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Apoptosis by 6-*O*-palmitoyl-L-ascorbic acid coincides with JNK-phosphorylation and inhibition of Mg^{2+}-dependent phosphatase activity

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Abstract

6-*O*-Palmitoyl ascorbic acid (PAA) has recently been used as a substitute for ascorbic acid because of its greater potency as an antioxidant. In detailed concentration response studies distinct cytotoxic effects of PAA at concentrations exceeding 100 μM were reported. Here we examined and further characterized this cytotoxicity. While ascorbic acid was tolerated well up to millimolar concentrations, PAA revealed an LC_{50} between 125 and 150 μM in rat GH_3 tumor cells. Morphological and biochemical observations suggested the induction of apoptosis at concentrations exceeding 125 μM with a prominent activation of caspase 3 at 250 μM after 4 hr. A subsequent pronounced fragmentation of DNA (DNA-ladder) was detected after 6 hr and was further enhanced after 12 hr. The activation of caspases and the cleavage of its substrate PARP was preceded by a distinct increase in the phosphorylation of stress activated JNK-kinases. This observation suggested that the agent affected signal transduction mechanisms regulating protein phosphorylation at serine/threonine residues in the cell. No effect of PAA on protein phosphatase 2A (PP2A)-like activity was observed while magnesium-dependent protein phosphatase activity, presumably PP2C, was inhibited concentration-dependently up to 75% at the respective concentrations. Thus, the cytotoxic, pro-apoptotic effect of PAA might be related to the inhibition of PP2C and the activation of JNK.

Keywords: 6-*O*-Palmitoyl ascorbic acid; Caspase activation; DNA-fragmentation; JNK phosphorylation; JNK-inhibitor SP600125; Phosphatase inhibition

1. Introduction

AsA is important for many functions in eukaryotic cells, e.g. as cofactor in collagen metabolism. The low stability of AsA in aqueous solutions has resulted in the search for more stable and potent AsA derivatives. PAA is such a derivative which could substitute for AsA at 5–10-fold lower concentrations in studies on collagen synthesis in cultured human fibroblasts [1]. Some cytotoxicity of PAA was observed at the used concentrations (5–20 μM) in medium containing low FCS (0.5%). At 10% FCS no cytotoxicity was reported up to 100 μM of PAA. The effects on collagen metabolism have been confirmed in human intestinal smooth muscle [2]. The cytotoxic effects of higher PAA concentrations led to experiments studying a potential anti-tumor activity which presented itself as a reduced DNA-synthesis and the release of membrane phospholipids in Ehrlich ascites tumor cells [3]. This activity was aggravated by simultaneous hyperthermia (42°). Later studies by the same group [4,5] further substantiated the concentration-dependency of this effect and described morphological features of treated cells suggesting the induction by apoptosis. In a previous study on potential inhibitors of protein phosphatases PP1 and PP2A and their related pro-apoptotic effect we have used Michael adducts of AsA or PAA [6]. In this context PAA as the parent substance was also studied. The present paper concentrates on the known cytotoxic properties of PAA observed in GH_3 rat tumor

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Abbreviations: CA, cantharidic acid; PP, protein phosphatase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DEVD-AFC, DEVD-7-amino-4-trifluoromethylcoumarin; AsA, ascorbic acid; PAA, 6-*O*-palmitoyl ascorbic acid; JNK, c-*jun* N-terminal kinases; FCS, foetal calf serum.

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cells at concentrations exceeding 125 μM. Cytotoxicity could be characterized by morphological and biochemical parameters to be the consequence of apoptosis induction associated with caspase activation and subsequent DNA-fragmentation. An early alteration of PAA treated cells undergoing apoptosis was the phosphorylation of JNK-kinases which has been shown to be a central mechanism for drug-induced apoptosis following the mitochondrial pathway of apoptosis [7]. Toxicity and caspase activation by PAA was concentration-dependently reduced by the JNK-inhibitor SP600125. Interestingly, inhibition of Mg^{2+}-dependent protein phosphatase activity, presumably PP2C, coincided with the concentration-dependency of the pro-apoptotic effect.

2. Materials and methods

2.1. Materials

Casein, AsA and PAA were obtained from Sigma Chemical Co. The JNK-inhibitor SP600125 was from Calbiochem. Culture media were supplied by CCPro. Antibodies for the detection of PARP and phosphorylated JNK were bought from Cell Signaling—New England Biolabs.

2.2. Cell culture

Rat pituitary GH_3 tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% FCS at 37° in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Viability testing—methylene blue-assay

The day before treatment 15,000 cells were plated per well in the inner 60 wells of 96-well plates. Treatment with the tested agents was done in complete DMEM (8% FCS) for 24 hr. Thereafter the medium was aspirated and 100 μL of fixative added (10% formaldehyde in 0.9% NaCl (v/v)) for at least 30 min at room temperature. The fixative was decanted and cells were stained with 100 μL of 1% methylene blue in 0.01 M sodium-borate buffer (w/v; pH 8.5) for another 30 min [8]. After decanting the dye solution the wells were washed four times with 0.01 M of sodium-borate buffer (pH 8.5). Thereafter 100 μL of 50% ethanol in 0.1 M HCl (v/v) were added to each well and the plates incubated for 10 min at room temperature. After shaking the plates, the dye content was determined by measuring absorption at 650 nm in a microplate reader (Molecular Dynamics). The OD of untreated cells was set to 100% and the treated samples referred to this value. Medium with corresponding concentrations of PAA was used to account for background values which were subtracted from the samples containing cells before viability was calculated with respect to control samples.

2.4. Analysis of DNA-fragmentation

One million cells were plated per well of a 6-well plate and treated 24 hr later with the respective agent for the indicated times. DNA-fragments were isolated according to the column isolation protocol described [9]. After neutral lysis of the cells with SDS, proteins and genomic DNA were precipitated with a CsCl solution and the DNA fragments in the supernatant isolated by the use of GFX columns (Pharmacia). DNA was separated by agarose gel electrophoresis on a 1.6% NuSieve gel (Biozyme). Fifteen microliters of sample were mixed with 3 μL of loading buffer containing orange G and loaded on the gels (Gibco Minigel, 6 cm × 8 cm). Gels were run for about 35 min at 90 V, stained with ethidiumbromide and documented with the Biodoc system (Biometra) after transillumination at 312 nm.

2.5. Fluorometric assay of caspase 3-like activity

Three hundred thousand cells were plated per well of a 24-well plate (Falcon). Treatment was started after 24 hr for the indicated period of time. Thereafter plates were spun for 10 min at 1500 rpm in a centrifuge (Eppendorf). Medium was aspirated and 250 μL lysis buffer (pH 7.4) consisting of 10 mM HEPES, 1% NP-40 (w/v), 0.01 mM digitonin, 0.1 mM EDTA, 1 mM DTT and 1 mM AEBSF were added per well. Plates were left on ice for 15 min. Thereafter cells were shock-frozen in liquid nitrogen and stored at −20° until the assay was performed.

After thawing the samples on ice, 50 μL of lysis buffer containing the substrate was added to give a final concentration of DEVD-AFC of 25 μM per well. The reaction was run in a water bath at 37°. The liberated amount of AFC was repeatedly measured in a fluorometer (Canberra Packard; $\lambda_{ex} = 440$ nm, $\lambda_{em} = 515$ nm). From the linear part of the increase in fluorescence the enzymatic activity was obtained and activity changes were expressed relative to the untreated cells.

2.6. Measurement of PP2A and PP2C activity in cell extracts

Phosphatase activity was measured in cytosolic extracts generated by sonification. Casein, a substrate of PP2A/C but not of PP1 [10], was used in a non-radioactive malachite green assay with colorimetric detection of liberated phosphate [11]. For the determination of PP2C activity samples were supplemented with 5 mM of Mg^{2+} acetate solution while PP2A-like activity was eliminated by the addition of 100 μM of cantharidic acid. The effect of the respective treatments is presented relative to the values observed with extracts from untreated controls.

2.7. Immunoblots

One million cells were plated per well of a 6-well plate. After 24 hr cells were treated with the respective agent for the indicated times, then suspended by careful scraping. The cell suspension was centrifuged for 3 min at 1000 *g* and 4°. After removal of the supernatant, the cell pellet was lysed by the addition of SDS-sample buffer [12] containing 125 mM Tris–HCl, pH 6.8; 4% SDS; 20% glycerol; 5% mercaptoethanol; 0.005% bromophenol blue. Sample buffer was added to the cell pellet in an approximated 1:3 ratio (volume cell pellet/volume sample buffer) followed by sonication on ice for 3×10 s (Bransson Ultrasonics). Samples were stored at −80° or immediately boiled for 5 min prior to gel electrophoresis, performed with aliquots of 150 μg protein per lane in homogenous 12% polyacrylamide gels. Proteins were then transferred onto PVDF-membrane (Millipore) by semi-dry blotting. Phosphorylation of JNK and cleavage of PARP was detected with antibodies supplied by Cell Signaling—New England Biolabs. Labeled proteins were visualized by the ECL-system (Amersham Biosciences).

3. Results

When comparing the effects of AsA and PAA on the viability of rat pituitary GH_3 cells, a distinct decrease in viability at PAA concentrations exceeding 125 μM ($LC_{50} \sim 150$ μM) was observed while AsA did not negatively affect the viability of the cells up to 1 mM (Fig. 1). At 50–100 μM PAA and 100–250 μM AsA absorption was slightly higher than under control conditions suggesting some induction of cell proliferation by both agents as had been reported [4]. The known inhibitor of protein phosphatase 2A (PP2A) cantharidic acid induced a comparable loss in viability at about 10-fold lower concentrations ($LC_{50} \sim 15$ μM; data not shown).

The morphological appearance of treated cells coincided with these observations. After 6 hr cell morphology was distinctly different in cells treated with 250 and 500 μM of PAA and 25 μM of CA when compared to the untreated cells (Fig. 2). No morphological change was apparent with 500 μM AsA or 125 μM PAA. While cells became rounded and condensed at 250 μM of PAA and 25 μM of CA, the cells treated with 500 μM of PAA appeared to be more severely damaged and presented signs of lysis and beginning cell disintegration. These impressions were further pronounced after 12 hr (data not shown). The appearance of cells incubated with 250 μM of PAA and 25 μM of CA represent the characteristic morphological features of cells undergoing apoptotic cell death.

In order to substantiate such a conclusion we evaluated the concentration-dependent effect of PAA on DEVDase (caspase 3/7) activity after treatment periods of 2, 4 and 6 hr (Fig. 3). While 500 μM of AsA did not activate caspase 3/7 at any of these time points, 125 μM of PAA or 25 μM of CA induced activity after 6 hr by 5–6-fold. A pronounced 45-fold elevation of caspase activity was observed with 250 μM of PAA after 4 hr which declined to about 30-fold after 6 hr. At the highest concentration tested (500 μM PAA) caspase activity was transiently increased by 6-fold after 4 hr and declined thereafter. This observation revealed that PAA at 250 μM activated caspase

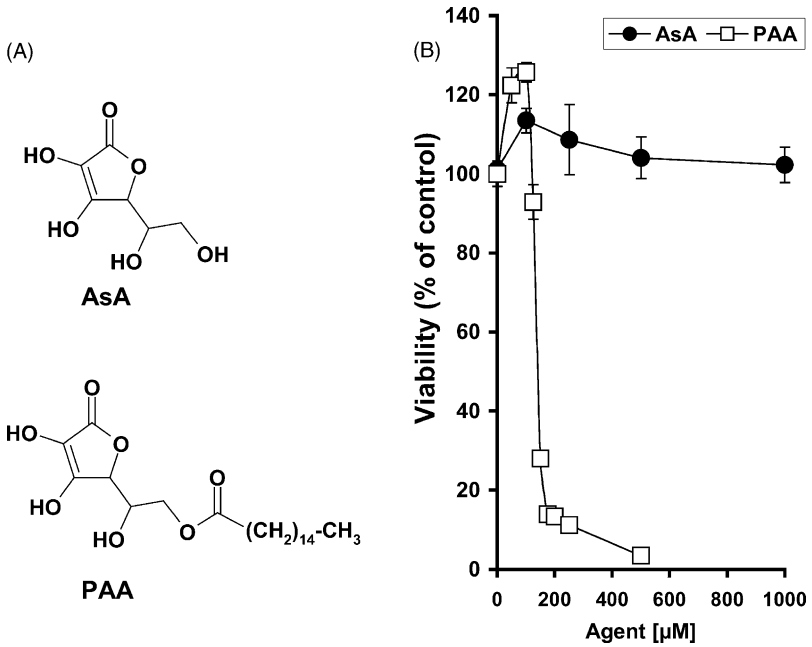

Fig. 1. (A) Structures of AsA and PAA. (B) The concentration-dependency of the potential cytotoxic effects of PAA and AsA after 24 hr of treatment was measured with the methylene blue assay. Mean ± SEM of N = 4–12 experiments.

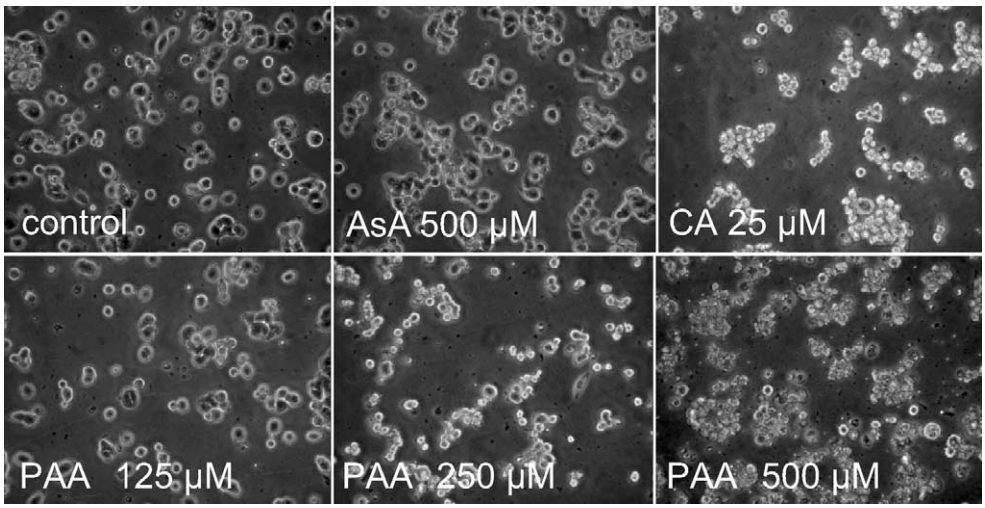

Fig. 2. Phase-contrast microscopy (magnification: 200×) of cells incubated for 6 hr under the respective conditions.

3/7 much more rapidly than CA. At 500 µM PAA either apoptosis progressed very fast into secondary necrosis or primary necrosis dominated. Such a rapid lysis of the cells presumably resulted in the loss of cytosolic caspase activity into the medium which was then lost during the generation of extract for measurement of caspase activity. Otherwise, caspase activity might also have been degraded by other proteases liberated during cell lysis. Both aspects would be consistent with rapid progression into secondary necrosis at the highest concentration of PAA.

Another feature of apoptosis is the subsequent activation of caspase activated DNase CAD [13] which results in the coordinated internucleosomal cleavage of DNA into fragments of about 200 base pairs or multiples of it. This DNA-fragmentation appears as a distinct ladder after separation on agarose gels. In accordance with the other data PAA at 125 µM revealed no distinct DNA-fragmentation after 6 hr and a faint laddering after 12 hr. At 250 and 500 µM PAA distinct DNA laddering was visible at both time points (Fig. 4) with a progression of band intensity to fragments of lower size after 12 hr. At 500 µM PAA DNAs of larger size appeared to be diminished after 6 and 12 hr (Fig. 4, arrows). This observation again supports the hypothesis of a very rapid apoptosis progression at the higher concentrations of PAA. With 25 µM of CA distinct fragment bands became apparent after 12 hr while after 6 hr only a faint laddering, slightly stronger than the one observed with 125 µM of PAA after 12 hr, was observed. Maximal effects of CA had been seen after 18–24 hr (data not shown). In accordance with caspase activation data the observations on DNA-fragmentation supported the conclusion that apoptosis with 250 µM of PAA progressed much faster than with 25 µM of CA.

An early feature of CA treated cells undergoing apoptosis is the observation that stress-activated JNK-kinases became phosphorylated and thus activated [14]. We checked the phosphorylation of JNK as the consequence of PAA (250 µM) treatment and compared it with the

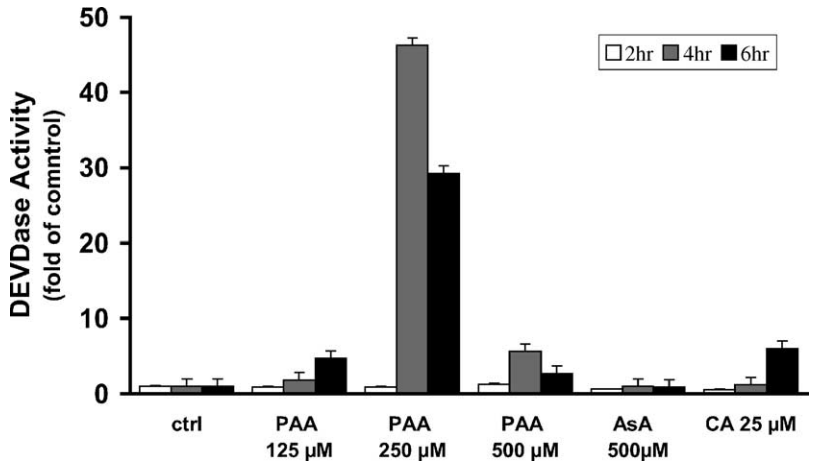

Fig. 3. DEVD-caspase activity was measured in cytosolic extracts of cells treated with the respective agents for 2, 4 and 6 hr. Mean ± SEM of N = 4–12 experiments.

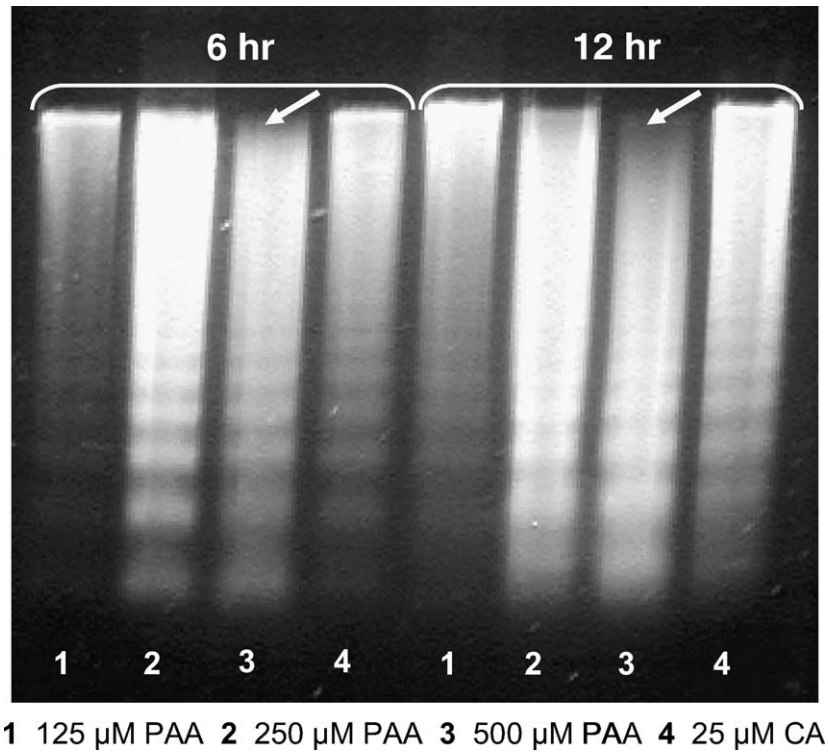

Fig. 4. DNA-fragmentation in cells treated for 6 and 12 hr with PAA and cantharidic acid.

known effect of CA. In addition, we checked for the cleavage of PARP as a result of caspase 3 activation (Fig. 5A and B). JNK phosphorylation became visible after about 1 hr of treatment with 250 µM of PAA (Fig. 5A) while cleavage of PARP was observed after 4 hr in PAA but not CA treated cells (Fig. 5A and B). Phosphorylation intensity after 4 hr appeared to be slightly less prominent than with 25 µM CA whereas on the other hand PARP cleavage was definitely earlier observed with PAA than with CA (Fig. 5B). This again supports our hypothesis of faster progression of apoptosis in PAA compared to CA treated cells. Addition of the selective JNK-inhibitor SP600125 [15] concentration-dependently reduced caspase 3/7 activation by 250 µM PAA (Fig. 6B). In addition, the rounded and condensed apoptotic appearance of cells treated with PAA alone was reduced by SP600125 (Figs. 6A and 7). Cells incubated with PAA and 75 µM SP600125 resembled the more irregular shape of control cells.

Both agents, PAA and CA, apparently increased phosphorylation of JNK suggesting an interference with mechanisms regulating reversible protein phosphorylation in the cells, albeit presumably by different mechanisms. In the case of cantharidic acid changes in protein phosphorylation are most likely the consequence of its well-known inhibition of PP2A. Therefore, we tested both agents for

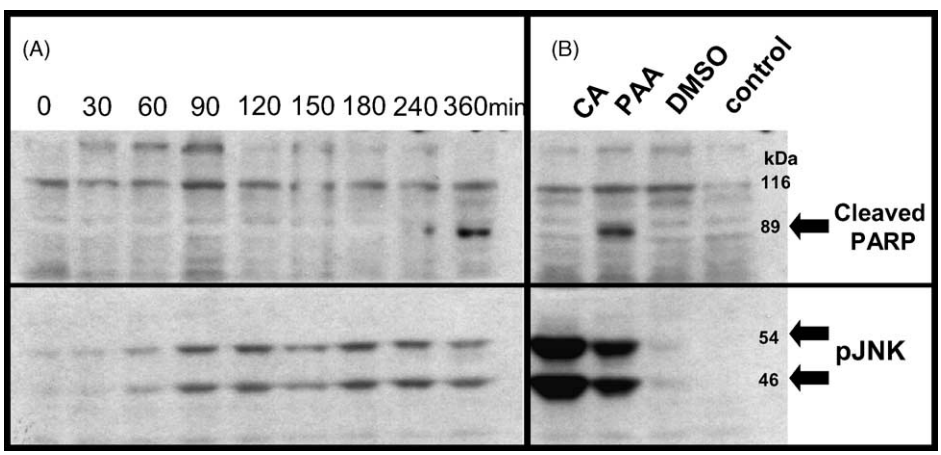

Fig. 5. Immunoblots of PARP cleavage and JNK phosphorylation. (A) Kinetics with 250 µM PAA. (B) Comparison of the effects of CA (25 µM) and PAA (250 µM) on PARP cleavage and JNK phosphorylation after 4 hr of treatment.

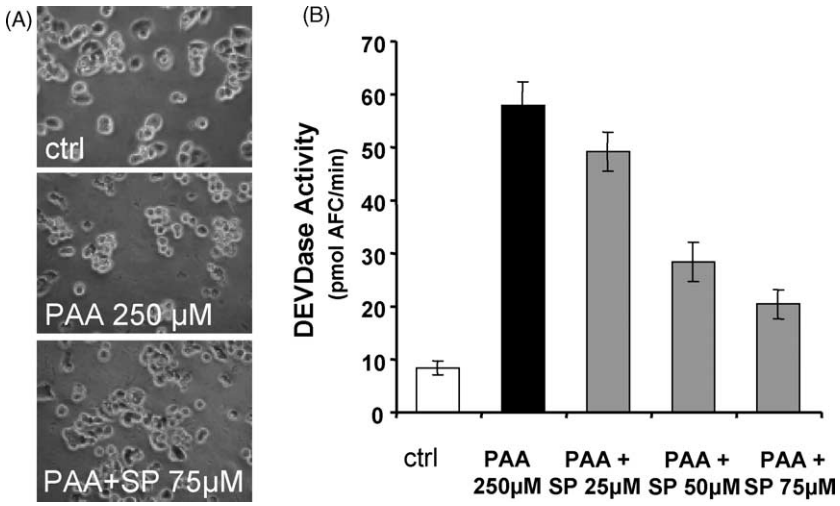

Fig. 6. Effect of JNK-inhibitor SP600125 on apoptosis induced by 250 µM PAA. Cells were treated for 4 hr as indicated. (A) Morphological appearance of cells exposed to the respective treatments. (B) Caspase 3/7 activity following the different treatments presented as picomoles AFC liberated per minute (pmol AFC/min). Mean ± SEM of N = 3 experiments.

PP2A inhibition in GH_3 cell extracts with a non-radioactive assay using casein as substrate [11]. While we observed a more than 80% inhibition with 100 µM of CA, no inhibition was observed with PAA between 250 and 1000 µM. In the presence of magnesium casein has been reported to be also a substrate of PP2C [10]. In addition, PP2C measurements are usually done in the presence of high concentrations of PP2A inhibitors, e.g. okadaic acid. In our case, we used cantharidic acid (100 µM) and tested for an effect of PAA on the remaining magnesium-dependent activity in the GH_3 extract. Interestingly, a concentration-dependent inhibition by PAA of the magnesium-dependent dephosphorylating activity of casein was observed. Fifty percent inhibition was seen at 250 µM which increased up to 75% at 1000 µM. Thus, a fairly good correlation between the concentration-dependencies of the observed cytotoxic, pro-apoptotic effect of PAA and the inhibition of the Mg^{2+}-activated protein phosphatase activity became evident.

4. Discussion

The data of the present paper for the first time clearly reveal that the known cytotoxic effect of PAA at concentrations exceeding 100 µM is apparently the consequence

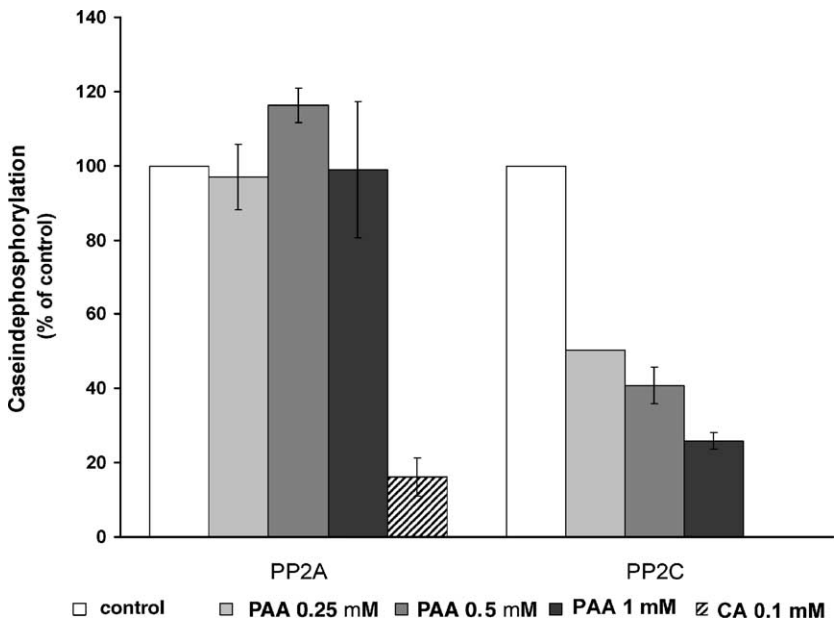

Fig. 7. Effects of PAA and CA on the dephosphorylation of casein by cytosolic extracts in the absence (PP2A) and the presence of 5 mM Mg^{2+} plus 100 µM cantharidic acid to completely inhibit PP2A (PP2C). Mean ± SEM of N = 3 experiments.

of apoptosis induction. This response presents itself with an early activation of caspases after 4 hr of treatment. Since this activity is preferentially cleaving after the sequence DEVD, these enzymes are most likely caspases 3 and 7 [16]. Caspase activation is followed by DNA-fragmentation after 6 hr. As early as 1 hr after the beginning of the treatment with 250 μM of PAA a definite increase of the phosphorylation of the 46 and 54 kDa bands of JNK-kinases was observed which remained increased for at least 6 hr. After 4 hr the cleavage of the caspase 3/7 substrate PARP could be detected in immunoblots. The activation of JNK has been described to be of major relevance for the mitochondrial pathway of apoptosis induction [7] which is targeted by most of the presently used anti-tumor agents. The conclusion that sustained JNK phosphorylation is responsible for the pro-apoptotic effect of PAA was supported by the observation that SP600125, a fairly selective JNK inhibitor, in the range from 25 to 75 μM concentration-dependently reduced caspase 3/7 activation by PAA.

The phosphorylation status and thus activity of this downstream kinases of the stress-activated kinase cascade is not only the consequence of activation of upstream kinases but might also be substantially affected by the activity of serine/threonine-phosphatases relevant for these enzymes. In former studies a raised JNK-phosphorylation within the first hour of treatment was observed in GH_3 cells treated with cantharidic acid [14], a well-characterized inhibitor of PP2A. Thus, PP2A appeared to be also involved in the regulation of the activity of the JNK-kinase cascade. No effect of PAA on PP2A activity was observed in the present paper. On the other hand, enzymatic dephosphorylation of casein can also be measured in the presence of 5 mM Mg^{2+}. The contribution of PP2A can be completely eliminated by the addition of cantharidic acid. In this case the phosphatase activity remaining is most likely PP2C activity. This activity was concentration-dependently inhibited by PAA with an IC_{50} of about 250 μM. This concentration response correlated well with the concentration response observed for the cytotoxic effect.

Interestingly, no selective inhibitors with high affinity for PP2C have been reported up to now. Some activity with IC_{50} values in the low millimolar range have been reported for levamisole and bromotetramizole [17]. Bromotetramizole at these concentrations also slowed the deactivation of CFTR channels [17] which reportedly are substrates of PP2C [18]. Apoptosis induction by levamisole was reported by another group [19]. Transfection and thus overexpression of some PP2C subtypes have suggested that PP2C overexpression resulted in the suppression of stress-activated p38 and JNK-MAPK pathway activity [20,21]. Thus, PP2C apparently behaves as a negative regulator of apoptosis involving JNK activation. A recent paper reports that apoptosis induction by the protein axin was dependent on JNK activation and could be attenuated by the overexpression of PP2Cα [22]. In the present paper GH_3 cells treated with pro-apoptotic concentrations of PAA displayed an early and sustained increase in JNK phosphorylation. This response might be the consequence of PP2C inhibition by PAA. Interestingly, in plants some fatty acids have been reported to specifically inhibit *in vitro* the plant enzyme MP2C, a PP2C-like phosphatase from alfalfa [23].

In conclusion, cytotoxicity observed with concentrations of PAA exceeding 125 μM were the result of apoptosis associated with caspase activation and the apoptotic DNA-ladder phenomenon. Apoptosis by PAA progressed much faster than that seen with the PP2A inhibitor cantharidic acid. Both, cantharidic acid and PAA increased JNK-phosphorylation before caspase activation was detectable. While cantharidic acid inhibited PP2A activity by more than 85%, PAA was only effective as a phosphatase inhibitor when PP2A activity was eliminated and in addition Mg^{2+} ions were added. Since Mg^{2+}-dependency of enzymatic activity dephosphorylating casein is a characteristic of PP2C enzymes we suggest that PAA acts via an inhibition of PP2C as an activator of JNK. Inhibitors of PP2C with high selectivity and affinity are still lacking. Therefore, this observation with PAA might provide the basis for the development of new PP2C inhibitors fulfilling these criteria.

Acknowledgments

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