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INHIBITION OF JNK AND P38 MAPK PHOSPHORYLATION BY 5-(ACETYLAMINO)-4-OXO-6-PHENYL-2-HEXENOIC ACID METHYL ESTER AND 4-PHENYL-BUTENOIC ACID DECREASES SUBSTANCE P-INDUCED TNF- α UPREGULATION IN MACROPHAGES

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Abstract

The interactions between the immune and nervous systems play an important role in immune and inflammatory conditions. Substance P (SP), the undecapeptide RPKPQQFFGLM-NH₂, is known to upregulate the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α . We report here that 5-(acetylamino)-4-oxo-6-phenyl-2-hexenoic acid methyl ester (AOPHA-Me) and 4-phenyl-3-butenic acid (PBA), two anti-inflammatory compounds developed in our laboratory, reduce SP-stimulated TNF- α expression in RAW 264.7 macrophages. We also show that AOPHA-Me and PBA both inhibit SP-stimulated phosphorylation of JNK and p38 MAPK. Furthermore, molecular modeling studies indicate that both AOPHA-Me and PBA dock at the ATP binding site of apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPKs upstream of both JNK and p38 MAPK, with predicted interaction energies of -7.0 kcal/mol and -5.9 kcal/mol, respectively; this binding overlaps with that of staurosporine, a known inhibitor of ASK1. Taken together, these findings suggest that AOPHA-Me and PBA inhibition of TNF- α expression in SP-stimulated RAW 264.7 macrophages is a consequence of the inhibition JNK and p38 MAPK phosphorylation. We have previously shown that AOPHA-Me and PBA inhibit the amidative bioactivation of SP, which also would be expected to decrease formation of pro-inflammatory cytokines. It is conceivable that this dual action of inhibiting amidation and MAPK phosphorylation may be of some advantage in enhancing the anti-inflammatory activity of a therapeutic molecule.

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Keywords

Substance P; TNF- α ; p38 MAPK; JNK; RAW 264.7 macrophage

1. Introduction

The interactions between the immune and nervous systems play an important role in immune and inflammatory conditions [1]. Substance P (SP), the undecapeptide RPKPQQFFGLM-NH₂, has been identified as a key signaling molecule whose functions span both the immune and nervous systems [2]. SP is a member of the tachykinins, a group of structurally-related peptide hormones which transmit signals by binding to the neurokinin (NK) family of G protein-coupled receptors, denoted NK-1R, NK-2R, and NK-3R [2, 3]. Although once considered to be found exclusively in the nervous system, tachykinins and their receptors have now been reported in many cell types such as human endothelial cells, human epithelial cells, and several human, mouse and rat immune cells [2].

SP exerts effects on many aspects of immune cell function. SP is known to upregulate the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α , and triggers generation of reactive oxygen species such as superoxide and hydrogen peroxide [4, 5]. SP binding activates cytokine production via mitogen-activated protein kinase (MAPK) signaling [6]. MAPKs are a family of proline-directed protein serine/threonine kinases which transmit signals from the surface of the cell to the nucleus via a cascade of intracellular phosphorylation events [7]. There are four families of MAPKs, the most studied being ERK, JNK, and p38 MAPK; each family consists of three tiers of kinases where sequentially a MAP kinase kinase kinase phosphorylates a MAP kinase kinase, which then phosphorylates a MAP kinase [7]. This process activates transcription factors such as NF- κ B [8], which in turn upregulates production of cytokines, such as TNF- α . TNF- α is a key signaling molecule in the inflammatory response and has been implicated in autoimmune diseases such as rheumatoid arthritis [9], ankylosing spondylitis [10], and Crohn's disease [11]. Pharmacological interruption of TNF- α signaling has shown to be an effective treatment for such diseases [12, 13].

SP, like all the tachykinins and roughly half of all peptide hormones, is initially synthesized as a glycine-extended precursor, which is then converted to the bioactive C-terminal amidated form [14]. Amidation is a two step process wherein the glycine-extended precursor (SP-Gly) is first acted on by peptidylglycine β -monooxygenase (EC 1.14.17.3) (PAM), the rate-limiting enzyme in the pathway, to form an α -hydroxyglycine intermediate [15]. Subsequently, peptidylamidoglycolate lyase (EC 4.3.2.5) (PGL) catalyzes the dealkylation of the α -hydroxyglycine intermediate to form the amidated peptide and glyoxylic acid [16, 17]. 5-(Acetylamino)-4-oxo-6-phenyl-2-hexenoic acid (AOPHA) and 4-phenyl-3-butenic acid (PBA) are turnover-dependent amidation inhibitors [15, 18, 19]. We have demonstrated that AOPHA-Me and PBA exhibit anti-inflammatory activity *in vivo* mediated by a non-COX inhibitory pathway [20, 21].

The effect of these anti-inflammatory agents on cytokine and MAPK signaling has not been determined. Therefore, this study was conducted to evaluate the effects of AOPHA-Me and

PBA on TNF- α expression and on phosphorylation of JNK and p38 MAPK in SP-stimulated RAW 264.7 macrophages.

2. MATERIALS AND METHODS

2.1. MATERIALS

RAW 264.7 cells were purchased from ATCC (Manassas, VA). SP, PBA, cell culture grade quality MTT and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture quality Pen/Strep, DMEM, PBS and bicarbonate were purchased from Cellgro. FBS (5 EU/mL) was purchased from Gibco (Grant Island, NY). TNF- α ELISA kits were purchased from e-Bioscience (San Diego, CA). 5-(Acetylamino)-4-oxo-6-phenyl-2-hexenoic acid methyl ester was synthesized as described previously [19]. Glycine-extended SP (RPKPQQFFGLMG-COOH) was synthesized as described previously [22]. Phospho-p38 MAP kinase (Thr180/Tyr182) polyclonal antibody, p38 MAP kinase polyclonal antibody, JNK polyclonal antibody, phospho-JNK (Thr183/Tyr185) polyclonal antibody, and anti-rabbit IgG alkaline phosphatase conjugated antibody were purchased from Cell Signaling Technology (Beverly, MA). Tween-20, TRIS-HCl, DC Protein Assay, SDS, nonfat dry milk, 25X alkaline phosphatase color development buffer, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT), protein molecular mass standards, and all electrophoresis and transfer buffer components were from Bio-Rad (Hercules, CA).

2.2. CELL CULTURE

RAW 264.7 cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells, when 70-80% confluent, were subcultured by scraping and plated at 10% confluence during each passage. For experiments, cells were seeded in either 96-well plates, 6-well plates, or 12 cm² dishes and grown overnight to 70-80% confluence. At least two hours before each experiment, cell media was exchanged for unsupplemented DMEM. LPS, SP, SP-Gly or PBA were dissolved in PBS and diluted in unsupplemented DMEM. AOPHA-Me was dissolved in DMSO and diluted in unsupplemented DMEM such that the final concentration of DMSO was less than 0.1%.

2.3. ELISA ASSAY FOR TNF- α

The concentration of TNF- α present in the media of RAW 264.7 macrophages was determined using a mouse TNF- α ELISA kit according to the instructions of the manufacturer.

2.4. WESTERN BLOT ANALYSIS FOR SIGNALING PATHWAY PROTEINS

RAW 264.7 cells were grown to 70-80% confluence in 12 cm² dishes, washed with PBS and extracted with a mixture of 2% SDS, 1mM PMSF, and 1:100 dilution of protease inhibitor cocktail. Lysed cells were scraped, transferred to microcentrifuge tubes, and sonicated for two, 15-second pulses at room temperature. Protein concentrations were determined using the Bio-Rad DC assay. Proteins were separated on 12.5% acrylamide SDS gels and transferred to PVDF membranes using a Trans-Blot Turbo system. Membranes were stained with Ponceau Red, scanned on an HPscanjet 4400C scanner and then incubated in non-fat

milk based block buffer for 1–2 hours. p38 MAPK, phospho-p38 MAPK, JNK or phospho-JNK antibodies were incubated separately with membranes in non-fat milk based block buffer overnight at 4°C. Immunopositive bands were detected using alkaline phosphatase-linked anti-rabbit secondary antibody, with development using BCIP/NBT as substrates. Blots were scanned on an HPscanjet 4400C scanner and band densities determined using UN-SCAN-IT software (version 5.1) from Silk Scientific, Inc. (Orem, UT). Two replicate blots were analyzed for each experiment. Antibodies used for the detection of phosphorylated forms of p38 and JNK only recognize these enzymes when they are dually phosphorylated at the key activation sites Thr180 and Tyr182 or Thr183 and Tyr185, respectively [23, 24].

2.5. CELL PROLIFERATION VIABILITY

Cell proliferation of RAW 264.7 macrophages was determined by use of a MTT assay. Living cells react with MTT to form formazan crystals. The MTT assay was conducted in 96-well plates, and 20 μ L of 5 mg/mL MTT in PBS was added to each well of the plate. Experiments were conducted in duplicate with three replicate samples per treatment. The plates were incubated for two hours, the supernatants were removed, and 200 μ L of DMSO was added each to each well to dissolve the formazan crystals. The absorbance of the dissolved formazan crystals was measured at 570 nm minus the absorbance at 670 nm.

2.6. CELL VIABILITY

Cell viability of RAW 264.7 macrophages was determined by use of a trypan blue exclusion assay. Cells were grown in 6 well plates as described in 2.2. Experiments were conducted in duplicate with three replicate samples per treatment. Cells were scrapped and automatically analyzed by a Vi-CELL instrument.

2.7. MOLECULAR MODELING

Autodock Vina was used for all docking in this study [25]. In general, parameters were kept at their default values. The size of the docking grid was $50 \times 60 \times 50$, which encompasses the entire apoptosis signal-regulating kinase 1 (EC 2.7.11.25) (ASK1), structure and the grid spacing was set at 1.0. Ligand structures were prepared using ChemBio3D Ultra 12.0 and individual PDB files were prepared for docking using AutoDock Tools. The 2CLQ ASK1 structure was obtained from the PDB [26]. To prepare the structure for docking, the ligand and all water molecules were removed and charges and non-polar hydrogen atoms were added using AutoDock Tools.

2.8. STATISTICAL ANALYSIS

Data were analyzed with a one-way analysis of variance and followed by Tukey's *post hoc* test. Values of *p* less than 0.05 were considered significant for all calculations. Statistical analyses were performed using Statistix for Windows v8.1.

3. RESULTS

3.1. SP BUT NOT SP-GLY STIMULATES TNF- α PRODUCTION IN RAW 264.7 MACROPHAGES

We investigated the effects of SP and SP-Gly on TNF- α production by RAW 264.7 macrophages. As shown in Figure 1, incubation with 1-100 μ M SP for 24 hours stimulated 70-1000 pg/mL of TNF- α production in a dose-dependant manner, and unstimulated control cell produced 37 pg/mL of TNF- α . In contrast, SP-Gly at similar concentrations had no effect on TNF- α production. To determine the onset of TNF- α upregulation by SP in macrophages, RAW 264.7 macrophages were incubated with 1 μ M SP or 10 μ M SP for 2, 4, 8, 12, 16, 20 and 24 hours. As shown in Figure 2, an increase in TNF- α concentration was apparent after about 4 hours, and reached a maximum at about 20 hours.

3.2. SP AND SP-GLY DO NOT ALTER RAW 264.7 MACROPHAGE PROLIFERATION AND VIABILITY

The effects of SP and SP-Gly on RAW 264.7 macrophage proliferation and viability were determined using MTT and trypan blue assays. Neither 20 μ M SP nor 20 μ M SP-Gly had any significant effect on cell proliferation or viability after 24 hours of incubation (data not shown).

3.3. INHIBITION OF SP-STIMULATED TNF- α PRODUCTION IN MACROPHAGES

Next, we investigated the effects of AOPHA-Me and PBA and on SP-stimulation of TNF- α production by RAW 264.7 macrophages. As shown in Figure 3, co-incubation of 0.01-1 mM PBA with 20 μ M SP for 24 hours reduced TNF- α expression by as much as 68%. It is apparent from Figure 3 that AOPHA-Me is more potent than PBA, since a reduction in TNF- α expression of 79% was obtained in the presence of only 10 μ M AOPHA-Me.

3.4. AOPHA-ME AND PBA DECREASE SP-INDUCED PHOSPHORYLATION OF JNK AND P38 MAPK IN RAW 264.7 MACROPHAGES

To assess protein loading, Ponceau Red-stained membranes were scanned and determined to possess even total protein loading (data not shown). Figure 4A shows the effects of SP treatment on p38 MAPK phosphorylation in RAW 264.7 macrophages in the absence and presence of AOPHA-Me. As shown in the figure, treatment with 20 μ M SP for 24 hours markedly increases phosphorylation of p38 MAPK. It has been demonstrated that phosphorylation at both Thr180 and Tyr182 is required for activation of p38 MAPK [24], and the antibody used for the detection of phosphorylated p38 MAPK is specific for the dually phosphorylated form of p38 MAPK. It is apparent that 10 μ M AOPHA-Me markedly inhibits SP-induced phosphorylation of p38 MAPK. As shown in Figure 4B, densitometric scans of the blots in Figure 4A reveal a 15.6-fold increase in the immunoreactive band density of phospho-p38 MAPK in SP-stimulated cells which is inhibited by 94% in the presence of AOPHA-Me.

Figure 4C shows the effects of SP treatment on JNK phosphorylation in the absence and presence of AOPHA-Me. As shown in the figure, treatment with 20 μ M SP for 24 hours markedly increases phosphorylation of JNK. It has been shown that phosphorylation at both

Thr183 and Tyr185 is required for activation of JNK [23], and the antibody used for the detection of phosphorylated JNK is specific for the dually phosphorylated form of JNK. It is apparent that 10 μ M AOPHA-Me markedly inhibits SP-induced phosphorylation of JNK. As shown in Figure 4D, densitometric scans of the blots in Figure 4C reveal a 2.8-fold increase in the density of phospho-JNK in SP-stimulated cells and an inhibition of 63% in the presence of AOPHA-Me.

Figure 4, panels E-H, illustrate similar experiments on phosphorylation of p38 MAPK and JNK in the presence and absence of PBA. Once again, densitometric scans of the blots from Figure 4E and 4G reveal that SP treatment gives rise to 9.3-fold and 2.9-fold increases in the densities of phospho-p38 MAPK and phospho-JNK, respectively, which are in turn inhibited by 84% and 91%, respectively, upon treatment with PBA.

3.5. EFFECT OF AOPHA-ME AND PBA ON RAW 264.7 MACROPHAGE PROLIFERATION AND VIABILITY

We investigated the effect of AOPHA-Me and PBA on RAW 264.7 macrophage proliferation and viability. Administration of 10-1000 mM PBA, or 10 μ M AOPHA-Me alone or with 20 μ M SP had no significant effect on cell proliferation or viability after 24 hours, as demonstrated by MTT and trypan blue assays (data not shown).

3.6. AOPHA-ME AND PBA VIRTUALLY DOCK IN THE ATP BINDING SITE OF ASK1

Bunkoczi et al. [26] have reported the crystal structure of apoptosis signal-regulating kinase 1 (ASK1) bound to the kinase inhibitor staurosporine. It has been demonstrated that inhibition of ASK1 results in decreased phosphorylation of JNK and p38 MAPK [27]. We therefore used the molecular modeling software, AutoDock Vina, to investigate whether or not our inhibitors would overlap with the bound staurosporine when docked to the ATP binding site of ASK1. To first validate the use of AutoDock Vina for this purpose, we docked staurosporine and observed it to be faithfully recapitulated with the crystallographically-determined binding of staurosporine.

Our results are illustrated in Figure 5. In Figure 5A, it is evident that AOPHA-Me (yellow) and PBA (green) exhibit predicted overlap with both the virtually bound staurosporine (gray) and the crystallographically-determined binding of staurosporine (black). Note that, in Figure 5B, the phenyl ring of both AOPHA-Me and PBA packs against the hydrophobic side-chains of the ASK1 residues Leu686, Val694, Ala707, and Leu810. This is in agreement with the crystallographically-determined binding of staurosporine which was shown by Bunkoczi et al. [26] to also pack with its five-ring system against these hydrophobic residues of the binding pocket. We calculate a binding energy of -7.0 kcal/mol for binding of AOPHA-Me to ASK1, a binding energy of -5.9 kcal/mol for binding of PBA to ASK1, and a binding energy of -6.9 kcal/mol for binding of ATP to ASK1. In addition, we calculate a binding energy of -12.0 kcal/mol for the binding of staurosporine to ASK1, which is expected as staurosporine is known to bind more tightly than ATP to a number of kinases [28].

4. DISCUSSION

Taken together, our results suggest that AOPHA-Me and PBA reduce SP-stimulated TNF- α production by preventing phosphorylation of JNK and p38 MAPK in RAW 264.7 macrophages. To demonstrate this, we first show that SP stimulates TNF- α expression in RAW 264.7 macrophages, as shown in Figures 1 and 2. We note that others have reported similar results with SP in primary culture [29] but, to the best of our knowledge, our findings represent the first report on the effect of SP on TNF- α production in RAW 264.7 macrophages. Next, we report that both AOPHA-Me and PBA attenuate SP-stimulated TNF- α expression in RAW 264.7 macrophages, as shown in Figure 3. Compounds that can modulate TNF- α signaling have been of particular interest in treating inflammatory diseases and indeed, we have shown that AOPHA-Me and PBA exhibit anti-inflammatory activity in rat models of both chronic and acute models of arthritis [20]. Lastly, we report here that AOPHA-Me and PBA both inhibit SP-stimulated phosphorylation of JNK and p38 MAPK, as shown in Figure 4. We note that Sun *et al.* [30] have reported that treatment with SP increased phosphorylation of p38, but not of JNK, in RAW 264.7 macrophages; however they used a much lower concentration of SP in their work.

These findings are in agreement with our previous work where we showed that PBA inhibits JNK phosphorylation in both Ras transformed WB and human lung carcinoma cells [31]. However, in contrast to our findings in SP-stimulated RAW 264.7 macrophages, it was also demonstrated that PBA activates p38 MAPK phosphorylation in both Ras transformed WB and human lung carcinoma cells [31]. It is conceivable that the differences between the cell lines used could account for the differing activities of PBA on p38 MAPK phosphorylation; we also note that these previous studies were conducted in the absence of SP-stimulation.

Upregulation of cytokines, such as TNF- α , in immune cells is generally controlled by the MAPK pathways [32]. MAPKs are activated by phosphorylation initiated by cell surface receptor signaling [32]. Phosphorylated MAPKs then activate several transcription factors such as NF- κ B, AP-1, CREB, c-Jun, and STAT1, leading to upregulation of cytokines [32]. Indeed, Azzolina *et al.* [6] have shown that SP upregulates TNF- α via activation of JNK and p38 MAPK in mast cells. Similarly, the link between JNK and p38 MAPK activation and TNF- α upregulation has also been demonstrated for LPS-activated RAW 264.7 macrophages [33, 34]. These findings support our conclusion that AOPHA-Me and PBA inhibition of TNF- α expression in SP-stimulated RAW 264.7 macrophages is a consequence of the inhibitory activities of AOPHA-Me and PBA on phosphorylation of JNK and p38 MAPK.

In contrast to our results with SP, we find that SP-Gly has no effect on TNF- α expression in RAW 264.7 macrophages, as shown in Figure 1. We had hypothesized that SP-Gly and SP would have similar activities, on the assumption that amidation of SP-Gly to SP would readily occur. It has long been assumed that the glycine-extended precursors of bioactive peptides have little or no bioactivity prior to amidation [14]. For example, SP-Gly-induced relaxation of rat aortic strips and stimulation of NO production in bovine endothelial cells is markedly reduced in the presence of inhibitors which prevent conversion of SP-Gly to SP [22, 35]. It is therefore apparent that, at least under our experimental conditions, RAW 264.7

macrophages are unable to amidate exogenous SP-Gly to a degree that would stimulate TNF- α production.

If RAW 264.7 macrophages are unable to amidate exogenous SP-Gly it raises the question of how our inhibitors, designed as amidation inhibitors, inhibit SP activation of JNK and p38 MAPK. We found through the use of molecular modeling software Autodock Vina that AOPHA-Me and PBA are predicted to bind to the ATP binding site of apoptosis signal-regulating kinase 1 (ASK1), a member of the MAPKs upstream of both JNK and p38 MAPK, with energies of -7.0 kcal/mol and -5.9 kcal/mol respectively. This is in comparison to ATP binding to the ATP binding site of ASK1 with an energy of -6.9 kcal/mol. Importantly, the phenyl ring of both AOPHA-Me and PBA packs against the hydrophobic side-chains of the ASK1 residues Leu686, Val694, Ala707, and Leu810. This is in agreement with the crystallographically-determined binding of staurosporine, a known ASK1 inhibitor, which was shown by Bunkoczi et al. [26] to also pack with its five-ring system against these hydrophobic residues of the binding pocket. Inhibition of ASK1 has been shown to inhibit the activation of JNK and p38 MAPK in pancreatic β cells and could therefore provide a plausible explanation for the effects of AOPHA-Me and PBA on MAPK signaling [27].

Small molecule inhibitors of non-receptor protein kinases which participate in intracellular signaling cascades are of current interest for the treatment of inflammatory conditions [36]. For example, several small molecule Janus kinase (JAK) inhibitors have proceeded through phase II and phase III clinical trials for the treatment of rheumatoid arthritis, irritable bowel syndrome, and psoriasis [37, 38]. Inhibition of the JAK pathway prevents the activation of several transcription factors and thereby impedes upregulation of a number of pro-inflammatory cytokines such as TNF- α and IL-6 [39]. We demonstrate here that AOPHA-Me and PBA inhibit the phosphorylation of JNK and p38 MAPK by SP and prevent the upregulation of TNF- α . Since none of the signaling molecules in the JNK and p38 MAPK cascades are presently known to be subject to amidative bioactivation, the activities of AOPHA-Me and PBA reported here are presumably unrelated to their activities as amidation inhibitors. On the other hand, pro-inflammatory amidated peptide hormones such as SP activate MAPK signaling; therefore inhibition of amidation would also be expected to lead to a decrease in pro-inflammatory cytokines such as TNF- α . It is conceivable that this dual action of inhibiting amidation and MAPK phosphorylation may be of some advantage in enhancing the anti-inflammatory activity of a therapeutic molecule.

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Highlights

- Substance P (SP) upregulates the production of pro-inflammatory cytokines
- The novel anti-inflammatory compounds AOPHA-Me and PBA inhibit amidative bioactivation of SP
- AOPHA-Me and PBA inhibit TNF- α expression and MAPK phosphorylation in SP stimulated macrophages
- Molecular modeling is consistent with inhibition of an upstream kinase by AOPHA-Me and PBA
- The novel dual action on amidation and MAPKs may enhance the therapeutic potential of these anti-inflammatory compounds

Figure 1.

SP but not SP-Gly upregulates TNF- α in RAW 264.7 macrophages. Cells were incubated with 0.1-100 μ M SP or 0.01-100 μ M SP-Gly for 24 hr. The cell media was analyzed for TNF- α with an ELISA kit, and the results are presented as the mean $\pm$ standard deviation. The data are representative of two independent experiments. n=4. * $p < 0.05$ versus control samples.

Figure 2.

Time dependent effect of SP on TNF- α expression in RAW 264.7 macrophages. Cells were incubated with 1 μ M or 10 μ M SP for 2, 4, 8, 12, 16, 20 and 24 hr. The cell media was analyzed for TNF- α with an ELISA kit, and the results are presented as the mean $\pm$ standard deviation. The data are representative of two independent experiments. n=4. * $p < 0.05$ versus control samples.

Figure 3.

Amidation inhibitors decrease SP-stimulation of TNF- α in RAW 264.7 macrophages. Cells were co-incubated with 20 μ M SP and 0.01-1 mM PBA or 0.01 μ M AOPHA-Me for 24 hr. The cell media was analyzed for TNF- α with an ELISA kit, and the results are presented as the mean $\pm$ standard deviation. The data are representative of two independent experiments. n=4. * $p < 0.05$ versus SP samples.

Figure 4.

Amidation inhibitors prevent SP-stimulated activation of p38 MAPK (Thr180/Tyr182 phosphorylation) (A and E) and JNK (Thr183/Tyr185 phosphorylation) (C and G) in RAW 264.7 macrophages. Cells were either left untreated (lanes 1 and 2), treated with 20 μ M SP (lanes 3 and 4), 10 μ M AOPHA-Me (lanes 5 and 6 in panels A and C), 1mM PBA (lanes 5 and 6 in panels E and G) or co-incubated with 20 μ M SP and either 10 μ M AOPHA-Me (lanes 7 and 8 in panels A and C) or 1 mM PBA (lanes 7 and 8 in panels E and G) for 24 hr. Whole cell lysates were extracted for total protein for Western blot analysis as described in Materials and Methods and an equal amount of protein was loaded in each lane. Panel A was loaded with 0.4 mg/mL of protein, panels C, E, and G were loaded with 0.1 mg/mL of protein. Identical treatments from replicate cultures are shown on each blot. Identical blots were either incubated with phospho-p38 MAPK or p38 MAPK antibodies in panels A and E and either phospho-JNK or JNK antibodies in panels C and G. The densitometric quantification of bands in scanned blots (B, D, F, and H) are presented as the mean $\pm$ standard deviation, and are representative of two independent experiments (*a* indicates *p*

<0.05 compared to SP, *b* indicates $p < 0.05$ compared to control). Phospho-p38 MAPK antibodies used are specific for Thr180/Tyr182 and phospho-JNK antibodies used are specific for Thr183/Tyr185.

Figure 5.

Docking of inhibitors into the ATP binding site of ASK1 using AutoDock Vina. A. Models of AOPHA-Me, PBA and staurosporine superimposed with the crystallographically-determined binding site of staurosporine. Crystallographic staurosporine, black; Virtual staurosporine, gray; AOPHA-Me, yellow; PBA, green. B. Superimposed models of AOPHA-Me, yellow, and PBA, green, shown with the main interacting residues of ASK1 in ball-and-stick representation. Note that the phenyl ring of both AOPHA-Me and PBA packs against the hydrophobic side-chains of the ASK1 residues Leu686, Val694, Ala707, and Leu810, in agreement with the crystallographically-determined binding of staurosporine [26].