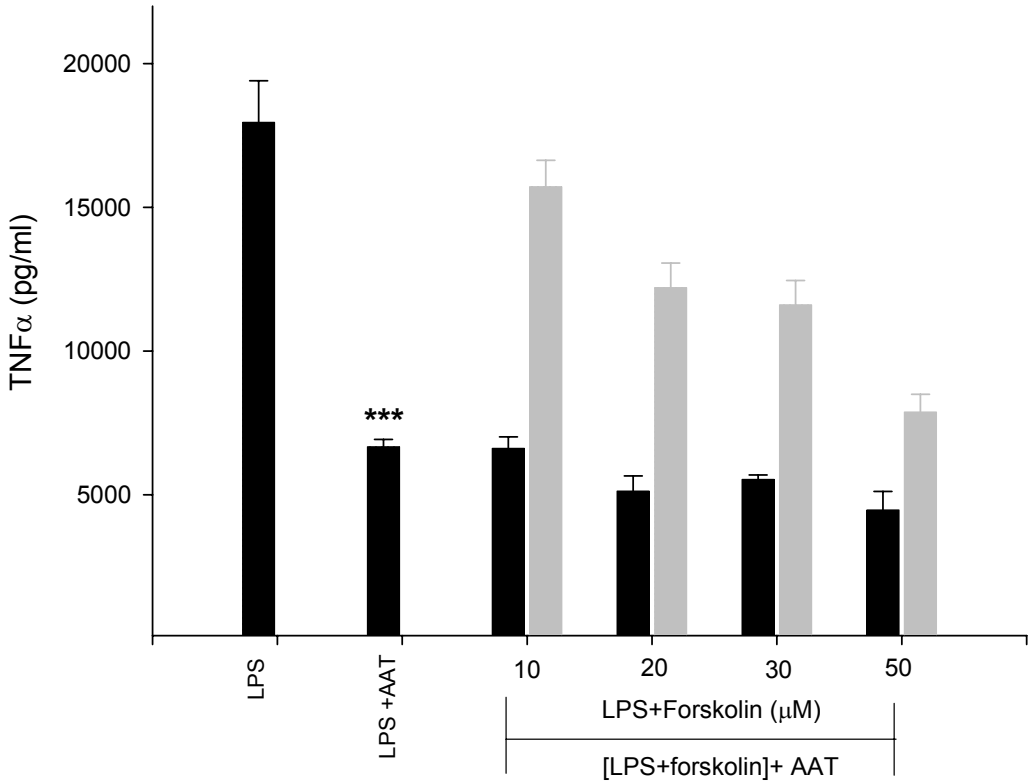

20000
15000
10000
5000
TNFα (pg/ml)
***
LPS
LPS +AAT
10
20
30
50
LPS+Forskolin (μM)
[LPS+forskolin]+ AAT

FIGURE 8