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Regulation of aldose reductase activity by tubulin and phenolic acid derivates

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ABSTRACT

In this work we demonstrate that aldose reductase (AR) interacts directly with tubulin and, was subjected to microtubule formation conditions, enzymatic AR activity increased more than sixfold. Since AR interacts mainly with tubulin that has 3-nitro-tyrosine in its carboxy-terminal, we evaluated whether tyrosine and other phenolic acid derivatives could prevent the interaction tubulin/AR and the enzymatic activation. The drugs evaluated have two characteristics in common: the presence of an aromatic ring and a carboxylic substituent. The 9 drugs tested were able to prevent both the interaction tubulin/AR and the enzymatic activation. In addition, we found that the induction of microtubule formation by high concentrations of glucose and the consequent activation of AR in cultured cells can be inhibited by phenolic acid derivates that prevent the interaction tubulin/AR. These results suggest that tubulin regulates the activation of AR through a direct interaction which can be controlled with phenolic derivates of carboxylic acids.

1. Introduction

AR belongs to the aldo-keto reductase (AKR) super family, and it is involved in the detoxification of many substrates, such as aliphatic aldehydes, monosaccharides and the products of lipid peroxidation [1,2]. The implication of AR activation in several pathologies has been widely studied, particularly in secondary complications of diabetes. It is also known that overexpression or increased activity of AR is involved in carcinogenesis and/or resistance to cancer drugs in different commonly occurring cancer tissues, e.g. breast, ovarian, rectal, hepatic and cervical cancers and in the hyperproliferation of vascular smooth muscle cells [1,3–7]. Moreover, activation of AR has been correlated with allergic airway inflammation in asthma, oxidative stress-induced inflammatory disorders and pulmonary fibrosis [1,8–13].

We previously demonstrated that acetylated tubulin (AcTub) associates with Na^+,K^+-ATPase (NKA) to form a complex that results in the inhibition of NKA enzyme activity [14–19]. More recently, we have shown that treating cells with high glucose concentrations induces polymerization of microtubules, increases the formation of the AcTub/NKA complex and inhibits NKA enzyme activity. All these effects appear to have resulted from increased levels of sorbitol produced by AR activation. AR activity increased when the enzyme associated with microtubules, resulting in an “upregulation cycle” between the formation of microtubules and AR activation [20]. In this work, we study how AR becomes activated through its interaction with tubulin. Our data demonstrate that AR interacts directly with tubulin and activates the enzyme. Moreover, we show that different drugs, which are chemically derived from phenolic acid, were able to prevent tubulin/AR interaction, suggesting that therapies blocking the association between tubulin and AR could be important in preventing AR activation.

2. Materials and methods

2.1. Tubulin purification

Brains from rats between 30 and 60 days old were homogenized at 4 °C in one volume of MEM buffer (0.1 M Mes/NaOH, pH 6.7, containing 1 mM EGTA and 1 mM $MgCl_2$). The homogenate was centrifuged at 100,000 × *g* for 45 min, and the pellet was discarded. Tubulin was purified as described by Ref. [21]. The concentration was adjusted to 1 mg/ml, and the tubulin was used immediately.

Abbreviations: AR, aldose reductase; NKA, Na^+,K^+-ATPase; 3-NTyr, 3-nitro-L-tyrosine; 3-NTyr-tub, 3-nitrotyrosinated tubulin; Tyr, tyrosine

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2.2. Recombinant AR production

AKR1B1 cDNA was isolated by RT-PCR, starting from human adrenal total RNAs. cDNAs were inserted into the Nde1 and *Eco*RI sites of the PET 28a vector (Novagen, Tebu, Le Perray-en-Yvelines, France) to produce N-terminal fusions with six histidine residues. This construct was kindly provided by Dr. Anne-Marie Lefrancois-Martinez, and recombinant AR was expressed and purified as described by her [22].

2.3. AR assay

Overexpressed and purified AR was assayed spectrophotometrically by the method of Tabakoff and Erwin [23]. The reaction mixture for this assay contained 7 μg protein, 0.1 mM NADPH, 1 mM D,L-glyceraldehyde, and 0.1 M sodium phosphate, pH 7.0, in a total volume of 3 ml. The reaction was initiated by the addition of aldehyde. The rate of NADPH oxidation was assessed as the decrease in absorbance at 340 nm for 20 min at 25 °C.

2.4. Interaction of tubulin with AR

Recombinant AR was linked to Ni^{+2}-NTA beads (Novagen) as described by Ganesan et al. [24]. AR-linked beads (0.2 ml) were washed with 1 ml of 10 mM Tris-HCl (pH 7.6) buffer containing 20 mM NaCl, and incubated with various concentrations of tubulin in a total volume of 400 μl for 30 min at 20 °C. The samples were centrifuged, and the precipitated materials were washed five times with the same buffer. Fractions (50 μl) of packed beads were resuspended in 50 μl Laemmli sample buffer, heated at 50 °C for 15 min, and centrifuged. Aliquots (20 μl) of the soluble fractions were subjected to SDS-PAGE.

2.5. Cell culture and treatment with glucose or phenolic acid

COS cells were grown in DMEM at 37 °C in a water-saturated atmosphere of air/CO_2 (19:1). For treatment with glucose or phenolic acid compounds, cells cultured to 90% confluence were rinsed with HEPES-FBS buffer (25 mM HEPES, pH 7.4, supplemented with 1 mM sodium pyruvate, 0.22% sodium carbonate, 10 mM glutamine, 100 mM NaCl, 10% FBS, 10 IU/ml penicillin, and 100 μg/ml streptomycin) and incubated for 2 h with the indicated compound.

2.6. Isolation of cytoskeletal tubulin

COS cells were washed at room temperature with microtubule-stabilising buffer (90 mM Mes, pH 6.7, 1 mM EGTA, 1 mM $MgCl_2$, 10% (v/v) glycerol) and extracted with 2.5 ml of the same buffer containing 10 μM taxol, 0.5% (v/v) Triton X-100, and protease inhibitors (10 μg/ml aprotinin, 0.5 mM benzamidine, 5 μg/ml *o*-phenanthroline, 0.2 mM PMSF) for 3 min at 37 °C with gentle agitation. The detergent extract (a cytosolic tubulin fraction including a membrane fraction) was separated, and the cytoskeletal tubulin fraction, which remained bound to the dish, was washed with pre-warmed microtubule-stabilising buffer and resuspended in sample buffer [25].

2.7. Immunofluorescence staining

Cultured cells were fixed on coverslips with anhydrous methanol at −20 °C, washed, incubated with BSA in PBS buffer, and stained by indirect immunofluorescence as described by DeWitt et al. [26].

2.8. Gel electrophoresis, immunoblotting and protein determination

Proteins were separated by SDS-PAGE on 15% polyacrylamide slab gels as described by Laemmli[25] and transferred to nitrocellulose sheets. Blots were reacted with various antibodies as indicated. Sheets were reacted with the corresponding anti-IgG conjugated with peroxidase and stained by the 4-chloro-1-naphthol method. Band intensities were quantified using the Scion imaging programme. Protein concentration was determined by the method of Bradford [27].

2.9. Materials

Nitrocellulose membrane, taxol, Triton X-100, SDS, L-tyrosine, 3-nitro-L-tyrosine, 3-hydroxy-4-methoxymandalic acid, 3-methoxy-4-hydroxymandelic acid, 4-hydroxy-3-methoxyphenyl lactic acid, vanilic acid, 3,4-dihydoxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid were obtained from Sigma-Aldrich (St.Louis, MO, USA).

2.10. Antibodies

Mouse anti-Ac-tubulin mAb 6-11B-1, mouse anti-α-tubulin mAb DM1A, anti-Tyr-tubulin mAb Tub1-A2, peroxidase-conjugated mouse IgG, and fluorescein-conjugated mouseIgG were from Sigma. Rabbit antidetyrosinated tubulin polyclonal Ab was prepared as described by Gundersen et al. [28].

3. Statistical analysis

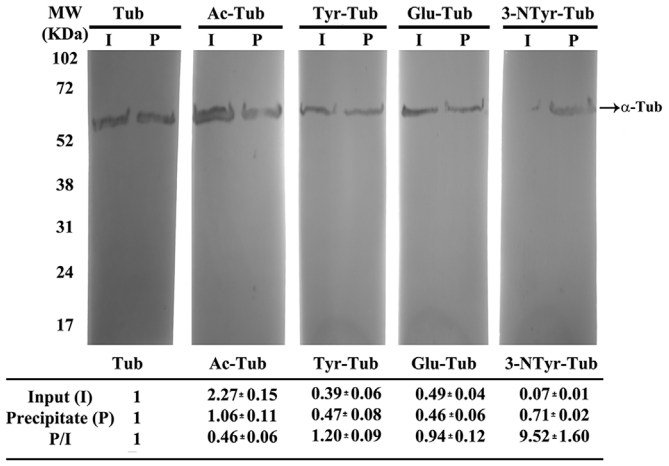
Results were expressed as mean ± SD. Student's t-test was used for comparison of two populations. Analysis of variance (ANOVA) was used for comparison when sample sizes differed between groups. Differences between means were considered statistically significant for p values ≤ 0.05.

4. Results

4.1. AR interacts directly with tubulin and has greater affinity for tubulin 3-nitro-tyrosinated

Given that tubulin affects AR activity [20], we speculated that both proteins could interact directly. To demonstrate this, we purified rat brain tubulin and AR overexpressed on a strain of *Escherichia coli*. Overexpressed AR bound to Ni^{+2}-NTA beads was incubated with tubulin and the precipitated material was analyzed by SDS-PAGE immunobloting with specific antibodies for total tubulin (Fig. 1). A simple visualization of the immunoblot shows that tubulin was found in the precipitated material (see line P for Tub, Fig. 1). This result demonstrates that there is a direct interaction between tubulin and AR linked to Ni^{+2}-NTA beads. As a negative control, the same experiment was carried out using an exopolyphosphatase of *Pseudomonas aeruginosa* instead of AR bound to Ni^{+2}-NTA beads, and no tubulin was found in the precipitated material (data not shown), which is evidence of the specificity of the interaction between tubulin and AR.

Tubulin is the substrate of different post-translational modifications [29]. Among the most studied modifications in β-tubulin we find polyamination, polyglycosylation, phosphorylation and acetylation of lysine 252. On the other hand, α-tubulin is codified with a tyrosine residue at its C-terminal end. After becoming associated with β-tubulin and polymerized into microtubules, this amino acid is removed to produce detyrosinated tubulin (or Glu-tubulin, so called because of exposed glutamic acid on its C-terminal end). The removal is catalysed by Tubulin Carboxipeptidase (TCP), an enzyme which has been recently characterized [30,31]. The action of tubulin tyrosine ligase (TTL) can re-add Tyrosine on the glutamic acid at the C-terminus of α-tubulin, then Tyr-tubulin is obtained again, and the tyrosination/detyrosination cycle goes on [32]. In addition to tyrosination and detyrosination, another posttranslational modification occurs on α-tubulin such as phosphorylation, acetylation, glutamylation, etc. It has been shown that all these modifications in α-tubulin are present in all cells and coexist in a proportion that depends on the physiological state in which the cell is found. In fact, the presence of nitric oxide in some diseases allows the



	Tub	Ac-Tub	Tyr-Tub	Glu-Tub	3-NTyr-Tub
Input (I)	1	2.27±0.15	0.39±0.06	0.49±0.04	0.07±0.01
Precipitate (P)	1	1.06±0.11	0.47±0.08	0.46±0.06	0.71±0.02
P/I	1	0.46±0.06	1.20±0.09	0.94±0.12	9.52±1.60

Fig. 1. Pos-translational modifications of tubulin associated with AR. Ni^{+2}-NTA beads linked to recombinant AR were incubated with Tub (200 μg) plus taxol 5 μM. The samples were precipitated and the Tub preparation ("Input") and precipitate were analyzed by immunoblotting to determine total Tub and different Tub modifications (acetylated, Ac-; tyrosinated, Tyr-; detyrosinated, Glu-; 3-nitrotyrosinated, 3-NTyr). Bands were quantified by densitometry using Scion Image software. "Input I" = ratio between Tub modifications and α-Tub in preparation (control of the content of each Tub modificaction present in the tubulin preparation used). "Precipitate P" = ratio between Tub modifications and α-Tub in precipitate. "P/I" = ratio between precipitate ratio and input ratio. Values shown are mean ± S.D. from three independent experiments.

nitration of free tyrosine, which can be incorporated into tubulin by TTL generating 3-nitrotyrosinated tubulin.

To establish which modifications of tubulin interact with AR, material precipitated with AR linked to Ni^{+2}-NTA beads was analyzed by immunoblot with specific antibodies for acetylated (Ac-Tub), tyrosinated (Tyr-Tub), glutaminated (Glu-Tub) and 3-nitrotyrosinated (3-NTyr-Tub) tubulin. Each tubulin modification was quantified in the tubulin sample (input, I) and in the AR precipitated material (P) (Fig. 1). Results indicate that AR interacts preferentially with the 3-NTyr-Tub. In effect, the tubulin that precipitated with AR bound to Ni^{+2}-NTA beads was ~10 times richer in 3-NTyr-Tub (see relationship P/I, Fig. 1). Taken all together, these results indicate that AR interacts directly with tubulin to form a tubulin/AR complex, and that AR has a higher affinity for interacting with the 3-NTyr-Tub than with others tubulin pos-translational modification.

4.2. Free 3-NTyr or tyr prevents tubulin/AR complex formation and AR activation

The C-terminal end of tubulin is the substrate in a cycle of detyrosination and tyrosination, where Tyr is eliminated from or added to tubulin, respectively [32]. In this cycle, Tyr can be replaced by 3-NTyr if the conditions of the medium allow the increment of this Tyr substitute. Because 3-NTyr-Tub is the main modification of tubulin involved in the interaction with AR, we hypothesized that either free amino acids, 3-nitrotyrosine (3-NTyr) or its analogue Tyr could prevent the formation of the tubulin/AR complex by competition with 3-NTyr-Tub for the interaction site present in AR. To evaluate this possibility, AR attached to Ni^{+2}-NTA beads was incubated with tubulin in the presence of Tyr or 3-NTyr and the precipitated material was analyzed by immunoblot using a specific antibody against α-tubulin. Results indicate that both amino acids can prevent the formation of the tubulin/AR complex in a concentration-dependent manner (Fig. 2A). Tyr had a more potent effect than 3-NTyr in preventing complex formation, because with 10 μM Tyr the tubulin which co-precipitated with AR was ~10 times lower than with the same concentration of 3-NTyr. When

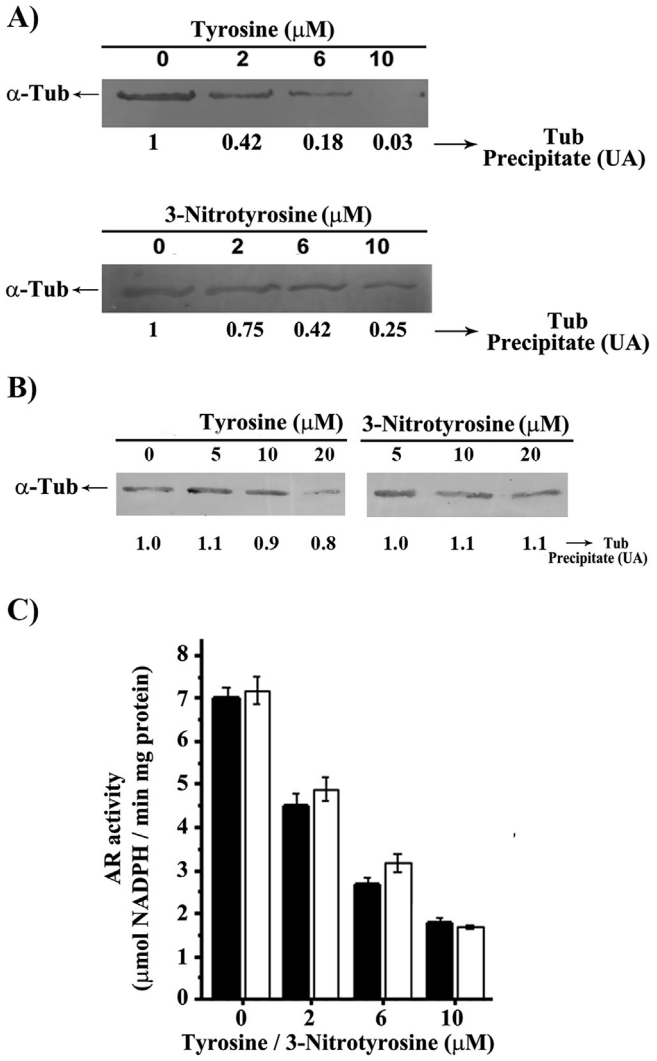


Fig. 2. Tyr and 3-NTyr prevent *in vitro* tubulin/AR association and stimulation of AR activity by tubulin. Purified rat brain tubulin and recombinant AR were used. Tubulin (Tub, 200 μg of prot.) was mixed with: (A) Ni^{+}2-NTA beads linked to AR previously incubated with Tyr or 3-NTyr. (B) Ni^{+}2-NTA beads linked to AR and then added Tyr or 3-Ntryr at the indicated concentrations. Precipitated materials were analyzed by western blot and stained with mAb DM1A. The volume of the sample analyzed was calculated to represent the same amount of tubulin in each case. (C) AR (7 μg) was incubated for 5 min at 37 °C in sodium phosphate buffer (final volume 1 ml) in the presence of Tyr (■) or 3-NTyr (□), then was added tubulin (200 μg of prot.), 5 μM taxol and incubated for 30 min at 37 °C. The samples were then processed for determination of AR activity as described in Materials and Methods. Basal AR activity was 1,2 ± 0.3 μmol NADPH/min mg of protein. The absence of tyr or 3-Tyr (concentration "0″) is the control both for the determination of association of both proteins and for the enzymatic activity. The values are mean ± SD from three independent experiments.

the preformed tubulin/AR complex was incubated in the presence of 3-NTyr, the amount of tubulin precipitated by AR was not affected, and in presence of Tyr only 20% of tubulin present in the complex was dissociated (Fig. 2B), but it was necessary to use twice the concentration of Tyr that was used to prevent complex formation (compare Fig. 2A and B). This indicates that the drugs are more efficient to prevent the tubulin/AR association than to dissociate the previously formed complex. Seeing that Tyr and 3-NTyr were able to prevent the formation of the Tub/AR complex, we analyzed how AR activity increased when induced by the association of the enzyme with tubulin, under conditions of polymerization at microtubules [20]. AR was incubated with tubulin

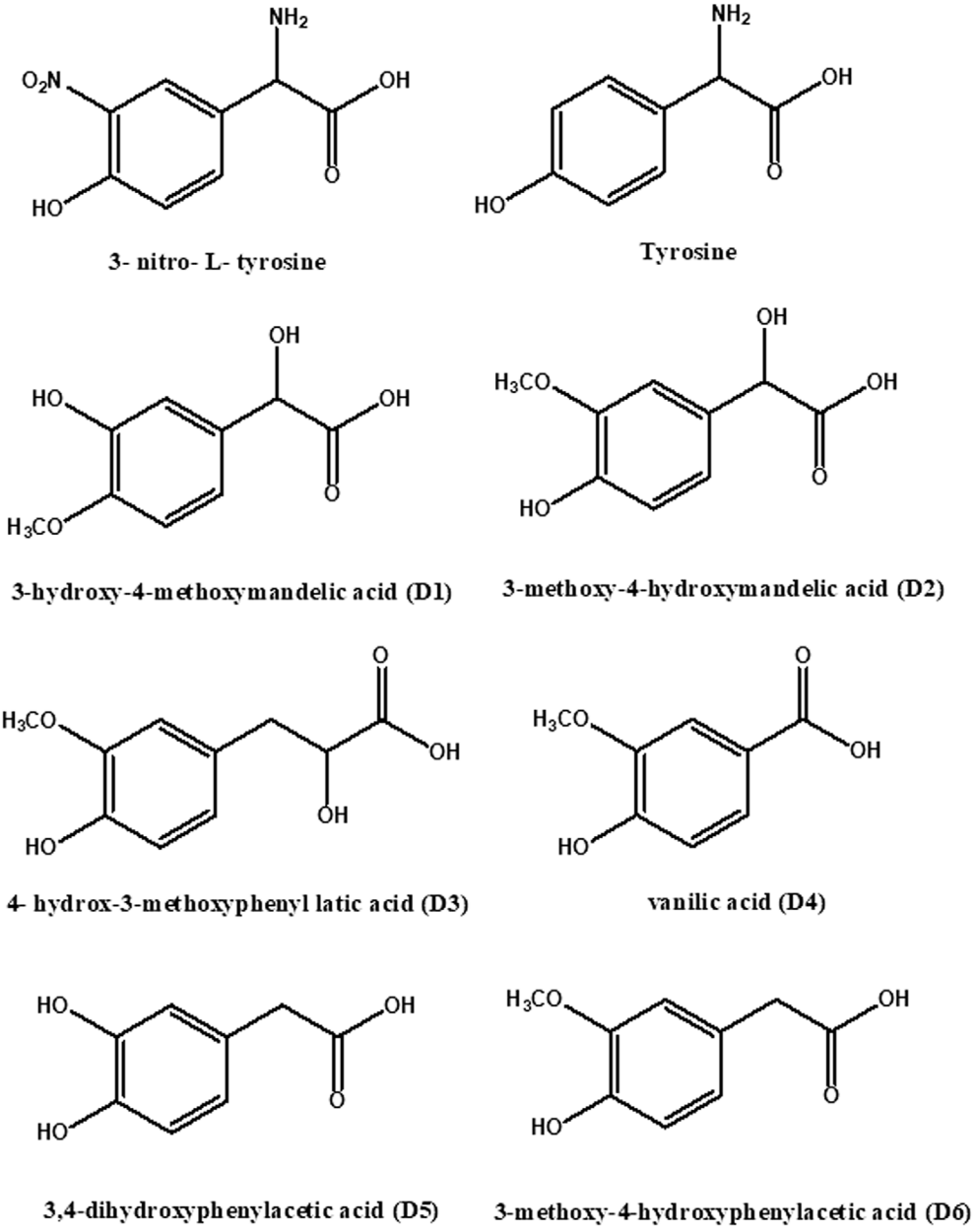


Fig. 3. Structures of phenolic acid derivates that prevent the formation of the tubulin/AR complex.

and taxol in the presence of different concentrations of Tyr or 3-NTyr, and AR activity was subsequently determined. As expected, under polymerization conditions tubulin activated AR more than 6 times, as was already observed [20]. On the other hand, in the presence of Tyr or 3-NTyr, AR activity decreased in a manner dependent on drug concentration (Fig. 2C). AR activity measured in the presence of 2, 6 or 10 μM Tyr or 3-NTyr decreased approximately 30%, 60% and 80%, respectively. Together, these data indicate that AR activity can be regulated by compounds able to prevent the association of tubulin with AR, such as Tyr or 3-NTyr. This represents a new mechanism of physiological regulation of AR activity by tubulin.

4.3. Effect of phenolic acid derivates on tubulin/AR complex formation and AR activation

Both 3-NTyr and Tyr have an aromatic and an acidic group in their structures. In order to evaluate the importance of these functional groups in preventing the formation of the tubulin/AR complex, we determined the ability of different phenolic acid derivates, with structures similar to 3-NTyr or Tyr, to regulate AR activity through their association with tubulin. The structure of Tyr, 3-NTyr and the compounds evaluated is shown in Fig. 3 and named as drug D1-6. An aliquot of AR was incubated with tubulin under polymerizing conditions in the presence of each drug. AR activity was also determined without drugs and without tubulin as controls. In the presence of tubulin, AR activity increased ~3.5-fold, while all compounds tested decreased to a greater or lesser extent the activation of AR caused by Tub/AR association (Fig. 4A). Tyr, 3-NTyr, D2, D3 and D4 totally reverted the effect of tubulin on AR activity, while the effects of D1, D5 and D6 were less remarkable: these compounds decreased the activation of AR by tubulin by 18%, 60% and 42%, respectively. If these drugs are able to prevent the activation of AR by tubulin, they should also be able to prevent the association between both proteins. To corroborate this, the association between the two proteins in the presence of the compounds was analyzed. For this, AR previously bound to Ni^{+2}-NTA beads was incubated with tubulin, in absence or in presence of 3-NTyr as a positive and

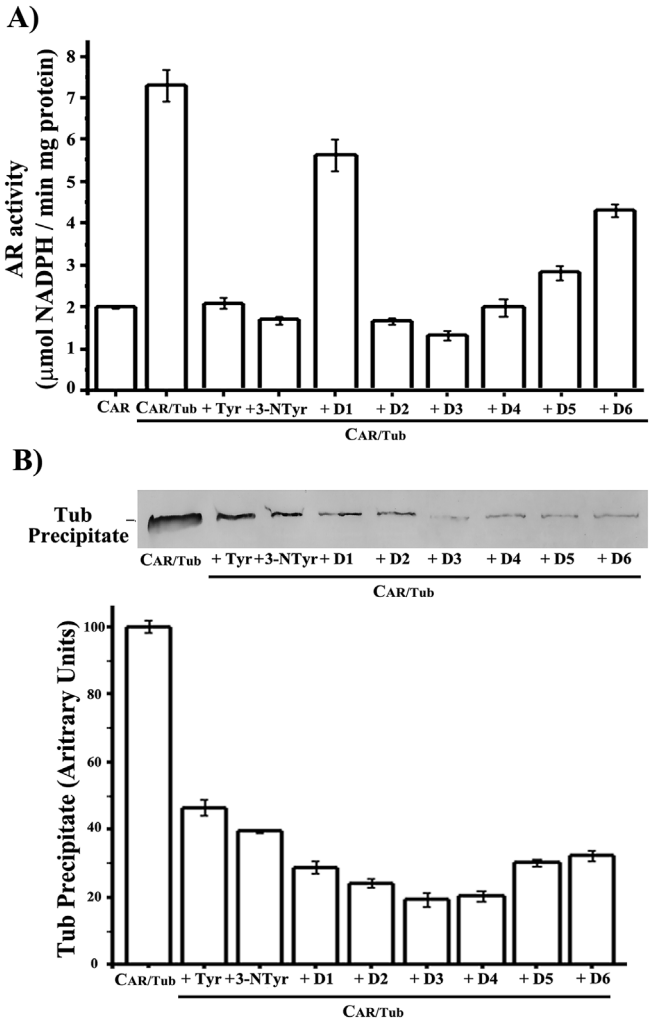


Fig. 4. Effect of phenolic acid derivates on tubulin/AR complex and stimulation of AR activity by tubulin. (A) AR (7 μg of prot.) plus taxol (5 μM) was incubated in absence (CAR, control AR activity) or presence (CAR/Tub, control AR activity and tubulina/AR association without phenolic acids compounds) of tubulin (100 μg of prot.). Where it is indicated in the figure, one of the six phenolic acids tested was added at 30 μM. Then, AR activity was determined as described in "Materials and Methods". (B) Ni^{+} 2-NTA beads linked to AR were incubated in presence (CAR/Tub) of tubulin with addition of 30 μM of the phenolic acid derivates indicated in the figure. Precipitated materials were analyzed by immunoblotting with mAb DM1A and Tub was quantified using Scion Image software. The values are mean ± SD from three independent experiments.

negative control of the association, respectively, and in the presence of the different phenolic acids (Fig. 4B). All the phenolic acids tested prevented the formation of the tubulin/AR complex, even more than Tyr and 3-NTyr. These results indicate that Tyr, 3-NTyr and phenolic acid derivates are able to prevent the formation of the tubulin/AR complex and the activation of AR by tubulin. Indeed, with all the compounds tested, tubulin/AR interaction was prevented and, to a lesser degree, so was the activation of AR by tubulin, although no strict proportionality between both was found.

4.4. Free phenolic-acid derived compounds inhibit microtubule formation induced by glucose in COS cells

We previously showed that in response to glucose, a vicious circle is established between tubulin polymerization and AR activation. This circle is blocked by preventing glucose-induced tubulin polymerization or AR activation [20]. Our hypothesis is that when AR interacts with tubulin, this induces AR activation and tubulin polymerization, which in turn is required for activation of AR, thus generating a vicious circle between these two metabolic pathways. Then, we reasoned that if we could prevent the formation of the tubulin/AR complex, tubulin polymerization would be lower. If phenolic acid derivaes prevent the formation of the tubulin/AR complex *in vitro,* they could also prevent the formation of this complex *in vivo* and therefore decrease the polymerization of tubulin to microtubules induced by glucose in the cells. To test our hypothesis, we determined the microtubule content in cells treated with phenolic acid derivates. COS cells were grown on coverslips in presence of phenolic acids derivates (Tyr, 3-NTyr and D1-D6 derivates) and with high glucose concentrations as inducers of tubulin polymerization. Then, tubulin content in the cytoskeleton was determined by indirect immunofluorescence (Fig. 5A). A simple visualization of the microtubule network of COS cells shows that microtubule mass increased in cells preincubated with glucose, but this effect was reverted if there were phenolic acid derivates in the medium. What is more, the microtubule content in some treatments seemed to be lower than in the control. On the other hand, Fig. 5 A shows that phenolic acid derivates decreased fluorescence intensity in the microtubule network, and that the structure was less organized, since fewer filaments were observed. To corroborate this result, we used western blot to determine tubulin content in the cytoskeletal fraction of COS cells (Fig. 5B). All compounds tested showed a decrease in tubulin content in the cytoskeleton, to a similar (Ntyr, D1, D5) or lesser extent (Tyr, D2, D3, D4, D4) than the control without glucose (compare CLG with the derived phenolic acids, Fig. 5B). These results suggest that phenolic acid derivates diminish glucose-induced tubulin polymerization, therefore preventing the formation of the tubulin/AR complex.

5. Discussion

This is the first report that shows that AR activity can be regulated *in vitro* by using phenolic acid derivates to prevent tubulin/AR interaction. Our findings indicate that: i-a complex between tubulin and AR is formed by direct interaction between both proteins, ii- AR activity increases ~6-fold when the enzyme interacts with tubulin and the complex becomes part of the microtubules, ii- 3-NTyr-tub is the preferred modification of tubulin for association with AR, and iv-free phenolic acid compounds prevent the formation of the tubulin/AR complex, and consequently also prevent AR activation.

AR activity is inhibited *in vitro* and *in vivo* by different natural and synthetic compounds of varied structure, all of which are referred to as ARI, aldose reductase inhibitors[29]. The causes of the activation of the enzyme are not very clear, although it has been proposed that nitration of Cys298 in the active site of AR, which increases in cells undergoing oxidative stress, is involved [33,34]. Ours is the first work that describes a mechanism through which AR is activated or inhibited by protein-protein interaction. The interaction between AR and tubulin occurs preferably when tubulin is 3-nitro-tyrosinated at the C-terminal end. These findings indicate that the presence of 3-NTyr at the C-terminal end of α-tubulin is important (perhaps essential) for the tubulin/AR interaction. However, such presence is not sufficient to activate the enzyme: the complex must be part of the microtubules for the enzyme to become activated. The nitro group of 3-NTyr incorporated into the C-terminal is involved in the interaction between tubulin and AR, but not in the activation of the enzyme. This means that there is no nitrosative process of AR involved in activating the enzyme, unless nitration occurs after the tubulin/AR complex becomes part of the microtubules. Another alternative is that once the tubulin/AR complex is established and the tubulin polymerizes, a structural modification occurs in AR and triggers its activation.

One aspect to note is that despite this apparent importance of the nitro group of 3-NTyr for the tubulin/AR interaction, Tyr is more efficient than 3-NTyr in abolishing the interaction between both proteins

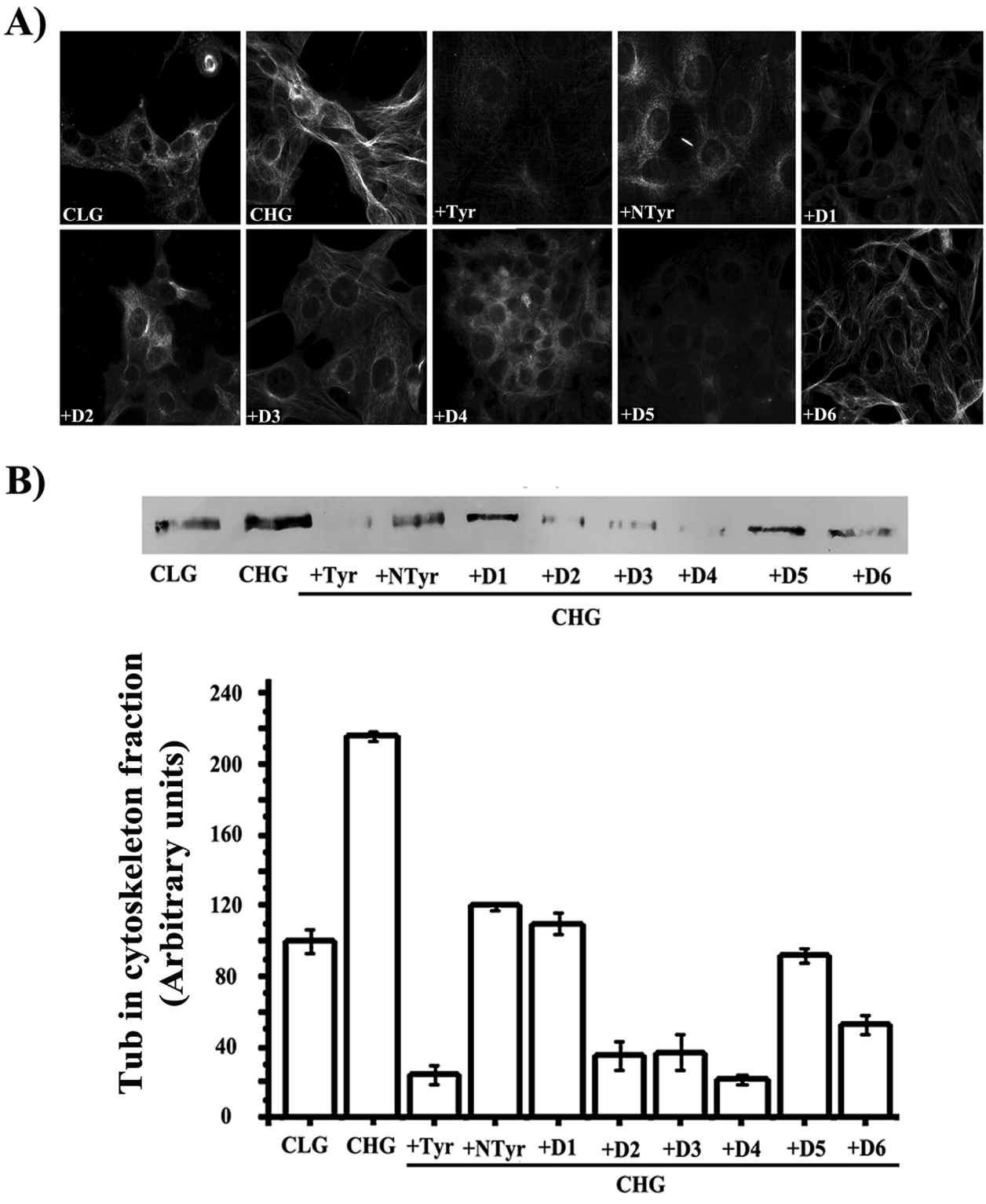


Fig. 5. Effect of phenolic acid derivates on tubulin polymerization induced by glucose in COS cells. Cells were incubated in glucose-free HEPES-FBS buffer at 37 °C for 2 h, added with high glucose (CHG, glucose 25 mM, control with high glucose concentration) or low glucose (CLG, glucose 5 mM, control with low glucose concentration) plus 300 μM of Tyr, NTyr, D1, D2, D3, D4, D5 or D6 and incubated for another 2 h. (A) Microtubules of the cells were visualized by immunofluorescence microscopy using mAb DM1A. (B) Cells were processed to isolate the cytoskeletal fractions as described in "Materials and Methods". Then, an aliquot of cytoskeletal fraction was subjected to SDS-PAGE for the determination of α-tubulin by immunoblotting with DM1A mAb (upper). Tubulin bands were quantified using Scion Image software, and values expressed as arbitrary units (lower). Results shown are mean ± SD from three independent experiments.

(Fig. 2). In addition, other acid phenolic compounds that do not possess nitro groups are also capable of abolishing the tubulin/AR interaction (Fig. 4). This suggests that AR prefers 3-nitrotyrosine tubulin to interact, but the presence of the nitrated phenolic ring is not essential to avoid the interaction between AR and tubulin. On the other hand, all phenolic acid derivatives blocked the tubulin/AR interaction but D1 and D6 were ineffective in preventing enzyme activation *in vitro* (Fig. 4). We think that this is due to an effect on the polymerization of

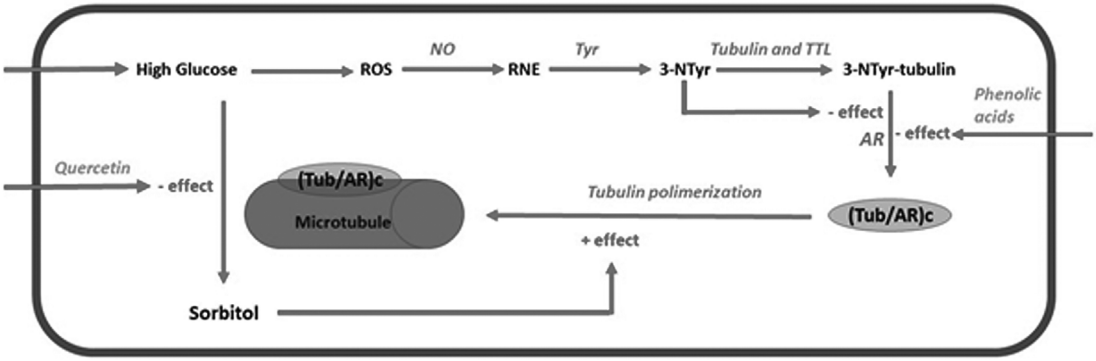


Fig. 6. Schematic representation of the activation mechanism of AR by tubulin. ROS: reactive oxygen species. RNE: reactive nitrogen species. NO: nitric oxide. (Tub/AR)c: tubulin/AR complex.

tubulin in the presence of AR and taxol. The interaction is determined under nonpolymerizing tubulin conditions, while the AR activity under taxol-induced tubulin polymerization conditions. Evidently, the induction of the polymerization of tubulin by taxol does not allow the blocking of the tubulin/AR interaction by D1 and D6, so that the enzyme is activated. In fact, D1 and D6 have similar effects to the other phenolic compounds in cells in culture (Fig. 5), where the polymerization of tubulin with taxol was not induced but with glucose. Although in this work we demonstrate a high preference for AR to bind tubulin 3-nitrotyrosinated and that this interaction can be blocked with phenolic acid derivatives, the mechanism of this new system of regulation of AR is not yet known in detail. In this moment we are conducting experiments to advance in this direction.

Because nitration of tyrosine is higher under conditions of oxidative stress [34], we suggest that the formation of the tubulin/AR complex could explain the activation of the enzyme in pathological conditions in which this stress is generated, such as diabetes and hypertension [35,36]. Although a nitration process is necessary to activate AR, we believe that it is not a direct nitration of the enzyme, but of the tyrosine in the C-terminal of tubulin. Tubulin is not an oxidant or nitrosative molecule, it simply interacts with AR through a domain not yet known. Once the tubulin/AR complex is formed, it has to be part of the microtubules for AR activation to take place [20] (Fig. 4). Our present results lead us to hypothesize that in pathologies where oxidative and nitrosative stress is produced, high Tyr nitration occurs and generates 3-Ntyr, which is then incorporated into the C-terminus of α-tubulin and forms 3-NTyr-tyrosinated tubulin (as Tyr does by tubulin tyrosine ligase [32]) or causes nitration of tyrosinated tubulin. The 3-NTyr-Tub formed under these conditions interacts with AR and triggers processes that lead to the activation of the enzyme and the production of microtubules, as summarized in Fig. 6. The resulting 3-NTyr-tub preferentially associates with AR, and when this complex is polymerized into the microtubules, the enzyme is activated. In turn, the increased sorbitol concentration generated by AR enhances tubulin polymerization [20]. A positive feedback loop is thus generated between enzyme activation and microtubule polymerization. This would explain the great AR activity in pathologies that include oxidative stress, such as diabetes, inflammatory processes and some types of tumor cells [28,37–40].

We previously demonstrated that the positive feedback loop between microtubule polymerization and AR activation can be partially blocked by the AR inhibitor quercetin [20]. Here, we show that this feedback circuit can be interrupted with drugs that prevent the association between tubulin and AR. Specifically, this paper shows that phenolic acids block the formation of the tubulin/AR complex and the activation of AR *in vitro* (Fig. 4). These compounds have been effective *in vitro* and in cultured cells. In fact, the formation of microtubules is induced in cultured cells subjected to high concentrations of glucose. This induction is blocked by quercetin [20], and similar results were found when COS cells were incubated with phenolic acid derivates (Fig. 5). These findings focus on derivates of phenolic acids as potential drugs that can reduce AR activity *in vivo* by blocking the tubulin/AR interaction.

The search for drugs capable of blocking the tubulin/AR interaction was based on the fact that the enzyme interacts preferentially with 3-nitro-tyrosinated tubulin. These drugs have a phenolic ring and an acid group in common (Fig. 3). If these drugs are able to block the tubulin/AR interaction, it is likely that they compete with the binding domain of both proteins, specifically with 3-Ntyr in the C-terminal end of α-tubulin. We think that phenolic acid compounds bind to the same AR domain that binds tubulin, thus blocking the interaction between AR and 3-Ntyr-Tub. This is because AR preferentially associates with tubulin that possesses a 3-NTyr residue in its C-terminal, so any structural molecule similar to 3-NTyr could potentially occupy the interactional domain of AR in order to join tubulin. All the compounds tested seem to fulfill this condition, which is a phenol group with a carboxylic acid. This does not rule out that other molecules may be able to block the interaction of both proteins: in fact, any molecule capable of blocking the tubulin/AR interaction will be able to reduce enzymatic activity. Since AR activation is one of the most important complications in diabetes, there is an intensive, ongoing search for new AR inhibitors suitable for human clinical therapy [41]. Our present findings provide the first clear evidence of a new mechanism for AR regulation, and present a series of phenolic acid compounds which are able to inhibit the tubulin/AR association, leading to reduced AR activity.

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