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Novel function of calreticulin: Characterization of calreticulin as a transacetylase-mediating protein acetylator independent of acetyl CoA using polyphenolic acetates*

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Abstract: Our earlier investigations culminated in the discovery of a unique membrane-bound enzyme in mammalian cells catalyzing the transfer of acetyl group from polyphenolic acetates (PAs) to certain functional proteins, resulting in the modulation of their activities. This enzyme was termed acetoxy drug:protein transacetylase (TAase) since it acted upon several classes of PAs. TAase was purified from rat liver microsomes to homogeneity and exhibited the molecular weight of 55 KDa. TAase-catalyzed protein acetylation by PAs was evidenced by the demonstration of immunoreactivity of the acetylated target protein such as nitric oxide synthase (NOS) with anti-acetyl lysine. The possible acetylation of human platelet NOS by PA as described above resulted in the enhancement of intracellular levels of nitric oxide (NO). PAs unlike the parent polyphenols were found to exhibit NO-related physiological effects. The N-terminal sequence was found to show 100 % homology with N-terminal sequence of mature calreticulin (CRT). The identity of TAase with CRT, an endoplasmic reticulum (ER) protein, was evidenced by the demonstration of the properties of CRT such as immunoreactivity with anti-calreticulin, binding to Ca^{2+} ions and being substrate for phosphorylation by protein kinase c (PKC), which are the hallmark characteristics of CRT. These observations for the first time convincingly attribute the transacetylase function to CRT, which possibly plays an important role in protein modification by way of carrying out acetylation of various enzymes through a biochemical mechanism independent of acetyl CoA.

Keywords: polyphenolic peracetates; calreticulin; nitric oxide synthase; biochemical mechanism; NADPH cytochrome P-450 reductase.

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INTRODUCTION

A large number of polyphenols are the phenolic derivatives of the oxygen-containing heterocyclics, such as coumarins, flavones, chromones, and xanthones. These compounds are widespread in the plant kingdom and are known to have a plethora of divergent biological activities, among them their antioxidant and radical scavenging effects have received much attention [1]. To our knowledge, polyphenolic peracetates (PAs), which are the acetyl derivatives of the parent polyphenols, have received little attention from the point of view of their biological activities. Aspirin-related compounds are the only group of phenolic acetates well known for their pharmacological action owing to the inhibition of cyclooxygenase protein by way of nonenzymatic acetylation leading to cessation of prostaglandin synthesis [2]. The enzymatic acetylation of proteins is known to involve the participation of specific acetyl transferases acting upon acetyl CoA in the transfer of acetyl group to the proteins; the familiar example being the histone acetyl transferase [3]. The other type of enzymatic acetylation of proteins in nature, but independent of acetyl CoA, was unknown until we discovered the novel enzyme acetoxy drug:protein transacetylase (TAase) from mammalian tissues and cells [4,5]. TAase was found to transfer the acetyl group from the model acetoxy drug 7,8-diacetoxy-4-methylcoumarin (DAMC) to the target protein glutathione-*S*-transferase (GST), resulting in the inhibition of activity of GST [5]. Later, several classes of PAs were found to elicit pharmacological effects in tune with their specificities to TAase, implicating the possible acetylation of the target protein. In this review, we describe the TAase-related biological actions of PAs and reveal the identity of TAase with the endoplasmic reticulum (ER) protein calreticulin (CRT).

RESULTS AND DISCUSSION

When DAMC was incubated with rat liver cytosol and liver microsomal TAase, it resulted in the time-dependent inhibition of GST activity [5]. The effect of DAMC was similar on liver microsomal cytochrome P-450 linked mixed function oxidases (MFOs) [6] while the incubation of DAMC with rat liver microsomes resulted in the profound irreversible activation of NADPH cytochrome c reductase [7]. The irreversible inhibition of GST [8] (rat liver cytosol) upon incubation with liver microsomes served as a method for the assay of TAase (Fig. 1) in that the extent of inhibition of GST represented the activity of TAase. Utilizing this elegant assay procedure, TAase was purified 40-fold (Table 1) from rat liver microsomes. The purity of TAase was further assessed by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. The molecular weight of TAase was found to be 55 KDa (Fig. 2). Furthermore, TAase was subjected to N-terminal amino acid sequence analysis. The sequence obtained when aligned with nonredundant Swiss-Prot Database Sequences revealed a perfect match with the N-terminal sequence of mature CRT. To confirm the identity of purified TAase, we employed a commercially available antibody against CRT that cross-reacted with TAase when an immunoblot analysis

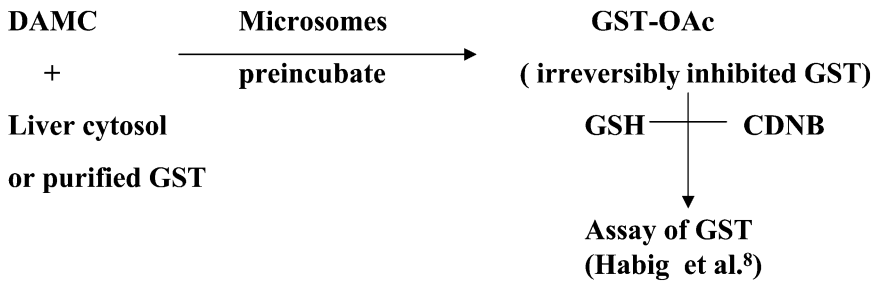

Fig. 1 Assay of TAase. Irreversible inactivation of GST by DAMC catalyzed by transacetylase offers a very convenient procedure for the assay of TAase in microsomes.

Table 1 Purification of rat liver TAase.

Step	Amount of protein (mg)	Total activity (units)	Specific activity units/mg	Fold purification	Percentage yield
Microsome	85	31 733	373	–	100 %
Solubilization	40	28 000	700	1.87	88 %
DEAE Sepharose	3	19 000	6 500	17.41	61 %
Q-Sepharose	1	14 600	14 600	39.11	46 %

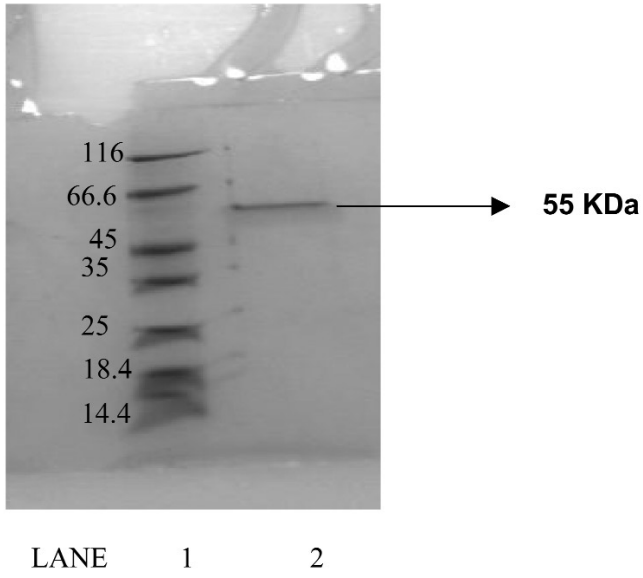

Fig. 2 SDS-PAGE of TAase. Lane 1: mol wt markers (kDa). Lane 2: purified TAase.

assay was performed (Fig. 3). Since this antibody was raised against the C-terminus of CRT peptide (DEEDATGQAKDEL), the presence of this sequence in TAase is convincingly implicated. An effort was made to probe whether the TAase exhibits the properties of CRT, a multifunctional protein [9]. We have established that TAase-like CRT is a calcium-binding protein (Fig. 4) as confirmed by its altered electrophoretic mobility [10]. Also, the addition of Ca^{2+} ions to the TAase assay mixture resulted in a drastic reduction of the TAase activity, and complete inhibition was observed at 3 μM concentration of Ca^{2+} ions (data not shown). These observations clearly point out that the active site of TAase is located in the calcium-binding domain of CRT. The purified TAase, like CRT, gets phosphorylated by protein

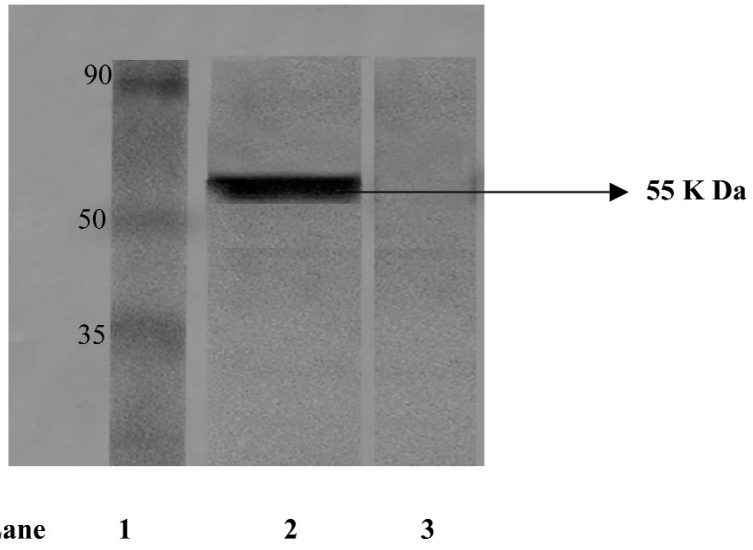

Fig. 3 Immunological identification of TAase using anti-calreticulin. Lane 1: mol wt markers. Lane 2: TAase immunoreacted with anti-calreticulin. Lane 3: BSA was blotted and treated in the same manner as TAase.

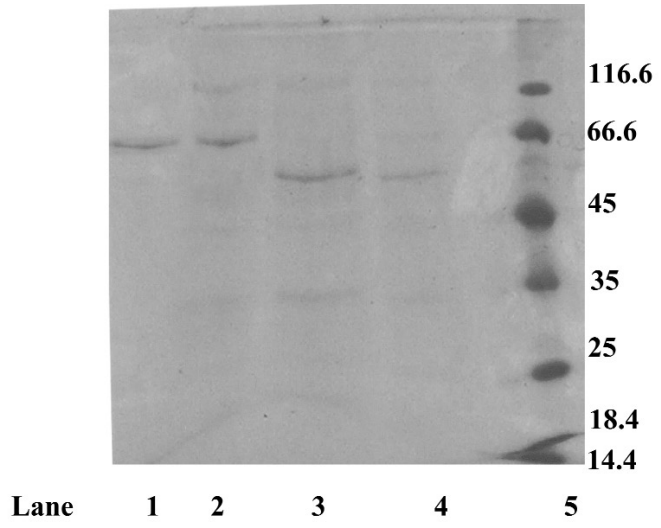

Fig. 4 Electrophoretic mobility shift on SDS-PAGE of proteins: Effect of Ca^{2+} ions (1 mM). Lane 1: BSA + 1 mM EGTA. Lane 2: BSA + Ca^{2+} ions. Lane 3: TAase + EGTA. Lane 4: TAase + Ca^{2+} ions. Lane 5: mol wt markers.

kinase c (PKC) (Fig. 5). The studies presented here emphasize that CRT possesses an important function in catalysis of the acetylation of functional proteins, thus showing the newer physiological roles of this unique protein. Our investigations presented here also describe CRT for the first time as an enzyme that mediates the protein acetylation independent of acetyl CoA. The studies reported here also purport the role of PAs as versatile donors of acetyl groups for the acetyl transferase function of CRT. The specificities of TAase for various PAs were in accordance with the quantitative–structure activity relationship (QSAR) that we had established for the microsomal bound TAase [11,12].

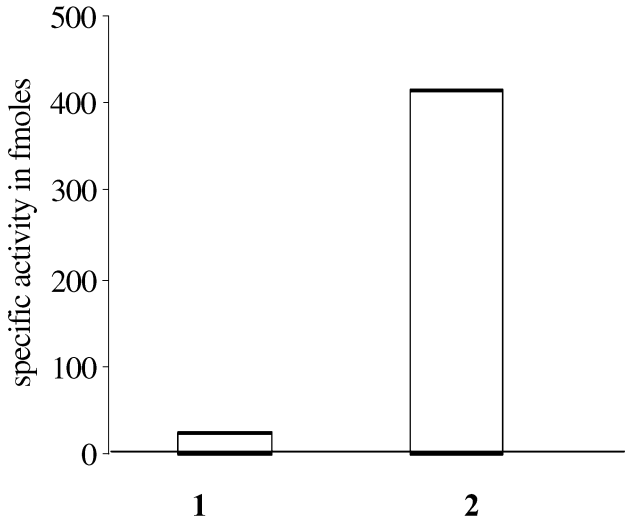

Fig. 5 PKC-catalyzed phosphorylation of TAase. 1: histone + ATP + $MgCl_2$ + PS + $CaCl_2$ + DTT + $\Upsilon P^{32}ATP$ (0.1 μCi). 2: TAase + ATP + $MgCl_2$ + PS + $CaCl_2$ + DTT + $\Upsilon P^{32}ATP$ (0.1 μCi). Lymphocyte lysate was used as the source of PKC. The values are mean of 3 experiments with variation less than 5 %.

BIOLOGICAL SIGNIFICANCE OF POLYPHENOLIC PERACETATES

As described above, the microsomal TAase catalyzes the activation of reductase by PAs. Nadler and Strobel [13] have reported the activation of the reductase upon nonenzymatic acetylation of the reductase protein by acetic anhydride. TAase-catalyzed acetylation of a target protein, namely, NADPH cytochrome P-450 reductase (referred to as reductase) by the model acetoxy drug, DAMC, was visualized by the western blot of the modified (acetylated) protein using anti-acetyl lysine (Fig. 6). These results established for the first time the enzymatic acetylation of reductase resulting in its activation. This con-

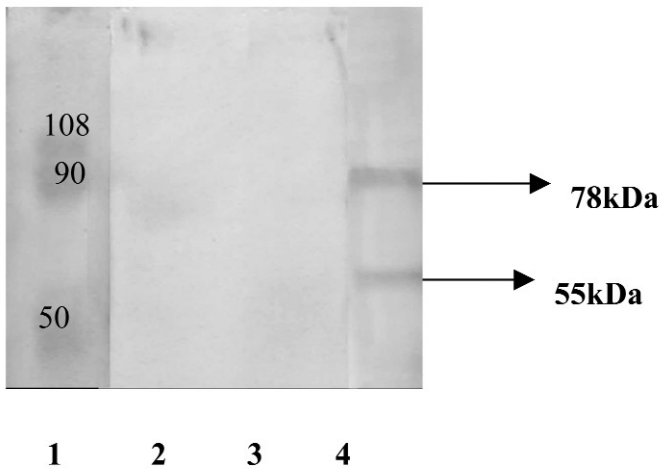

Fig. 6 Western blot using polyclonal antibody against acetyl lysine to demonstrate TAase-catalyzed acetylation of NADPH cytochrome P-450 (CYP) reductase by DAMC. Lane 1: mol wt markers (kDa). Lane 2: control (NADPH CYP reductase + DAMC). Lane 3: control (TAase + NADPH CYP reductase + DMSO). Lane 4: acetylated CYP (TAase + NADPH CYP reductase + DAMC).

cept of reductase activation by PAs was thought applicable to nitric oxide synthase (NOS) since NOS bears a domain of NADPH reductase. Efforts were made to examine whether PAs could activate NOS in cells such as blood platelets. Accordingly, PAs were indeed found to cause profound activation of NOS leading to enhanced NO levels in platelets (Fig. 7). Furthermore, it was thought interesting to find whether PAs could cause NO-related physiological effects. For this purpose, the effect of PAs on adenosine 5'-diphosphate (ADP)-induced platelet aggregation was investigated. The preincubation of platelet with PAs prevented effectively the ADP-induced platelet aggregation (Fig. 8), while parent polyphenols were found to be ineffective.

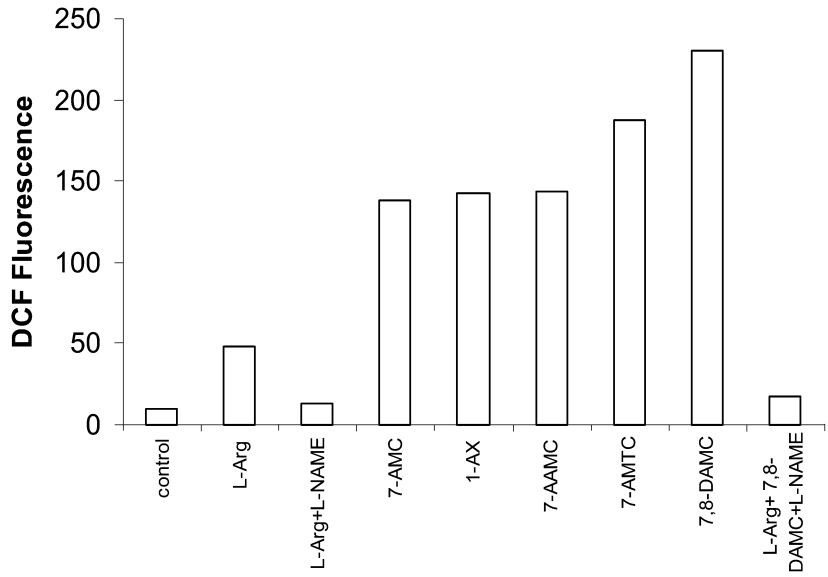

Fig. 7 Platelet was incubated separately with several classes of PA (100 μM) along with arginine, the DCF fluorescence due to the formation of NO was measured. Activation of human platelet NOS by PAs 7-AMC (7-acetoxy-4-methylcoumarin); 1-AX (1-acetoxy xanthone); 7-AAMC (7-acetoxy-3-acetyl-2-methyl chromone); 7-AMTC (7-acetoxy-4-methyl thiocoumarin); DAMC [14].

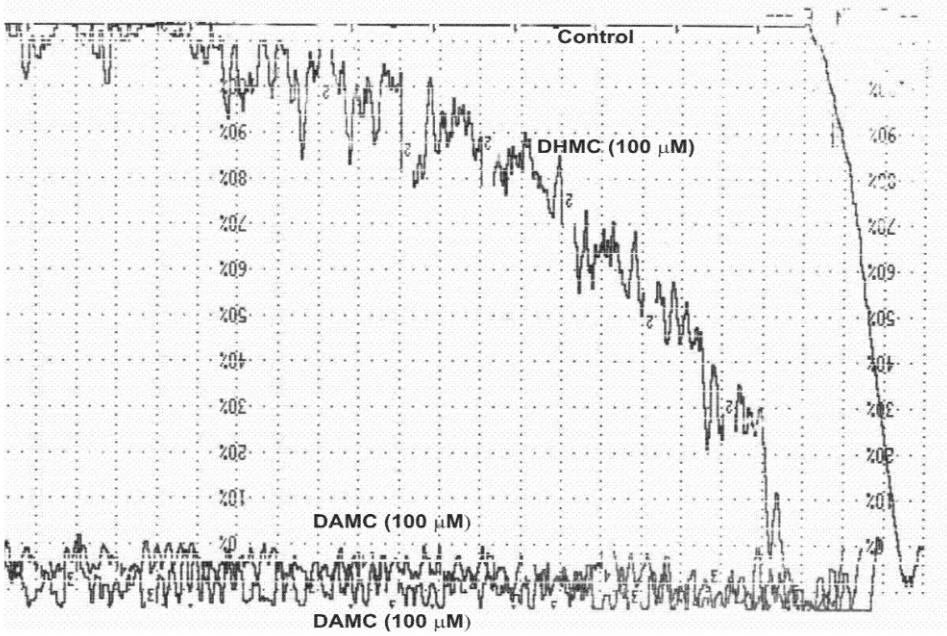

Fig. 8 Comparison of inhibition of ADP-induced platelet aggregation by DAMC and DHMC.

CONCLUSIONS

The investigations carried out in our laboratory unravelled the role of the enzyme TAase catalyzing the transfer of acetyl group(s) from PAs to certain functional proteins. TAase was purified and characterized from rat liver, and the protein showed 100 % homology with CRT, a major microsomal calcium binding protein of ER. The detailed studies on TAase-mediated NOS acetylation independent of acetyl CoA revealed its pharmacological significance. All these observations, for the first time, convincingly attribute the transacetylase function to CRT. Hence, this transacetylase function of CRT plays an important role in protein modification by way of acetylation independent of acetyl CoA.

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