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THE LIPOPOLYSACCHARIDE DETOXIFYING PROPERTIES OF CALF INTESTINAL ALKALINE PHOSPHATASE: AN IN VITRO STUDY

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ABSTRACT

This thesis provides an overview of the role of intestinal alkaline phosphatase (IAP) and lipopolysaccharide (LPS) in liver fibrosis and investigates the anti-inflammatory properties of IAP in vitro. IAP is an enzyme primarily expressed in the intestines, playing a vital role in nutrient absorption and gut health. LPS, an endotoxin found in gram-negative bacteria, triggers an inflammatory response when leaked from the intestines into the portal vein and subsequently into the liver. In liver fibrosis, LPS-induced inflammation leads to excessive extracellular matrix production and fibrotic tissue formation. Studies suggest that IAP can detoxify LPS by dephosphorylating its lipid A component. The thesis focuses on exploring additional factors that may enhance the detoxification of LPS by IAP through nitric oxide (NO) assays, p-nitrophenyl phosphate (pNPP) assays and co-immunoprecipitation. IAP was unable to significantly reduce inflammation caused by LPS on RAW 264.7 cells. With pNPP assays it was found that IAP had a higher activity in deproteinized and regular fetal bovine serum (FBS), compared to delipidated FBS. Additionally, during the immunoprecipitation experiment, several proteins co-eluted with IAP and IAP+LPS from FBS. There was no big visual difference on the SDS-page gel between the sample containing IAP compared to IAP + LPS. Further analysis using LC-MS is required to identify and quantify these co-eluted proteins of interest. These findings suggest that lipids and proteins in the cellular environment may play a role in modulating the activity of AP.

INTRODUCTION

INTESTINAL ALKALINE PHOSPHATASE

Alkaline phosphatase (AP) is an enzyme that plays an important role in various biological processes. It is involved in numerous physiological functions and is found in many tissues throughout the body, including the liver, bone, intestines, kidneys, and placenta. AP is used as a diagnostic marker for several diseases. [1]

AP, found in mammals, is a glycoprotein enzyme. AP operates as dimers, with two Zn2+ ions and one Mg2+ ion occupying the active site, which is crucial for its enzymatic function. Additionally, these metal ions play a significant role in shaping the structure of the AP monomer and indirectly govern interactions between subunits. Since AP is encoded by multiple genes, several isoforms of AP exist with slightly different properties [1]. The major isoforms include tissue-nonspecific alkaline phosphatase (TNAP), intestinal alkaline phosphatase (IAP), and placental alkaline phosphatase (PLAP). TNAP is present in most tissues, while IAP is predominantly found in the intestines, and PLAP is primarily expressed in the placenta. This thesis focuses on IAP and its role in liver fibrosis.

The primary function of AP is to catalyze the hydrolysis of phosphate groups from molecules such as nucleotides, proteins, and alkaloids, under alkaline conditions. It acts upon a wide range of substrates, such as p-nitrophenyl phosphate (pNPP) and phosphoesters, converting them into inorganic phosphate and alcohol or phenol products.

The different AP's have several unique physiological functions:

TNAP is critically involved in bone mineralization. vitamin B6 metabolism, and neurogenesis. It is also able to detoxify LPS and nucleotides [2,3] .

IAP is primarily expressed in the intestines and plays a significant role in nutrient absorption and gut health. It dephosphorylates various compounds, including lipids, carbohydrates, and bacterial endotoxins, contributing to their digestion and detoxification[1].

PLAP is found in the placenta and is involved in fetal development. It participates in the transport of nutrients, phosphate, and calcium across the placental barrier [4]

CLINICAL SIGNIFICANCE:

AP is widely used as a diagnostic marker for liver and bone disorders. Elevated levels of AP in the blood can indicate liver diseases such as hepatitis, cholestasis, or liver tumors. Increased AP levels are also observed in bone diseases like osteoporosis, Paget's disease, and bone metastasis. AP is routinely measured as part of liver function tests and bone-related investigations. Additionally, AP is used in combination with other enzymes and biomarkers to evaluate the overall health status of the liver and skeletal system[5]. Moreover, right now recombinant AP is investigated as a treatment for acute kidney injury caused by sepsis, due to its ability to detoxify lipopolysaccharide (LPS) and adenosine tri- and di-phosphates [6] .

In summary, AP is an essential enzyme involved in various physiological processes, including bone mineralization, intestinal function, and placental development. Its measurement and analysis serve as valuable tools in the diagnosis and monitoring of liver and bone disorders. Moreover, it exerts anti-inflammatory effects by detoxifying LPS.

ROLE OF LPS IN LIVER FIBROSIS

LPS is an endotoxin found in the outer membrane of gram-negative bacteria. When released into the body, LPS can trigger a strong immune response and inflammation. [7]

The LPS molecule consists of three parts: the lipid A part, the core oligosaccharides, and the O-antigen. Some variation is observed between different LPS molecules, this is mainly found in the length of the O-antigen. In some cases the O-antigen is completely absent, [8] which is referred to as rough LPS (rLPS) whereas LPS with O-antigen is called smooth LPS (sLPS). Due to the shorter polysaccharide chain, rLPS tends to form larger, more lipophilic aggregates. [9]

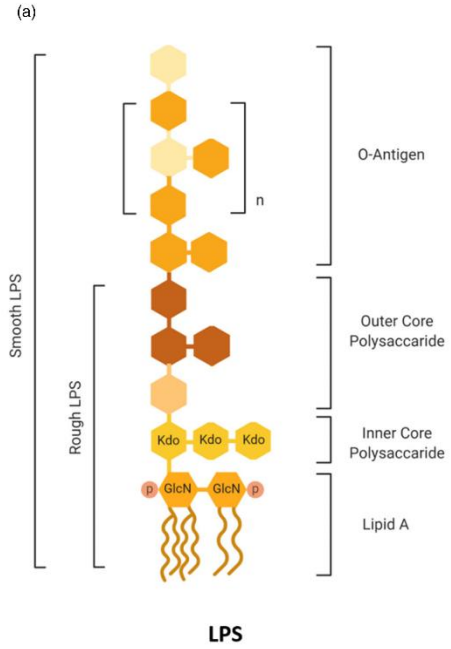

Figure 1. schematic overview of the LPS molecule[9]

The presence of gram-negative bacteria in the intestinal tract of healthy individuals ensures the presence of LPS. However, when the intestinal epithelium is compromised, for example due to alcohol consumption, intestinal permeability is increased, facilitating the translocation of LPS into the systemic circulation. Subsequently, LPS leaks into the portal vein, from which it can enter the liver. This event initiates a cascade of cellular responses and inflammatory processes. [10]

Upon encountering LPS, the resident macrophages of the liver, known as Kupffer cells, are stimulated and trigger an inflammatory response. These cells recognize LPS through Toll-like receptor 4 (TLR4) and activate intracellular signaling pathways, leading to the production and release of pro-inflammatory mediators such as tumour necrosis factor-α, interleukin-6 and nitric oxide (NO).[11–14]

The inflammation induced by activated Kupffer cells triggers the activation of hepatic stellate cells, which promotes the production and accumulation of excessive extracellular matrix proteins, including collagen. This process ultimately results in liver fibrosis.[15]

When LPS is present in human circulation it forms lipophilic aggregates. These micelles exert a less inflammatory response than single LPS molecules. To enhance TLR4 detection of LPS, two proteins, LPS binding protein (LBP) and cluster of differentiation 14 (CD14), are required. LBP is secreted by hepatocytes into the bloodstream and increases more than 10-fold during infections [16]. It extracts a single LPS monomer from LPS vesicles and delivers it to CD14. CD14, which is often membrane-bound through a glycosylphosphatidylinositol anchor, has a hydrophobic pocket that binds the lipid A part of an LPS monomer. The LPS molecule is then transferred to the TLR4-MD2 complex. Upon activation by the lipid A part of LPS, the TLR4-MD2 complex dimerizes which leads to the activation of two signaling pathways depending on their association with MyD88/TIRAP or TRIF/TRAM adaptor proteins [17,18]. Via these pathways, NFkB is activated, which increases inducible Nitric Oxide Synthase (iNOS) expression. iNOS catalyzes the production of NO from L-arginine[14].

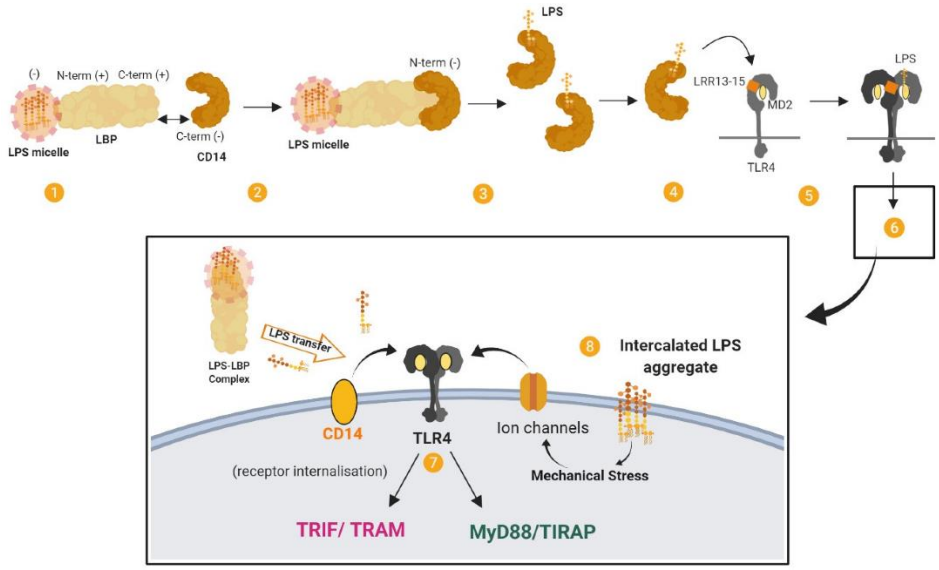

Figure 2. LPS transport, and TLR4 activation [17]

In vivo studies show promising results when AP is used to treat inflammatory diseases. In liver fibrosis, LPS is a key player inducing inflammation, which results in fibrotic tissue. It is hypothesized that AP detoxifies LPS, by removing at least one phosphate group from the lipid A part, thereby creating mono-phosphoryl lipid A (MPLA). MPLA has 1000 times less toxicity compared to di-phosphoryl lipid A (DPLA). Even though intravenous administration of AP has an LPS-detoxifying effect in vivo the exact mechanism in vitro is not well established [19,20].

Besides LPS dephosphorylation, other anti-inflammatory properties of AP have been described. AP can dephosphorylate adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), thereby creating adenosine, a powerful anti-inflammatory mediator[21–23].

Therefore, this study investigates the anti-inflammatory properties of AP in vitro. This is done by experiments with RAW 264.7 cells, to mimic liver Kupffer cells. These cells are stimulated with LPS, AP and AMP. Next to that crucial components in serum that might influence AP are investigated.

In short, it is known that AP detoxifies LPS. However, it has not been possible to demonstrate this in vitro. We hypothesize that an extra unknown factor is needed to detoxify LPS. In this thesis, we aim to find out what this might be by performing several in vitro experiments.

METHODS

RAW 264.7 CELLS

RAW 264.7 cells obtained from the American Type Culture Collection (ATCC) were employed for the experiment. The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Gipco 31966), supplemented with 10% Fetal Bovine Serum (FBS) sourced from (Seraba, S-FBS-SA-015), 2mM L-glutamine (Invitrogen), and 10 µg/mL gentamycin (Invitrogen). In cases where alternative conditions /media were utilized, these conditions are explicitly stated. The cells were cultured in T25 and T75 flasks and incubated in a controlled environment with a temperature of 37°C and 5% CO2.

NO ASSAY

To quantify the production of nitric oxide (NO), the amount of nitrite ions (NO_2-) was measured. Initially, cells were detached from a T25/T75 flask by scraping and seeded into a 96-well plate at a density of 1×10^5 cells in 200 µL per well. These cells were then allowed to grow overnight. After a 24-hour period, the culture medium was removed and replaced with fresh medium containing the desired concentrations of LPS (Coli, Sigma L2880), DPLA (Re 595 Minnesota, Sigma L0774), MPLA (Re 595 Minnesota, list biological laboratories #401), cIAP (AM-Pharma) or AMP. LPS was dissolved in milli-Q water. DPLA was dissolved in DMSO. In the experiments where DPLA was used along with LPS, the LPS was also dissolved in DMSO. When preincubation is mentioned, that means that the medium containing the stimuli was preincubated for a specified duration at 37°C and 5% CO2 before being added to the cells. Following medium replacement, the cells were incubated for the specified time at 37°C and 5% CO2.

After incubation, 100 µL samples were collected from the supernatant of the cell culture. To these samples, 100 µL of Griess reagents (composed of Griess A: 1000 mg sulphanilamide + 5.53 mL phosphoric acid + 94.47 mL distilled water and Griess B: 100 mg N-naphthyl ethylene diamine + 100 mL distilled water, freshly mixed in a 1:1 ratio) were added. Following this, the absorbance of these samples was measured at 550 nm using a microplate ELISA reader. Nitrite concentrations were determined by comparing the absorbance values of the samples with a standard curve generated using $NaNO_2$ in medium (table 1), with concentrations ranging from 0 to 100 µM. The results are expressed as the mean and standard deviation.

Table 1 dilution of $NaNO_2$ calibration standards

Standard	$NaNO_2$	Medium
100 mM (stock)	0.69 g in 100 ml MQ-water	
100 µM	2 µl of stock	1.998 ml
50 µm	1 ml 100 µm $NaNO2_2$	1ml
25 µm	1 ml 50 µm $NaNO2_2$	1ml
12.5 µm	1 ml 25 µm $NaNO2_2$	1ml
6.3 µm	1 ml 12.5 µm $NaNO2_2$	1ml
3.1 µm	1 ml 6.3 µm $NaNO2_2$	1ml
1.6 µm	1 ml 3.1 µm $NaNO2_2$	1 ml
0.8 µm	1 ml 1.6 µm $NaNO2_2$	1 ml
0 µM	0	1 ml

MTT ASSAY

In order to assess cell viability, an MTT tetrazolium assay was conducted. Following the completion of the NO assay, the excess 100µL solution was extracted from the cells. Subsequently, 200µL of tetrazolium solution was directly added. The tetrazolium solution was formulated by dissolving tetrazolium in serum-free DMEM medium (Gipco 31966), resulting in a final concentration of 0.255 mg/ml. After an incubation period of 10 minutes at 37°C with 5% CO2, the tetrazolium solution was removed. To dissolve the cells and achieve a uniform solution, 200µL of DMSO was added and resuspended to create a homogenous solution. The absorbance was then measured at 570nm. The obtained results were presented as a percentage of cell survival relative to the average absorbance of wells containing only 10% FBS (without stimuli) minus the blank (200 µl DMSO), which was assigned a value of 100%. The standard error of the mean was included for the samples.

PNPP ASSAY

AP activity was measured using the pNPP assay. In this assay AP dephosphorylates a phosphate group from PNPP, thereby creating PNP which can be measured at 405 nm. The pNPP assay was first used for confirming the actual amount of AP that was going to be used in the assays. The amount of AP that was being used for the experiments is expressed in units per liter (U/l). So, the amount of active enzyme needed to be verified, this was done by a pNPP assay. Secondly, the assay was used to measure the AP activity in different types of media.

MEASUREMENT OF ENZYME CONCENTRATION

The following assay components were prepared. A 0.05 M ammediol buffer solution was prepared by diluting the ammediol stock solution in deionized water. A 100 mM MgCl2 stock solution was prepared by dissolving the appropriate amount of MgCl2 powder in deionized water. A 10 mg/ml p-nitrophenyl phosphate (pNPP) stock solution was prepared by dissolving the pNPP powder in deionized water. A 0.1 M sodium hydroxide (NaOH) solution was prepared by diluting the NaOH stock solution in deionized water.

Next, 930 µl of the 0.05 M ammediol buffer was pipetted into each tube. Then, 20 µl of the 100 mM MgCl2 solution was added to each tube, followed by 50 µl of the 10 mg/ml pNPP solution. Finally, 25 µl of different AP concentrations were added so an activity concentration curve could be made.

The contents of each tube were gently mixed by shaking, and then the tubes were incubated at 37°C for 30 minutes. An incubator was used to maintain the desired temperature.

The reaction was stopped by adding 5 ml of the 0.1 M NaOH solution to each tube.

The extinction of each tube was measured at 405 nm within 30 minutes after adding NaOH using a spectrophotometer/microplate reader. A blank containing only the buffer and pNPP was used to calibrate the spectrophotometer and account for any background absorbance.

The AP activity or concentration of the enzyme in the samples was calculated based on the absorbance values with the following formula for the spectrophotometer:

$$\frac{\Delta A(sample-blanco)*6.025*1000}{30*18.5*0.025}$$

For samples measured in a 96-well round bottom plate a conversion factor of A*1.34 was used. This value was obtained by experiments comparing the absorbances measured of the spectrophotometer compared to that of the microplate reader.

MEASUREMENT OF AP ACTIVITY IN SERUM

To measure AP's activity in serum, a known amount of AP was added to different concentrations of either pooled human serum (HS) or FBS. To check whether certain components in these sera influenced AP activity. They were either delipidated, deproteinized, or remained untreated. So, six different sera were employed: HS (TCS BioSciences cat. nr. CS100-500), human serum delipidated (HSD) (TCS BioSciences cat. nr. DR750-500) and human serum deproteinized (HSDP) and FBS, fetal bovine serum delipidated (FBSD) (Capricorn scientific, FBS-DL-12A), and fetal bovine serum deproteinized (FBSDP).

The pNPP assay was carried out the same way as the AP activity measurement, with the one difference that the ammediol buffer was swapped for one of the above-mentioned sera, as specified in the experiment. Since we wanted to check the activity of AP at physiological pH, the serum was diluted in a 0.5M tris buffer of pH 7.4, unless otherwise specified. The data is expressed as a percentage, where the starting point is set at 100% activity. The starting point equates to 0% serum, so it only contains 0.5M tris buffer which is the same for every condition.

DEPROTEINIZATION OF SERUM:

150 ml of TCA was added to 1 ml of serum, and the mixture was incubated on ice for 30 minutes. Subsequently, the mixture was centrifuged at 10,000 g for 10 minutes. Following centrifugation, the supernatant was transferred to a new tube. To achieve a 10% serum concentration, the serum was diluted using tris 0.5M. Lastly, the pH was adjusted to 7.4.

IMMUNOPRECIPITATION

An alkaline phosphatase solution of 1000 U/l was obtained by adding cIAP (AP-pharma) to DMEM with 10% FBS.

A mixture of 200 µl of serum and 3µl of primary antibody against alkaline phosphatase (Rockland, 200-4135) was incubated overnight at 4°C with gentle mixing.

20 µl of protein A agarose beads slurry (50%) was added to the medium-antibody mixture. The mixture was incubated for an additional 2 hours at 4°C with gentle mixing. This binds the agarose beads to the antibody, which, in turn, was bound to the alkaline phosphatase.

The mixture was centrifuged at 6,000 rpm × g for 60 seconds at 4°C, to pellet the agarose beads.

The pellet was washed three times with 500 µl of washing buffer (10mM tris, 150 mM NaCl, 1% triton x-100, protease inhibitor cocktail (Roche, 11836170001).

In between the washing steps the sample was kept on ice.

To elute the protein from the agarose-antibody complex, urea elution buffer (7 M Urea, 20 mM Tris pH 7.4, and 100 mM NaCl) was added in 2–5 volumes, and the mixture was rotated for 30 minutes at room temperature with frequent agitation before undergoing gentle centrifugation.

The process was repeated twice more to ensure the complete release of the captured complex from the beads. The beads were then pelleted, and the supernatant, containing the proteins of interest was removed to a new tube (figure 1).

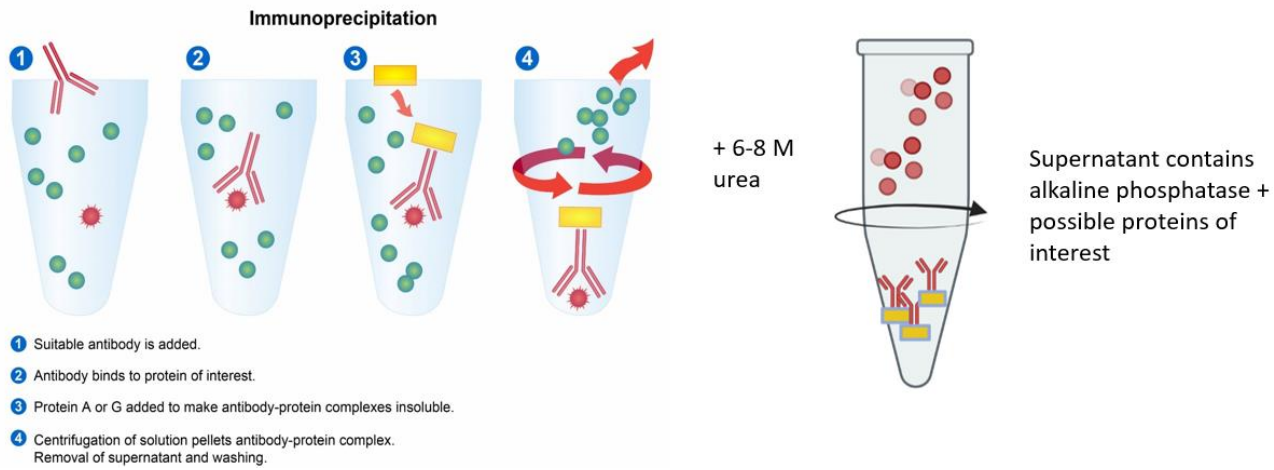

Figure 3. Schematic overview of immunoprecipitation.

Finally, 4X SDS sample buffer (25 ml 0.5M Tris-HCL pH 6.8, 20 ml glycerol, 4 g SDS, 3.1 g DTT, 1 mg bromophenol blue, mate to final volume of 50 ml with water) was added at a 1:4 ratio to 30 µl sample. The sample was then heated to 95°C for 5 minutes and was loaded onto a 10% gel for SDS-PAGE followed by silver staining.

The remainder of the sample containing the eluted proteins was measured with mass spectrometry for further analysis.

PROTEIN CONTENT

The protein content of the sample was determined by the DC protein assay of Bio-Rad.

A series of protein standard solutions were made with bovine serum albumin, ranging in concentration between 0-2 mg/ml. The dilution was done in the same elution buffer as used in the immunoprecipitation (IP).

20 µl of reagent S was added to 1 ml of reagent A and subsequently mixed to obtain reagent A'. 5 µl of standards and samples were pipetted into a flat bottom 96 well plate. First 25 µl of reagent A' was added to each well. Secondly, 200 µl of reagent B was added to each well. The plate was mixed for 5 seconds and after 15 minutes the absorbance was read at 650 nm.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

To identify the eluted proteins after immunoprecipitation, Liquid Chromatography-Mass Spectrometry (LC-MS) was performed. This process consists of several steps.

The first step in LC-MS-based proteomics involves protein digestion. Proteins are typically digested into smaller peptides using enzymes such as trypsin. This digestion step generates a complex mixture of peptides that can be analyzed by LC-MS. Remaining non protein residues such as detergents or buffer salts are removed by cleaning the peptides using solid phase extraction. The peptide mixture is then resuspended in an aqueous buffer suitable for LC-MS.

The peptide mixture is separated using liquid chromatography (LC), which utilizes a stationary phase (usually a reversed-phase column) and a mobile phase (solvent system). LC separates peptides based on their hydrophobicity and charge. After that the peptides are sprayed into the spectrometer as charged ions. The ionized peptides are detected by the spectrometer, which separates them based on their m/z ratio and measures their abundance.

The acquired mass spectrometry data is then processed and analyzed using specialized software tools. This includes peptide identification by matching the acquired mass spectra against protein sequence databases. Finally, statistical analysis methods are employed to determine protein abundance and protein interactions [24,25]

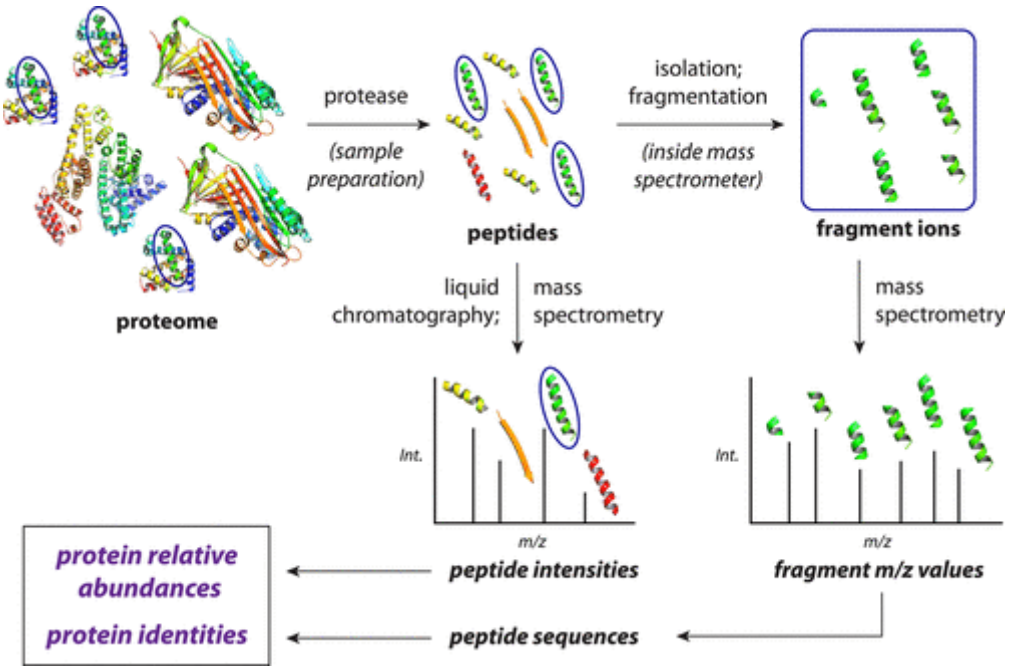

Figure 4. different steps in LC-MS proteomics. First protein digestion, then separation based on hydrophobicity using LC, after that detection of m/z ratio and abundance by mass spectrometry [25]

STATISTICS

GraphPad Prism 9.5.1 (GraphPad Software, USA) was used to perform statistical analysis and to make graphs. Differences between groups were considered statistically significant at a level of $p < 0.05$.

Ordinary one-way ANOVA was used for graph 6a, 7 and 11

Mixed effects analysis was used for graph 9a

Repeated measures ANOVA was used for graph 15 and 16

RESULTS

NO ASSAY

First, we looked at how much LPS was needed for the maximum NO response. We did not want to use more LPS than the minimum amount needed for maximal response, so any amount of LPS that would later be dephosphorylated would immediately be observed as a decreased NO response. This was done by testing several concentrations of LPS on the RAW 264.7 cells. A positive correlation between LPS concentration and NO production was observed (Figure 5a). The maximum NO response was achieved at 10 ng/ml LPS as can be seen in Figure 5b.

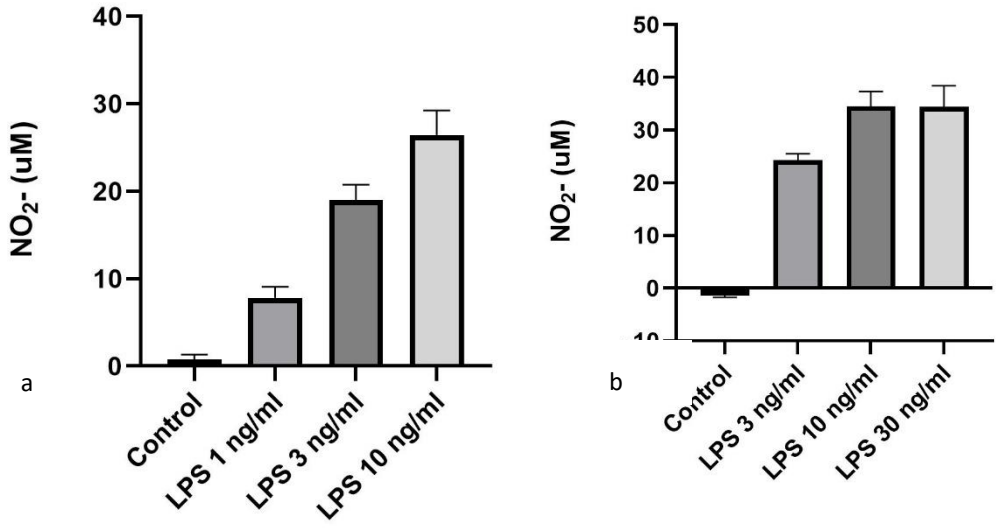

Figure 5. NO2- production by RAW cell after 20-hour stimulation. Positive correlation between increasing LPS concentration and NO production (5a). Maximum NO response is achieved at 10 ng/ml LPS (5b). Data are presented as mean +- SD (intra-assay variability) n=1.

EFFECT OF IAP ON LPS STIMULATED RAW 264.7 CELLS

After we established the amount of LPS that was going to be used, different amounts of AP were assessed to detoxify the LPS. We used concentrations of 500 U/l and 1000 U/l. During physiological circumstances serum AP values are between 20 U/l to 140 U/l, during inflammation AP values are around 300 U/l and can go up till 1000 U/l [1,26]. Since we induce the maximum NO response in RAW 264.7 cells, we also use the maximum AP concentration of 1000 U/l in the cell experiments. As can be seen in Figure 6 no significant reductions could be observed with both 500 U/l and 1000 U/l. So, we hypothesized that the LPS could already trigger the TRL4 receptors of the RAW 264.7 cells before a phosphate group could be removed by AP. Therefore, we added a preincubation step. Here we allowed AP for a given amount of time to dephosphorylate LPS in the medium that was later added to the cells.

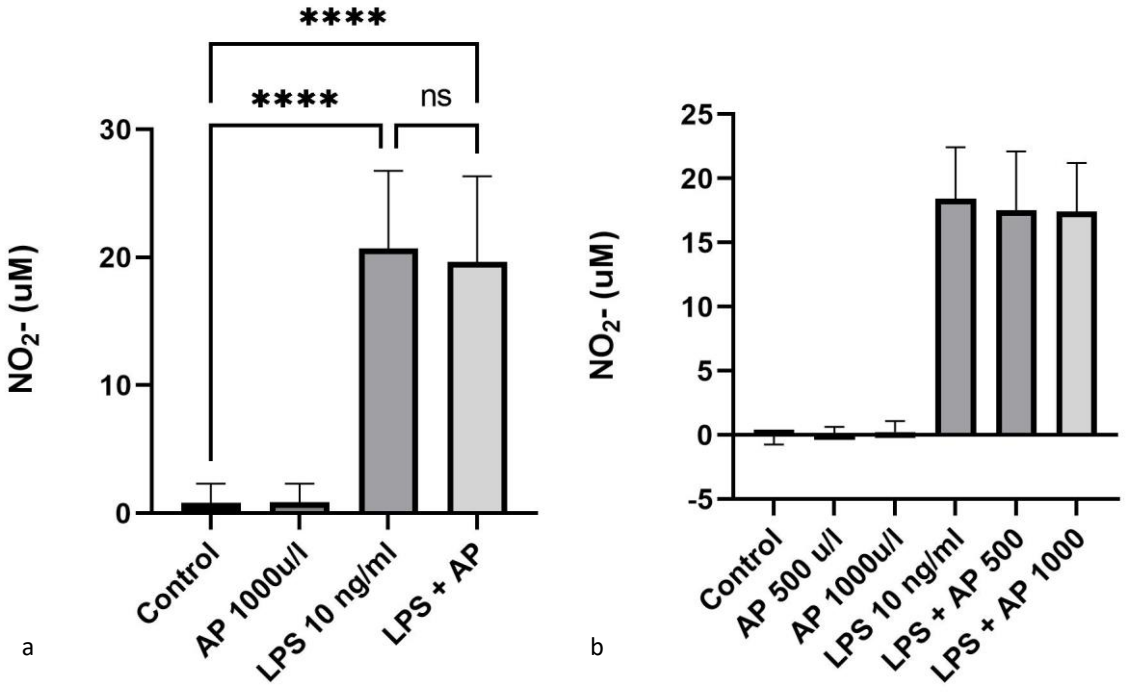

Figure 6. Effect of 1000 U/l AP on 10 ng/ml LPS during 20-hour incubation time. AP did not significantly reduce the NO production caused by LPS on RAW 264.7 cells. Data is expressed as mean + standard deviation (SD) n=5 (6a) and n=2 (6b).

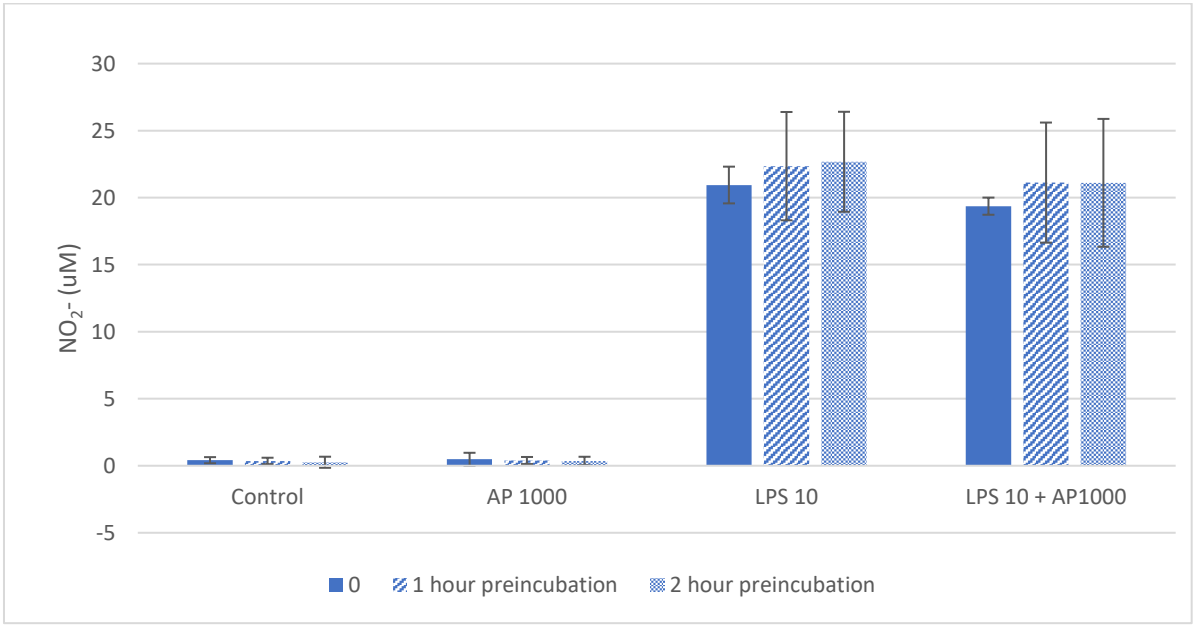

Figure 7. Effect of 1000 U/l AP on 10 ng/ml LPS during 22-hour incubation time with respectively 0-, 1- and 2-hour preincubation time. AP did not significantly reduce the NO production caused by LPS on RAW 264.7 cells. Statistical significance is analyzed with ordinary one-way ANOVA, data is expressed as mean + standard deviation (SD), n=3.

We introduced a preincubation step of 1 and 2 hours before incubating the cells for 20 hours as can be seen in Figure 7. Some reduction in the NO response was seen at 0-, 1- and 2-hour preincubation time. However, this effect was minimal and not significant. So, we concluded that AP could not dephosphorylate LPS very efficiently in the conditions provided.

ACTIVITY OF IAP IN 0.3% SERUM

To check the activity of AP in the serum, a pNPP-assay was developed where AP's activity was checked in regular FBS, deproteinized and delipidated FBS all ranging in concentrations between 0-10% serum. It was found that in a concentration of 0.1-0.3% serum, the activity of AP was optimal. Therefore, we performed another NO assay with a preincubation step in 0.3% serum to check whether AP could dephosphorylate LPS more efficiently. We added AP and LPS in a 10-fold concentration to 0.3% FBS in DMEM. After preincubation, the media were diluted 10 times with 10% FBS in DMEM. So, the cells still got sufficient FBS to survive on. As can be seen in Figure 8, some reduction in NO is observed, however the effect is still minimal.

Figure 8. **Effect of 500/1000 U/l AP on 10 ng/ml LPS during 22-hour incubation time and 0-, 1-, 2- and 3-hour preincubation in 0.3% FBS. (n=1, data expressed as mean).**

EFFECT OF IAP IN DPLA-STIMULATED RAW 264.7 CELLS

Besides LPS we also looked at the inflammatory properties of DPLA, since this is the inflammatory part of LPS which triggers the TRL4 receptor complex [17,18]. We found out that to exert the maximum NO response with DPLA we had to use between 150-300 ng/ml DPLA. Therefore, we performed experiments with 150 ng, 200 ng and 300 ng DPLA. Both 500 U/l and 1000 U/l of AP did not cause any significant reduction in NO production by DPLA stimulated RAW 264.7 cells as can be seen in Figure 9. There is even a slight increase in NO production. As an extra control MPLA was also added (Figure 9a). This is the expected product after dephosphorylation caused by AP. In all experiments MPLA did not induce any NO response at all, DPLA together with AP did induce a significant NO response. This means that DPLA has not been reduced to MPLA, therefore we conclude that DPLA is not dephosphorylated by AP in this experiment.

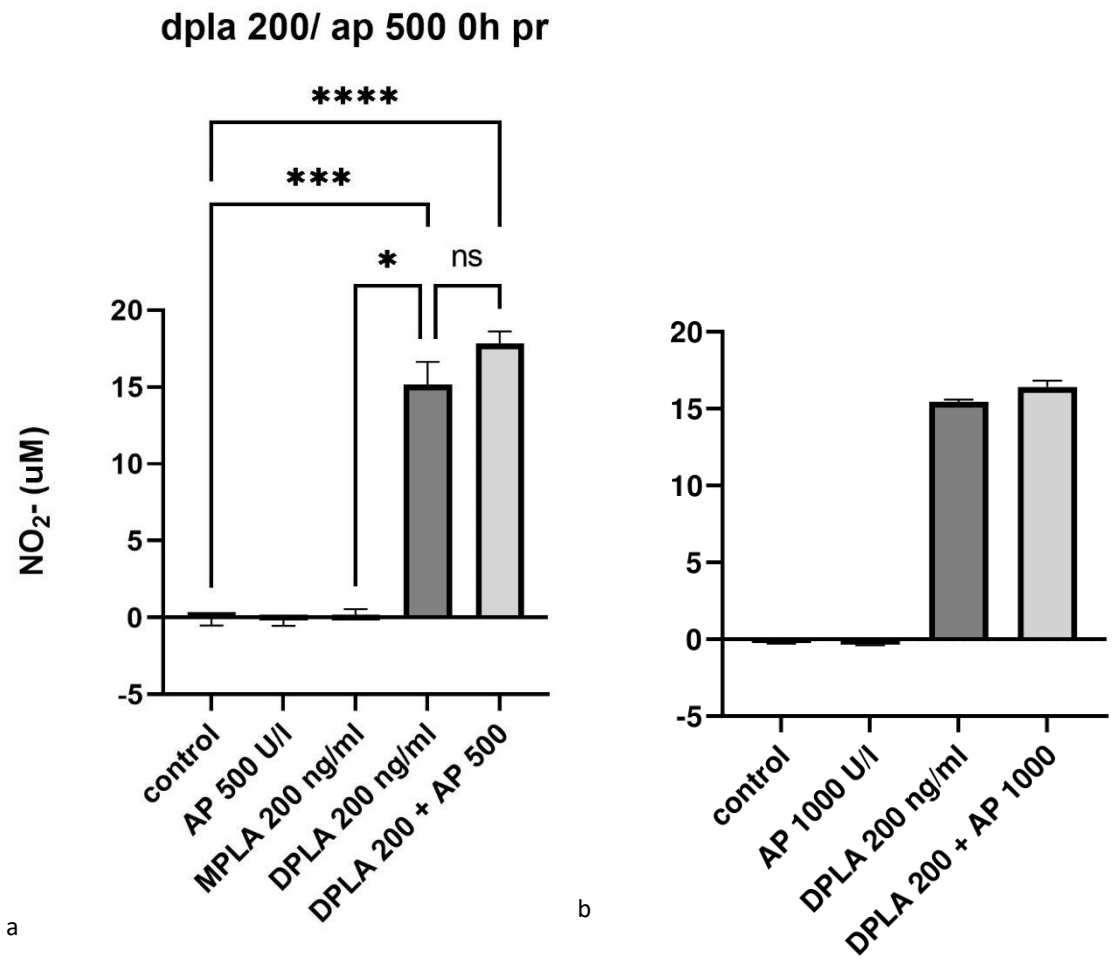

Figure 9. (a) Effect of 500 U/l AP on 200 ng/ml DPLA during 20-hour incubation time. AP did not significantly reduce the NO production caused by DPLA on RAW 264.7 cells. (n=3, data expressed as mean + standard deviation). (b) Effect of 1000 U/l AP on 200 ng/ml DPLA during 20-hour incubation time. AP did not significantly reduce the NO production caused by DPLA on RAW 264.7 cells. (n=2, data expressed as mean + standard deviation)

As with LPS, we also checked whether preincubation time might enhance dephosphorylation of DPLA. The strongest NO-inhibitory effect of AP was observed at 1000 U/l AP and 300 ng/ml DPLA during 1-hour preincubation time as can be seen in figure 10. However, this experiment was only performed twice. In Figure 11, the concentration of DPLA was reduced to 150 ng/ml, to check whether the NO production could be reduced even more. However, the decrease in NO is less compared to Figure 10.

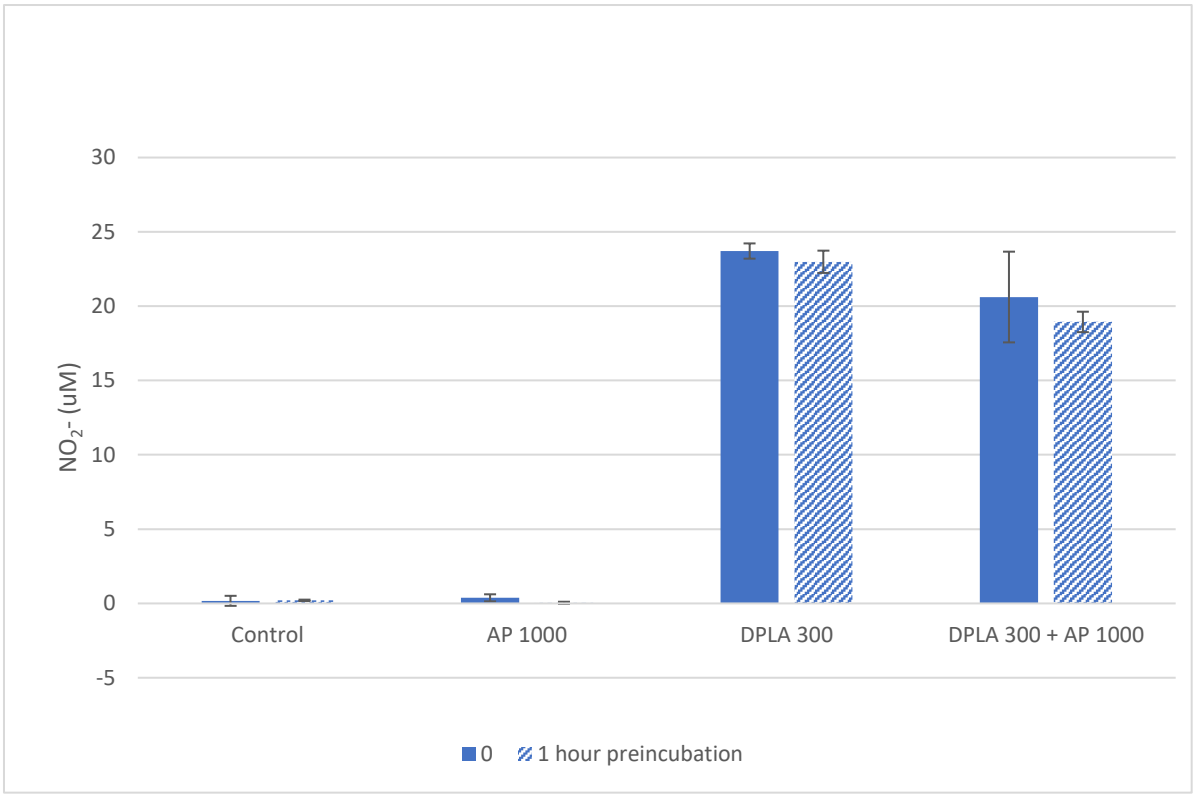

Figure 10. Effect of 1000 U/l AP on 10 ng/ml LPS during 21-hour incubation time. Visible reduction of NO response is observed at 0- and even more at 1-hour preincubation. Data is expressed as mean + standard deviation (SD), n=2.

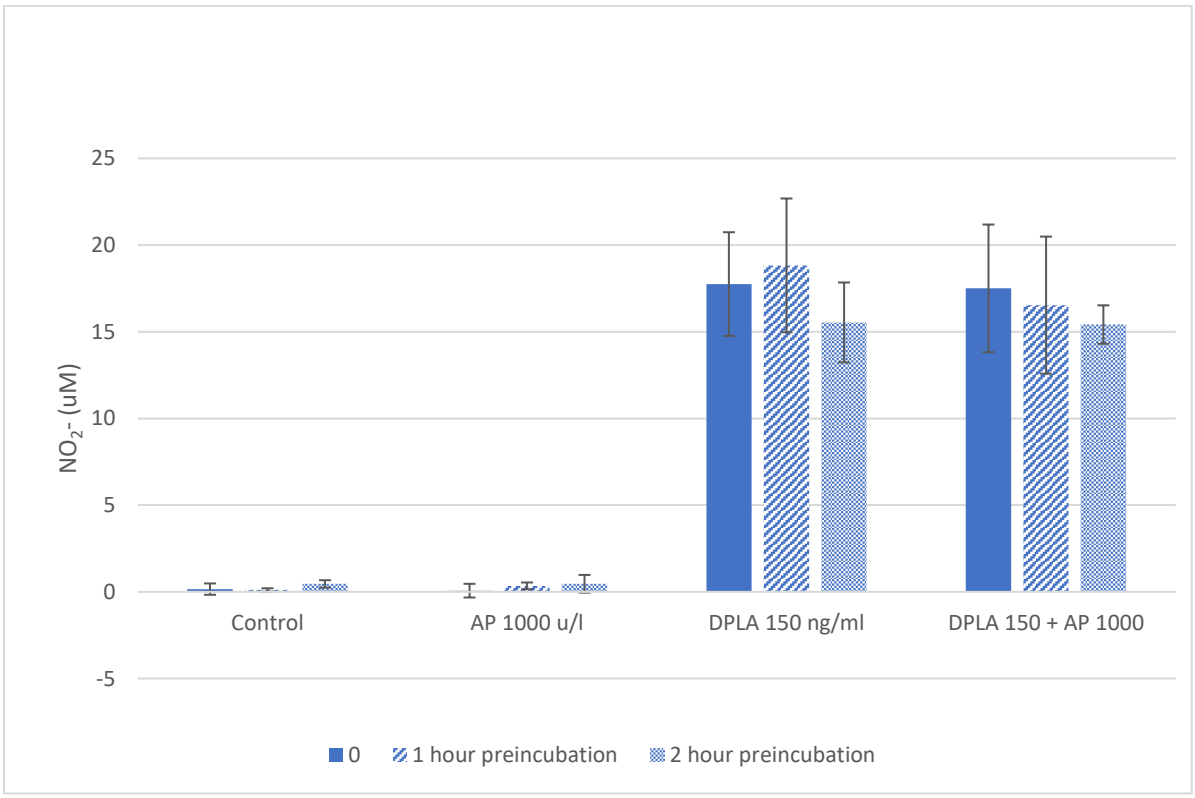

Figure 11. Effect of 1000 U/l AP on 150 ng/ml DPLA during 21-hour incubation time. AP did not significantly reduce the NO production caused by DPLA on RAW 264.7 cells. (n=3, data expressed as mean + standard deviation).

Next, we increased the preincubation time even more to see if this could benefit the lipid A detoxification (Figure 12). Here, we found something interesting, over time the NO response induced by DPLA decreases irrespectively of the addition of AP. This also occurred with incubation of just DMEM with no FBS. So, no serum components could influence this degradation or detoxification. After 22-hours preincubation, the DPLA did not induce any NO_2- production at all. This effect was not observed with LPS which induced a similar NO response at 0-, 5.5- and 21-hour preincubation.

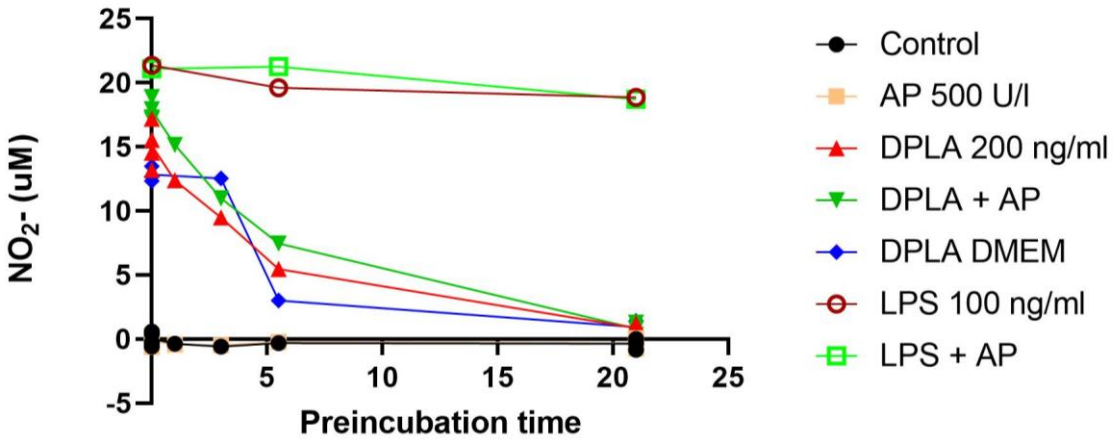

Figure 12. Decrease of anti-inflammatory effect of DPLA over time. The NO-response induced by DPLA decreases with an increased preincubation time. DPLA with AP, or DPLA added to just DMEM (no FBS) show a similar breakdown pattern. Measured data points are the shapes depicted in the legenda.

ADENOSINE MONOPHOSPHATE

As mentioned before, AP might also be able to exert anti-inflammatory properties beside LPS dephosphorylation. Therefore, as seen in Figure 13, we added adenose monophosphate (AMP), and checked if AP would be able to convert AMP to adenosine, which is known for its anti-inflammatory properties. A significant reduction in NO response was seen when AP was added to AMP and LPS, compared to just AMP and LPS. However, this experiment must be repeated at least twice more to get statistical significance.

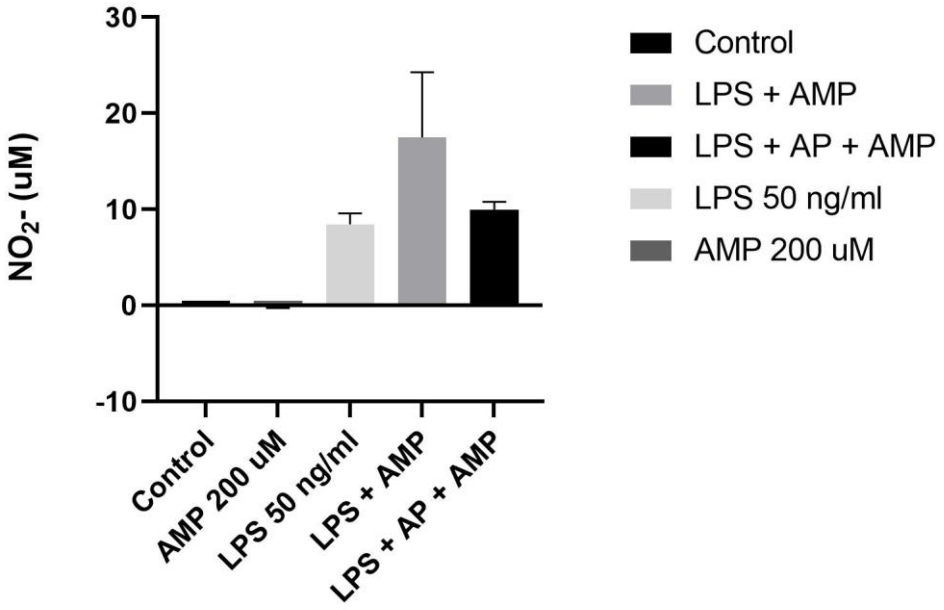

Figure 13. Effect of 500 U/l AP on 50 ng/ml LPS (dissolved in DMSO) and 200 µM AMP during 18-hour incubation time, (n=1, data expressed as mean + intra assay SD).

Figure 14a shows another experiment where cells are stimulated with 10 ng/ml LPS, 500 or 1000 U/l IAP and 200 µM AMP with 0- and 1-hour preincubation. Cells that are incubated with LPS + AMP, produce more NO_2- compared to just LPS. Whilst AMP on its own does not enhance NO production. Preincubation time reduces the NO production in the cells treated with LPS and AMP, the addition of 500 U/l IAP causes an even larger reduction of NO_2-. To check whether LPS, IAP and AMP play a role in viability, an MTT assay was performed on these same cells as well. Figure 14b shows that all samples treated with LPS show a decreased viability in comparison with the samples without LPS. IAP or AMP do not enhance cell viability in this experiment.

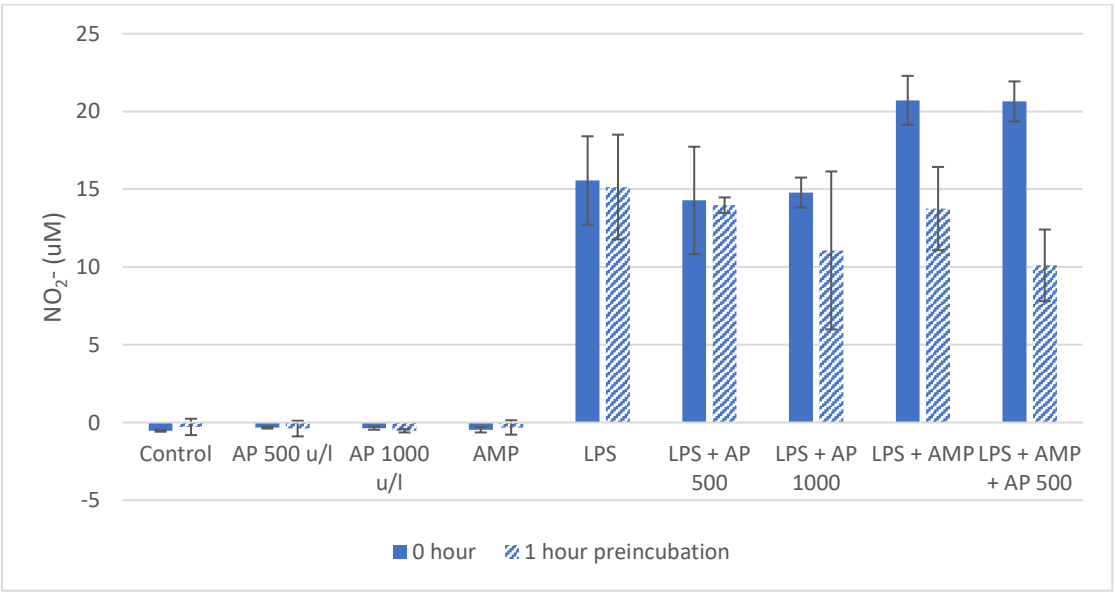

Figure 14a. NO_2- production after 20-hour stimulation with 10 ng/ml LPS, 200 µM AMP, 500/1000 U/l IAP. (n=1, data expressed as mean + intra assay SD).

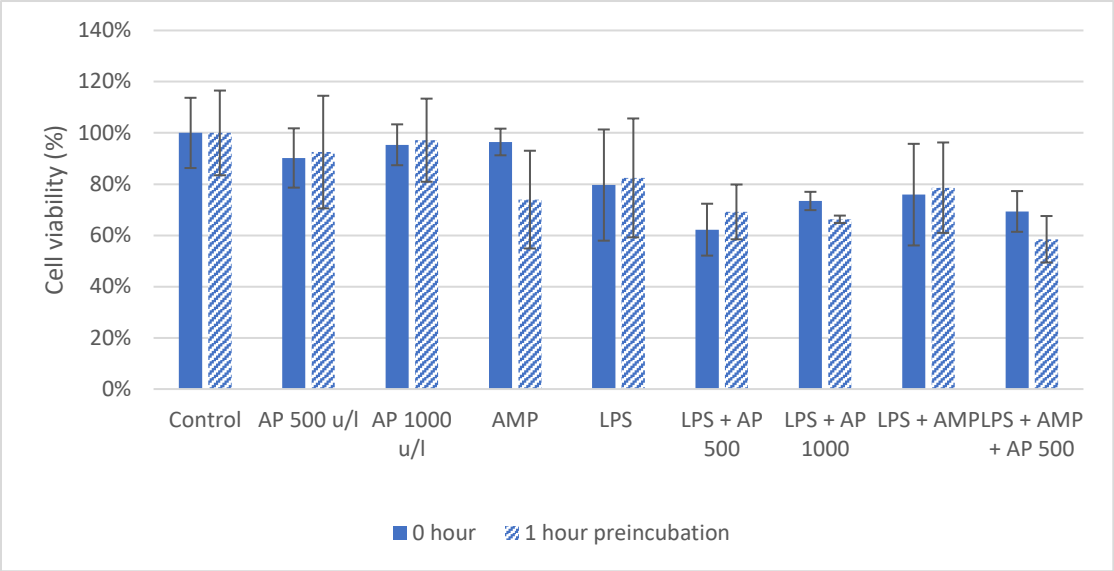

Figure 14b. Cell viability after 20-hour stimulation with 10 ng/ml LPS, 200 µM AMP, 500/1000 U/l IAP. (n=1, data expressed as mean + intra assay SD).

PNPP-ASSAYS

To check the activity of AP in the serum we developed a pNPP-assay where the activity of AP's was checked in regular serum, deproteinized and delipidated serum all ranging in concentrations between 0-10% serum. We used these different conditions to check whether there are certain proteins or lipids present in serum that might influence AP's dephosphorylating abilities.

First, we measured the activity of AP in FBS (Figure 15). A significant difference between delipidated and deproteinized FBS and delipidated FBS vs FBS was seen with $P<0.05$. Also, several trends can be observed. At a concentration between 0.1-0.3% FBS, AP seems to be 5-10% more active compared to 0% serum. Moreover, in 0,1-0.3 delipidated FBS this enhanced activity is around 15%. After this peak the activity drops to 75% at an FBS concentration of 10%. In the case of delipidated FBS, the activity drops back to similar activity as observed at 0% serum.

The activity of AP decreases to 87% in 0.1% deproteinized FBS, after this trough, the activity is similar to FBS.

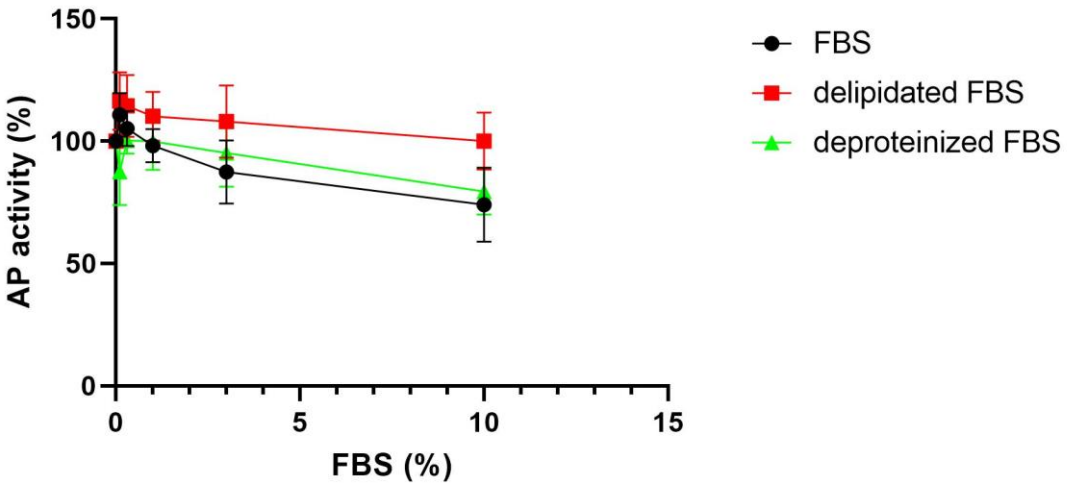

Figure 15. The activity of AP in FBS, delipidated FBS and deproteinized FBS. A significant difference in activity is observed between delipidated and deproteinized FBS, and between delipidated FBS and FBS ($P<0.05$). Data are presented as mean ± SD, n=3.

Besides the activity of AP in FBS, also AP activity in human serum (HS) was observed (figure 16). In HS, AP activity peaks at 0.3% serum with around 127 % activity, after which it decreases to 80 % at 10 % serum. Delipidated HS shows a similar pattern with a peak at 0.1% serum of 107.5 %. Deproteinized HS, however, exhibits a more linear curve where the activity increases along with the serum. No statistical significance was observed due to some high standard deviations caused by three outliers in de data.

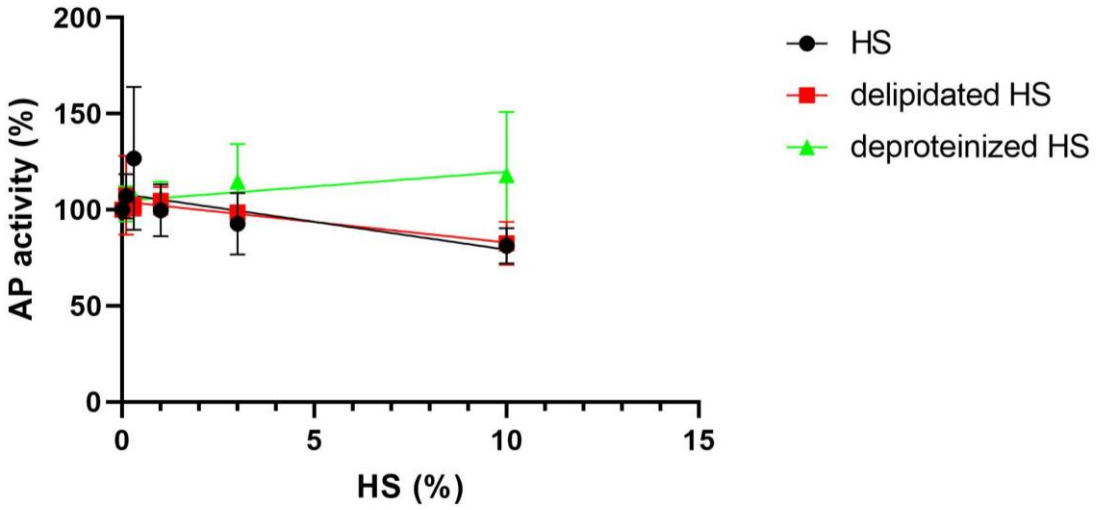

Figure 16. The activity of AP in HS, delipidated HS and deproteinized HS. No significant difference in activity is observed between the distinct types of serum. Data are presented as mean ± SD, n=3.

IMMUNOPRECIPITATION

In FBS and delipidated FBS, we saw an increased activity between 0.1 and 0.3% serum, the opposite was observed in deproteinized FBS. Therefore, we hypothesized that this increased activity is due to a protein that at a certain concentration enables AP to work more optimally. Also, the activity of AP was overall significantly lower in deproteinized FBS compared to delipidated FBS or FBS. This means that by either adding serum proteins or removing serum lipids, AP activity is enhanced. To test which proteins might play a role in the action of AP we developed an immunoprecipitation (IP) protocol.

We tested two different samples, the first sample contained 1000 U/l cIAP in FBS, and the second sample contained 1000 U/l cIAP + 10 ng/ml LPS. We chose to add LPS, to find out which proteins might play a role in the detoxification of LPS along with AP.

After silver staining the SDS-page gel, several bands were observed as is depicted in figure 17. In the samples containing serum and antibody 2 thick bands were observed at 50 and 20 kDa. At those places often denatured antibody is seen. The lane consisting of the control sample shows one band at 70 kDa and one at 140 kDa. There is also a thicker band at 70 kDa seen at the other samples. We expect these bands to represent cIAP, since the cIAP protein consists of a dimer of 140 kDa. The other bands are most likely co-eluted proteins from the serum. There is no big visual difference observed between the AP sample compared to the AP+LPS sample. Further LC-MS is needed to identify and quantify the co-eluted proteins of interest.

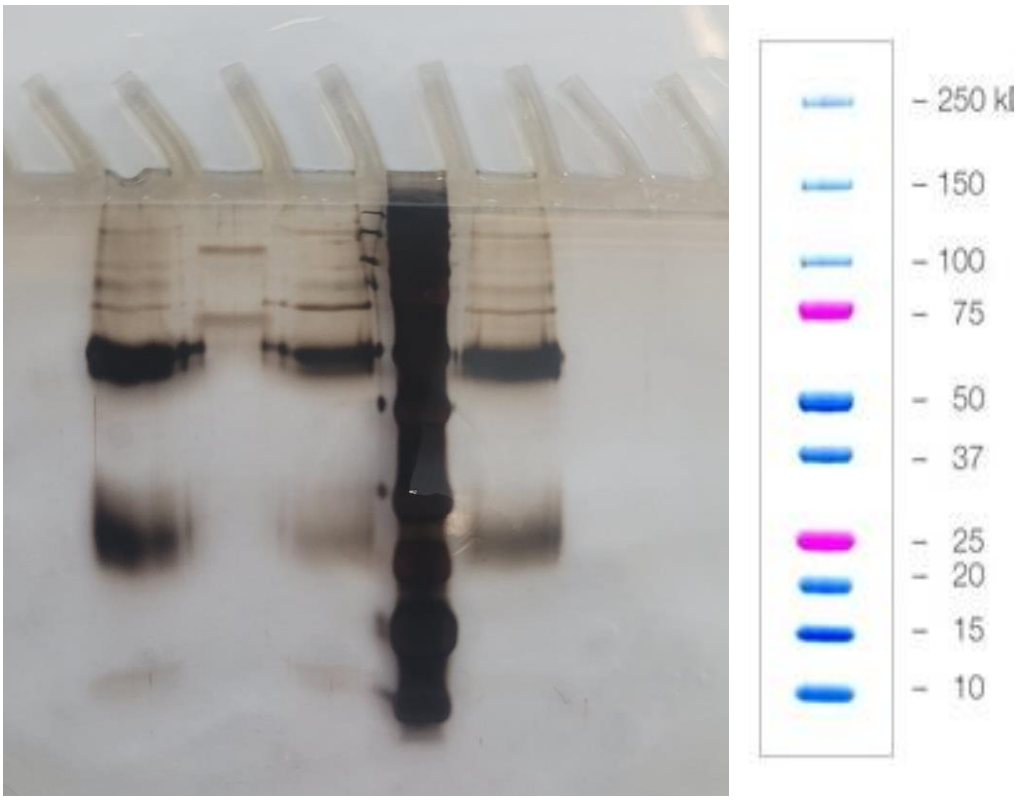

Figure 17. SDS-Page gel after silver staining. From left to right: control (just FBS), AP 1000 U/l in FBS, AP 1000 U/l in water, protein ladder, AP 1000 U/l + LPS 10 ng/ml.

DISCUSSION

Research shows that the lipid A part of LPS (DPLA), binds to the TRL4 receptor complex and induces an inflammatory response in macrophages [17,18]. In our experiments, after stimulation of RAW 264.7 cells with LPS and DPLA, an NO response was observed. 10 ng/ml LPS was the minimum amount of LPS required to generate the maximum NO production. In our experiments, AP was not able to significantly inhibit this NO response. In the case of 1000 U/l AP and 300 ng/ml DPLA, the biggest reduction in NO production was observed, especially after one hour of preincubation. However, this experiment was only performed twice. In all cases, LPS and DPLA caused the RAW 264.7 cells to produce NO, whereas with MPLA, the dephosphorylated lipid A part of LPS, this was never observed. Therefore, we conclude that any dephosphorylation of LPS or DPLA would result in a reduction of NO production. Since that did not happen, we conclude that in these circumstances AP was not able to dephosphorylate any significant amounts of DPLA or LPS. A recent study found out that cIAP had minimal dephosphorylation activity against unmodified enteric LPS molecules [27]. They concluded that in order for the 1- and 4'-lipid A phosphate to become susceptible to hydrolysis, first the ester linked primary acyl chains on lipid A need to be de-O-acylated. If this does not happen first, then it is not possible for AP to break down LPS. It might be possible that in our investigation the de-O-acylation did not happen and that subsequently the AP could not remove one of the phosphate groups. This might explain that AP did not inhibit the inflammatory response. Moreover, without the assistance of accessory proteins, highly aggregated LPS may not be readily available for interaction with IAP. These accessory proteins play a crucial role in exposing the phosphate groups of lipid A. In the presence of serum, LBP, sCD14, and albumin all play essential roles in breaking down LPS aggregates and facilitating its presentation to the TLR4/MD2 receptor complex [28,29]. We did not test if these proteins were present and in adequate quantity in the FBS we used, since LBP and sCD14 expression is only greatly upregulated during inflammation. In low concentrations, these proteins enhance LPS

mediated inflammatory response whereas in high concentrations these proteins decrease LPS bioactivity [30]. We hypothesize that this is due to presenting LPS to AP so it could be dephosphorylated.

Next to that we also found that the NO response of DPLA was negatively correlated with the preincubation time, after 22 hours of preincubation the NO response caused by DPLA was close to zero. This effect was also observed when the DPLA was added to DMEM without any FBS. This gives reason to think that DPLA dephosphorylates in DMEM, or in some other way loses its potential to exert an inflammatory response. Firstly, this can happen through hydrolysis of the disaccharide part. The toxicity of Monosaccharide substructures of lipid A is significantly lower compared to LPS, with a 10^7-fold reduction. Also, modifying the fatty acid composition of lipid A can result in a less toxic form of lipid A. The bioactivity of lipid A is influenced by both the number and arrangement of fatty acids attached to the disaccharide. For instance, lipid A substructures containing only four hydroxymyristic acids coupled to the glucosamine disaccharides exhibit up to a 10^7-fold decrease in toxicity compared to the original hexa-acyl synthetic lipid A.[31] These findings suggest also other possible methods to detoxify LPS.

In the few experiments we performed with IAP on top of LPS and AMP, an enhanced anti-inflammatory effect was observed. Stimulation of RAW 264.7 cells with AMP + LPS increased NO-production compared to stimulation with just LPS. However, after 1-hour preincubation a reduction in nitrite production was observed, and this effect was even more pronounced with the addition of IAP. This effect could be attributed to the dephosphorylation of AMP to adenosine by IAP. Adenosine is a powerful anti-inflammatory mediator[32]. The reduction of NO after just preincubation (without addition of IAP) might be attributed to AMP dephosphorylation by AP already present in the FBS. The increased NO production observed with AMP + LPS compared to just LPS, might be due to AMP-activated protein kinase (AMPK) activation by AMP. AMPK in turn increases endothelial nitric oxide through phosphorylation of endothelial nitric oxide synthase (eNOS) [33,34] While RAW 264.7 cells are not endothelial cells, but macrophages, the mechanism by which AMPK increases NO production may be similar in macrophages.

It must be noted that the experiments with AMP were not repeated so no statistics were performed. However, it looks like AP can dephosphorylate AMP to adenosine and thereby decrease the NO production. This mechanism could contribute largely to the anti-inflammatory properties AP exhibits in vivo.

The MTT assay that was performed showed that cell viability was decreased in LPS treated cells. Addition of AMP or IAP to LPS-treated cells did not increase cell viability. It has to be noted that this experiment was only performed once. The experiment should be repeated at least twice more to see if the reduced viability is statistically significant.

To check the activity of AP in the serum we developed a pNPP-assay where AP's activity was checked in regular FBS, deproteinized and delipidated FBS all ranging in concentrations between 0-10% serum. Here we found that in a concentration of 0.1-0.3% FBS, the activity of AP was optimal. At a 10% FBS concentration (which was also used during cell experiments) we saw that indeed the activity of AP was reduced compared to 0 % serum. This indicates that there are certain components in the serum that inhibit or stimulate AP depending on the concentration. Delipidated FBS showed a similar pattern compared to FBS. Deproteinized FBS had significantly lower activity than delipidated FBS, exhibiting a trough instead of a peak at 0.1-0.3 % serum. Given all these results it seems highly likely that there are certain lipids in FBS that limit AP's activity, whereas certain proteins stimulate its activity. Especially if these proteins are diluted at 0.1-0.3% concentration compared to normal. According to literature the inhibition due to lipids might be caused by long-chain phospholipids that exert inhibitory effects on AP [35,36]. Proteins present in FBS that increase the activity of AP could be LPS binding protein and sCD14 [29,37]. In HS, similarities and differences were observed compared to FBS. Regular HS showed a similar pattern as FBS with a peak at a concentration of 0.1-0.3%. This peak was also observed in delipidated HS. However, AP in delipidated HS had higher activity than deproteinized HS, the opposite of what was seen in FBS. Moreover, a correlation between increased AP activity and increased deproteinized HS was observed. This

makes it seem that in HS proteins inhibit AP, whereas lipids increase its activity. This difference can be attributed to bovine AP behaving differently in the serum of a different species (cIAP was used both in FBS and HS). In a follow-up study, human IAP should be used in human serum, to account for this and to get a better understanding of the activity of AP in humans. It is important however to keep in mind that pNPP was used as a substrate for AP instead of DPLA and LPS, so the optimal conditions for lipid A dephosphorylation may differ.

Based on the results of the pNPP-assays we wanted to assess which proteins might play a role in the action of AP. Therefore, we developed a co-immunoprecipitation (IP) experiment. We tested two different samples, one with AP and one with AP + LPS, to test which other proteins might play a role in the detoxification of LPS along with AP. After running and staining an SDS-page gel we observed several bands that indicated that we successfully eluted other proteins along with AP.

CONCLUSION

To summarize, we performed multiple in vitro experiments. With LPS and DPLA we induced an inflammatory response in RAW 264.7 cells. AP did not significantly inhibit the inflammatory response induced by LPS. To assess whether lipids or proteins might influence AP's activity, we performed a pNPP assay with FBS, delipidated FBS and deproteinized FBS. The activity of AP was significantly higher in delipidated FBS compared to regular and deproteinized FBS. Therefore, we conclude that certain lipids might inhibit AP, whereas proteins enhance AP's activity. Finally, we co-eluted several proteins along with AP out of FBS in an immunoprecipitation experiment. There was no big visual difference between the sample containing AP compared to AP + LPS. Further LC-MS is needed to identify and quantify the co-eluted proteins of interest. Follow-up studies could also focus on experiments with AMP or other adenosine phosphates on top of LPS and AP. The findings may contribute to a better understanding of the mechanisms underlying the anti-inflammatory properties of IAP in liver fibrosis and contribute to the development of innovative treatments.

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