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Acetoxy drug: Protein transacetylase catalyzed activation of human platelet nitric oxide synthase by polyphenolic peracetates

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Abstract—An enhanced intracellular level of Nitric oxide (NO) is essential to ameliorate several pathological conditions of heart and vasculature necessitating the activation of NOS. We have projected in this report the acetylation of eNOS by polyphenolic peracetates (PA) catalyzed by the novel enzyme acetoxy drug: protein transacetylase (TAase) discovered in our laboratory as an unambiguous way of activating NOS which results in the manifestation of physiological action. The human platelet was chosen as the experimental system in order to validate the aforementioned proposition. PA caused profound irreversible activation of platelet NADPH cytochrome *c* reductase mediated by TAase. The convincing biochemical evidences are presented to show that PA could cause acetylation of the reductase domain of NOS leading to the activation of eNOS in tune with their specificities to platelet TAase. As a result, the enhanced level of NO due to activation of platelet eNOS by PA was found to inhibit the ADP-induced platelet aggregation. The present studies highlight for the first time the role of PA as the novel potent agent for enhancing the intracellular NO levels.

1. Introduction

Accumulated evidences strongly suggest the presence of non-adrenergic, non-cholinergic (i-NANC) inhibitory nerves in many organ system and blood vessels. Nitric oxide (NO) is an established candidate for the mediation of i-NANC related physiological effects.[1] Therefore, there is considerable amount of interest in understanding the mechanisms involved in the activation of Nitric oxide synthase (NOS) leading to the alterations in the intracellular NO levels. Posttranslational modification of NOS, especially the phosphorylation of NOS involving various protein kinase(s), has received much attention.[2] Furthermore, the phosphorylation of NOS at different sites mediated by different protein kinases may evoke diverse responses. Phosphorylation of NOS, suggested to be one of the pathways for its activation, is beset with limitations. For example, phosphorylation of eNOS at Ser-1179 leads to activation, while phosphorylation of tyrosine leads to inhibition.[3,4] In order to overcome these complexities, we propose the acetylation of NOS by polyphenolic acetates (PA) as an effective approach to activate NOS thereby enhancing NO levels in human platelet. We have in this report demonstrated the role of acetoxy drug: protein transacetylase (TAase), a unique membrane-bound enzyme identified by us,[5,6] in mediating the possible transfer of acetyl groups from PA to platelet NOS resulting in the enhancement of NO levels in platelet. A positive correlation was found between the specificities of various

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PA for platelet TAase and the efficacy of the former to activate platelet eNOS leading to NO-mediated inhibition of platelet aggregation. The enhancement of intracellular NO level by this novel mechanism further strengthens the concept evolved by us of acetyl CoA independent enzymatic protein acetylation.[7,8]

2. Results

In the present investigations, the characterization of TAase catalyzed reaction (Fig. 1) in platelet was carried out by examining the specificity of various PA (Fig. 2) to platelet TAase. Further, the TAase mediated activation of NADPH cytochrome *c* reductase and NOS by PA, and the effects of PA on ADP-induced aggregation of platelet were also studied with specific reference to the role of TAase. The kinetic parameters of TAase presented in Table 1 demonstrate the specificities of various PA to TAase. The specificity of monoacetoxychromone (**4**) was found to be equivalent to that of monoacetoxycoumarin (**2**). It is evident from the data (Table 1) that for TAase, compound (**1**) exhibited the least K_m value followed in order by compound (**3**) < (**5**) < (**4**) ≈ (**2**). Results depicted in (Fig. 3) demonstrated that the TAase mediated inhibition of GST by compound (**1**) was nearly abolished when PHMB was included in the preincubation mixture.

2.1. TAase mediated activation of platelet NADPH cytochrome *c* reductase by PA

Platelet was preincubated with PA, followed by the addition of substrates of cytochrome *c* reductase, namely NADPH and cytochrome *c*, in order to assay the reductase activity. The results documented in (Fig. 4) indicated that the platelet reductase was remarkably activated by various PA in a manner similar to their specificities to TAase (Table 1). Accordingly, the PA caused the activation of platelet reductase in the following order: compound (**1**) > (**3**) > (**5**) > (**4**) ≈ (**2**) (Fig. 4).

2.2. RT-PCR

An attempt was made to examine the expression of NOS in human blood platelet. For this purpose, RNA was isolated from platelet (Fig. 5A) and RT-PCR was performed. The results (Fig. 5B) indicated the preponderance of eNOS and no iNOS under the present conditions.

2.3. TAase mediated activation of platelet NOS by PA

Platelets were incubated with L-arginine in order to measure the activity of NOS and a sensitive flow cytometric method was employed. The data presented are fluorescence intensities and the values are means of four measurements with an error less than 5%. It is evident that platelet when incubated with increasing concentrations of L-arginine led to enhanced activity of NOS (Fig. 6) as seen by DCFH oxidation resulting in the fluorescence due to DCF which is a measure of the NO levels. The platelet preincubated with PA and L-arginine exhibited a significantly higher level of NO as compared to that

Table 1. Substrate specificity for acetoxy drug protein: transacetylase

PA	K_m (μM)	V_{max} (units)
DAMC	1050	400
7-AMTC	1422	222
1-AX	1818	188
7-AAMC	1588	200
7-AMC	1612	190

The details are outlined under Section 4.1. Values are means of four experiments with variations less than 2%.

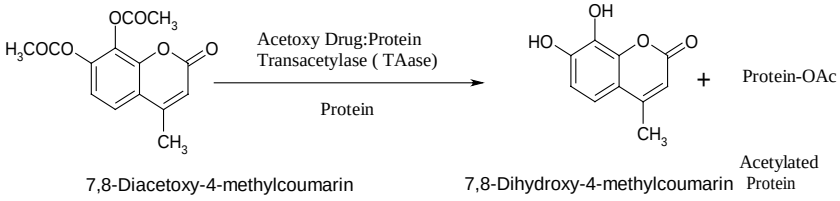

Figure 1. TAase catalyzed reaction.

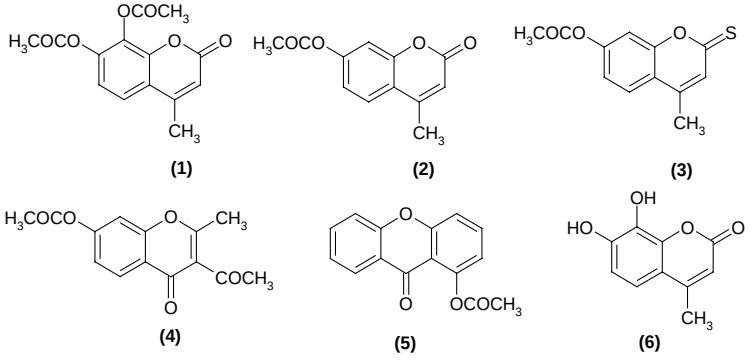

Figure 2. Structures of test compounds.

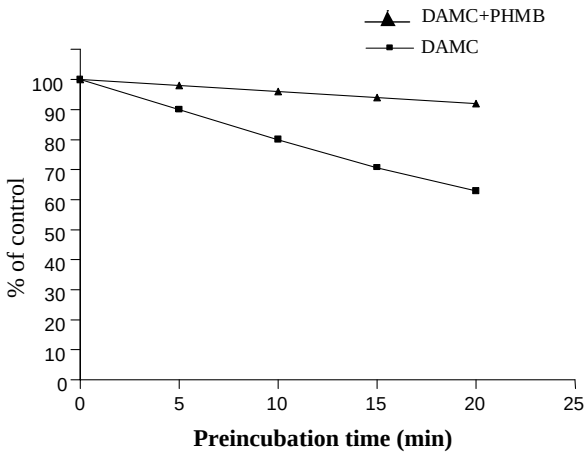

Figure 3. TAase activity in platelet: effect of PHMB. Platelet lysate(50 μg protein) was preincubated with DAMC (100 μM) along with cytosol (10–12 μg protein) for different periods and TAase was assayed as described under Section 4.1. The concentration of PHMB (where added) was 10^{-2} M. The values are means of three observations with variation less than 5%.

of L-arginine alone signifying the activation of platelet NOS (Fig. 6). It is clear from the data presented in Figure 7 that the preincubation of platelets with polyphenol like compound (**6**) other than PA itself failed to enhance the levels of NOS. Preincubation of platelets with L-arginine and L-NAME (an inhibitor of NOS) resulted in drastic reduction in the levels of NO compared to cells incubated with L-arginine alone, implying that the reactive species oxidizing DCFH to DCF under the conditions was indeed NO (Fig. 6). Similarly, the enhancement of platelet NO levels due to DAMC was also profoundly reduced when L-NAME was included in the reaction mixture (Fig. 6). Several classes of PA were also found to be effective in activating NOS (Fig. 8) in the following order: (**1**) > (**3**) > (**2**) ≈ (**4**) ≈ (**5**).

2.4. Demonstration of TAase catalyzed acetylation of eNOS by DAMC (Western blot)

The immunoblot shown in (Fig. 9) highlights the interaction of polyclonal anti-lysine with eNOS that was incubated with DAMC and purified rat liver TAase. In the controls, eNOS, when incubated in the absence of DAMC and TAase, failed to show any interaction with the primary antibody, showing that eNOS was not acetylated. These observations clearly document the TAase catalyzed acetylation of eNOS by DAMC.

2.5. TAase mediated inhibition of ADP-induced platelet aggregation by PA

ADP-induced platelet aggregation was significantly reduced by compound (**1**) at 100 μM, while (**6**) had very little effect under similar conditions (Fig. 9). Another acetoxycoumarin, 7-AMTC, was also found to be effective albeit to a lesser extent as compared to DAMC (Fig. 10). These results have clearly pointed out that PA is very effective in causing the inhibition of platelet aggregation in tune with their specificities to TAase.

2.6. Demonstration by confocal microscopy of enhanced NO production in platelet due to PA

The remarkable activation of NOS by compound (**1**) leading to an enhancement in the NO levels in the platelet is clearly shown in Figure 11. Under these conditions, platelet incubated with arginine and compound (**1**) exhibited green fluorescence significantly higher than the cells incubated with arginine alone (Figs. 12 and 13).

3. Discussion

The previous studies carried out in our laboratory demonstrated[5,19] the irreversible inhibition of several liver microsomal cytochrome P-450-linked mixed function oxidases (MFO) in vitro by a model PA, compound (**1**). DAMC-mediated irreversible inhibition of rat liver MFO activity was distinct from the action of the classical suicidal inhibitors of P-450 such as chloramphenicol, secobarbital, 21-chlorinated steroids, *N*-acetyl aminobenzotriazoles in that compound (**1**) needed no prior oxidative metabolism unlike the other inhibitors.[20–23] Compound (**6**), the deacetylated product of (**1**), failed to inhibit MFO and also the 7,8-diacetoxy-4-methylcoumarin mediated mechanism based inhibition of MFO was effectively abolished by thiol blockers such as PHMB and iodoacetamide.[19] These observations strongly suggested the existence of an enzyme in liver microsomes, which possibly transferred acetyl groups from (**1**) to cytochrome P-450 apoprotein leading to

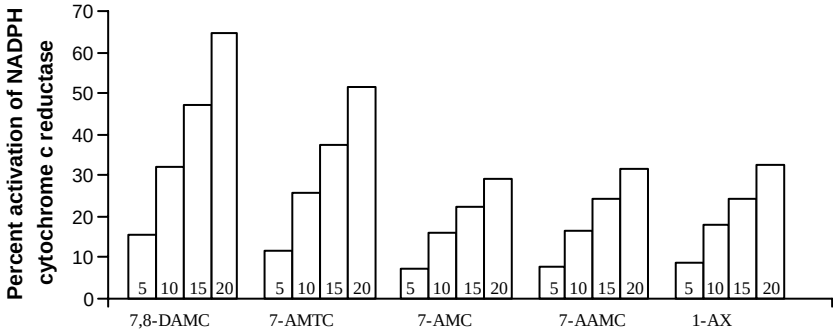

Figure 4. TAase catalyzed irreversible activation of NADPH cytochrome *c* reductase by PA. Preincubation time (min) indicated inside the bar. The control received DMSO in place of PA. The increment in the activity of NADPH cytochrome *c* reductase due to preincubation with PA over the control is expressed as percent activation. Concentration of the PA was 25 μM. Values are means of three observations with variation less than 2%.

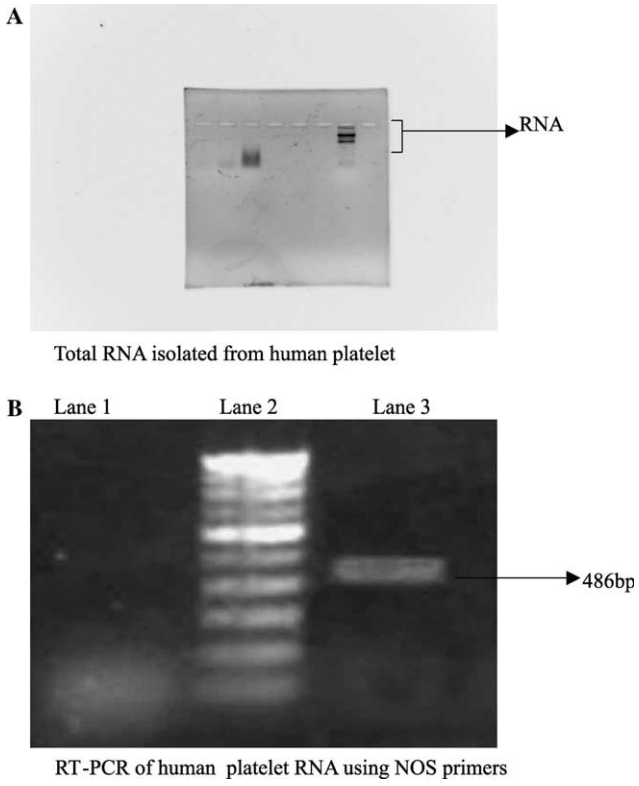

Figure 5. (A) Total RNA isolated from human platelet. (B) RT-PCR of human platelet RNA using NOS primers. Lane 1, RT-PCR using iNOS primers. Lane 2, DNA ladder. Lane 3, RT-PCR using eNOS primers.

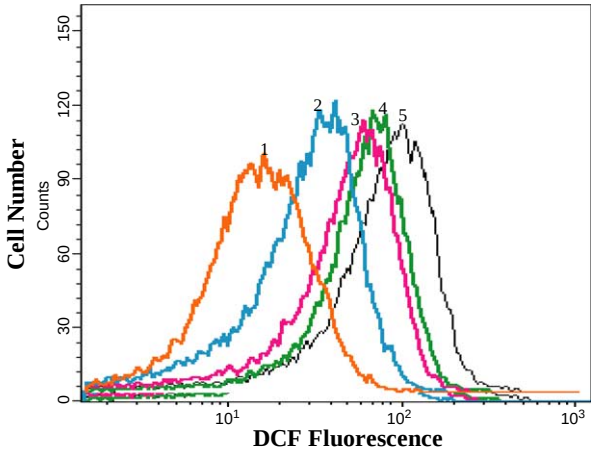

Figure 6. Effect of increasing concentration of L-Arg on human platelet NOS. Platelets were incubated with DCFH-DA for 30 min followed by the measurement of fluorescence due to intracellular. DCFH oxidation. Several concentrations of L-Arg were incubated separately with DCFH-DA and fluorescence was measured. The increment in fluorescence due to L-Arg represents the formation of NO. 1, control; 2, L-Arg (50 μM); 3, L-Arg (100 μM); 4, L-Arg (150 μM); 5, L-Arg (200 μM).

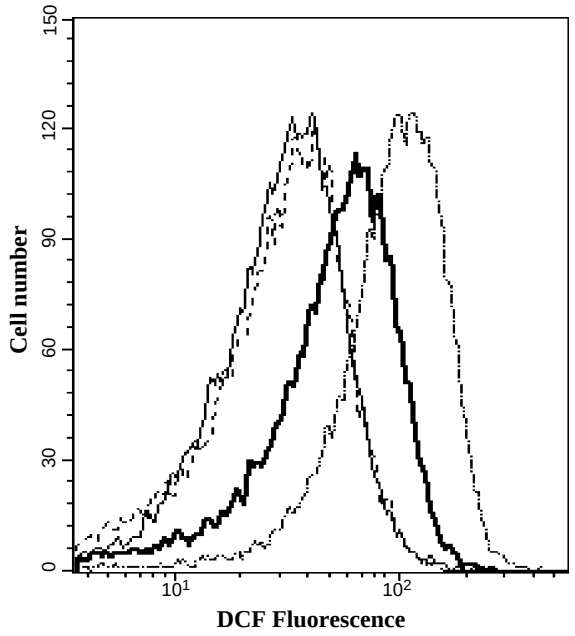

Figure 7. Activation of platelet eNOS by DAMC: effect of L-NAME. Details of NOS assay procedure given under Section 4.1. Solid light line, control; solid dark line, arginine; dotted and line, Arg + DAMC; dotted only, Arg + DAMC + L-NAME.

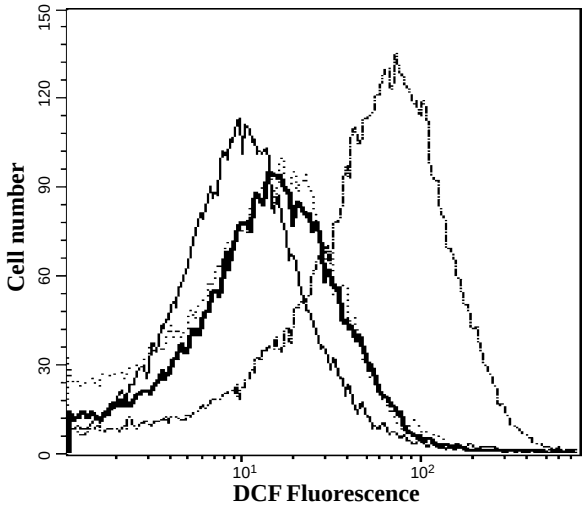

Figure 8. Effects of DHMC and DAMC on platelet eNOS. Solid light line, control; solid dark line, arginine; dot and line, Arg + DAMC; dotted only, Arg + HMC.

inhibition of P-450-catalyzed MFO reaction.[24,25] The enzyme was termed TAase in general, since apart from DAMC a large number of PA and related compounds were found to be acted upon by this enzyme. In the earlier study, an effort was made to examine whether any other protein in the P-450 cycle was modified by (**1**) catalyzed by microsomal TAase in a manner similar to P-450-linked MFO. Compound (**1**) contrary to its inhibitory action on hepatic P-450 caused kinetically discernible hyperbolic activation of liver microsomal NADPH cytochrome *c* reductase.[16] Compound (**1**) was also found to be competent in causing substantial activation of reductase of rats in vivo. Since the aforementioned work carried out in our laboratory firmly established the TAase catalyzed profound activation of NADPH cytochrome *c* reductase by PA, it was considered worthwhile to examine whether NOS which bears a domain of the reductase could similarly be activated leading to en-

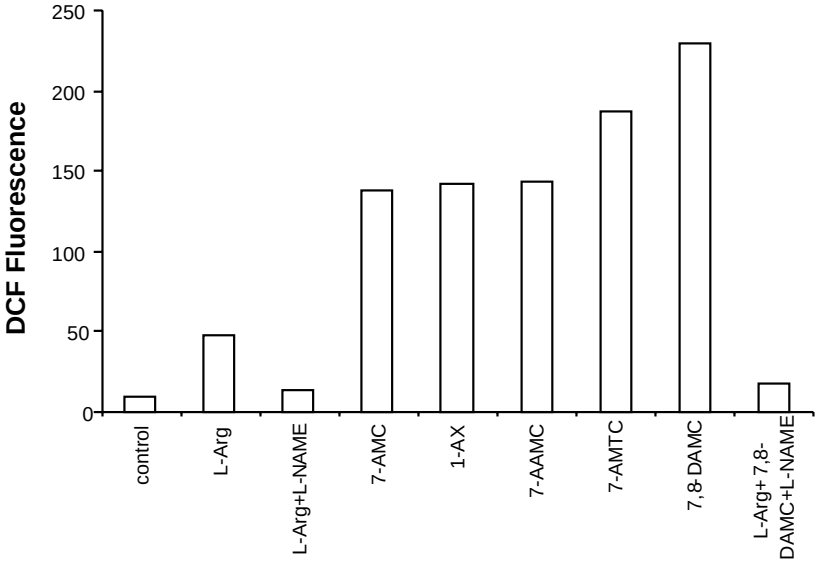

Figure 9. Platelet was incubated separately with several classes of PA (100 μM) along with arginine, the DCF fluorescence due to the formation of NO was measured. The details and abbreviations are given under Section 4.1.

Lane1: Control (eNOS+7,8-Diacetoxy-4-methyl coumarin)

Lane2: Control (TAase+eNOS+Dimethylsulfoxide)

Lane3: Acetylated eNOS (TAase+ eNOS+7,8-Diacetoxy-4-methylcoumarin)

Figure 10. Western blot using polyclonal anti-acetyl lysines. Lane 1, control (eNOS + 7,8-diacetoxy-4-methylcoumarin). Lane 2, control (TAase + eNOS + dimethylsulfoxide). Lane 3, acetylated eNOS (TAase + eNOS + 7,8-diacetoxy-4-methylcoumarin).

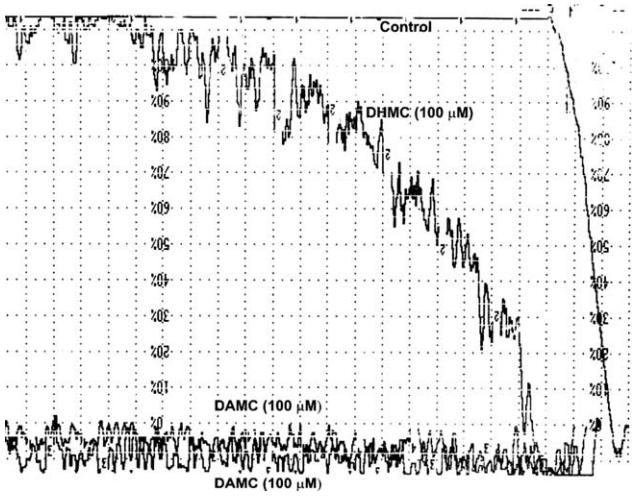

Figure 11. Comparison of inhibition of ADP-induced platelet aggregation by DAMC and DHMC. Details and abbreviations described under Section 4.1.

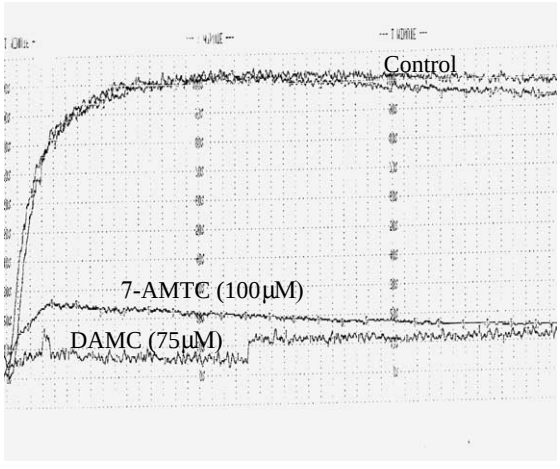

Figure 12. Inhibition of ADP-induced platelet aggregation by 7-AMTC and DAMC. (Details and abbreviations described under Section 4.1.)

hanced intracellular production of NO. In order to investigate this proposition, we chose human platelet as the suitable experimental system. Human platelet is recognized as a suitable model for investigation related to NO pathway.[26] As a first step, the presence of TAase in platelet was confirmed by the inhibition of cytosolic glutathione *S*-transferase (GST) activity by PA.[5] Human platelets were found to have considerable TAase activity and the specificities for various PA were in the

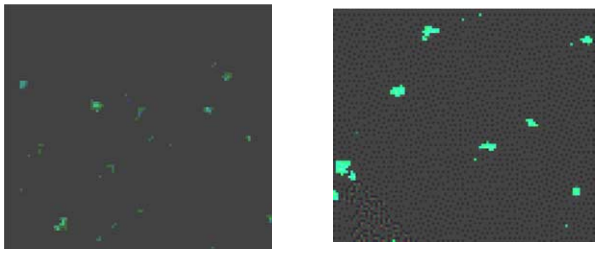

Figure 13. Demonstration by confocal microscopy of enhanced production of NO in platelets by compound (**1**) in comparison with compound (**6**), the deacetylated product which served as a control.

order (**1**) > (**3**) > (**5**) > (**4**) ≈ (**2**). The fact that the thiol-blocking agent PHMB blocked the TAase catalyzed inhibition of GST confirmed that the platelet TAase was a thiol sensitive enzyme as seen in the case of hepatic microsomal enzyme.[19] After having established the presence of TAase in platelet, an effort was made to examine the influence of Compound (**1**) and other PA on NADPH cytochrome *c* reductase. The results clearly indicated the time dependent activation of platelet reductase upon incubation with the test compounds. It was observed that the substrate (PA) that yielded high activity of platelet TAase (as measured by inhibition of GST) was equally effective in causing the activation of reductase (Fig. 4). After having convinced ourselves that PA activates the platelet reductase in the manner described above, we then proceeded to investigate the influence of PA on NOS of platelet. The result of the present study has very clearly established that platelet NOS is activated by the test compounds (PA) in tune with their specificities to platelet TAase. Accordingly, Compound (**1**) having the highest specificity for TAase activated NOS to the maximum extent. While the other PA showed varying degrees of capability to activate NOS in platelet. (**6**), the deacetylated product of (**1**), when incubated with platelet in the presence of L-arginine, failed to activate NOS, pointing the finger toward the acetyl groups of (**1**) in causing the profound activation of NOS. Our earlier work documented the time dependent inhibition of cytosolic GST by (**1**), catalyzed by partially purified TAase from buffalo liver. GST 3-3, when incubated with purified buffalo liver TAase and DAMC, was found to undergo acetylation at multiple sites as demonstrated by MALDI-TOF and LC/MS/MS studies.[7,8] Accordingly, N-terminal proline and 6-lysines: Lys-51, -82, -124, -181, -191, and -210 were found to be acetylated. It is thus logical to postulate, based on the aforementioned observations that PA catalyzed by TAase can as well acetylate the platelet eNOS. NADPH cytochrome P-450 reductase domain of platelet NOS is possibly the target for TAase catalyzed acetylation, as the activation of the former is now firmly established by our investigations.[16,24,25] There are many reports on the modification of NOS.[27] The phosphorylation of eNOS catalyzed by protein kinase Akt[3] is known to result in the activation of NOS activity. The enhanced electron flux owing to the phosphorylation of the reductase domain is ascribed to be the reason for enhanced activity of NOS. Nadler observed that the acetylation of purified reductase by acetic anhydride resulted in the activation of reductase activity.[28] Hence, it is plausible to infer that the TAase catalyzed acetylation of the reductase domain of platelet NOS could similarly account for the observed significant activation of NOS. Protein acetylation is often seen as a phenomenon, either independent or as adjunct to protein phosphorylation, that leads to the control of physiological events.[29] The multiple regulation of p53 tumor suppressor protein by phosphorylation and acetylation can be viewed in this context.[30,31] The fact of the matter is that PA cause significant activation of platelet eNOS (Fig. 3) catalyzed by TAase. The enhanced formation of NO in platelet leads to the increased production of cAMP, which causes degranulation resulting in the platelet aggregation. These studies purport for the first time a possible modification of NOS by way of enzymatic acetylation catalyzed by TAase resulting in the activation of platelet NOS. Such a conclusion was ratified by the observation that the incubation of purified eNOS with compound (**1**) and purified liver TAase resulted in the formation of the acetylated eNOS, which was found to avidly interact with the antibody against protein bearing acetylated lysines. These investigations have also projected for the first time PA as the novel potent activators of NOS that can lead to substantial release of NO as visualized by confocal microscopy (Fig. 11) leading to the inhibition of platelet aggregation. To our knowledge, no such xenobiotic acetates (PA) other than aspirin related compounds have been reported in the causation of significant physiological effects by way of acetylation of the target protein. Another remarkable distinction is that the action of PA is enzyme (TAase) mediated, while the effect of aspirin is solely non-enzymatic in nature. We have presented in this paper a unique mechanism for the enhancement of intracellular NO levels by PA, which can culminate in the expression of physiological effect, viz., inhibition of platelet aggregation.

4. Experimental

4.1. Materials and methods

4.1.1. Synthesis and characterization of polyphenolic peracetates (PA). 7,8-Dihydroxy-4-methylcoumarin (**6**), 7,8-diacetoxy-4-methylcoumarin (**1**), 7-acetoxy-4-methylcoumarin (**2**), 7-acetoxy-4-methylthiocoumarin (**3**), 1-acetoxyxanthone (**5**), and 7-acetoxy-3-acetyl-2-methylchromone (**4**) were synthesized as per the published procedures.[9–11] The *Indian Patent* has been filed for the compound (**1**) as the enhancer of intracellular NO levels.

4.1.2. Chemicals. Reduced glutathione (GSH), 1-Chloro-2, 4-dinitrobenzene (CDNB), NADPH, cytochrome *c*, dichlorofluorescin diacetate (DCFH-DA), *N*-nitro-L-arginine methyl ester (L-NAME), L-arginine and adenosine diphosphate (ADP), and anti-rabbit HRP conjugated secondary antibody were procured from Sigma Chemical, St. Louis, MO (USA). Sodium *p*-hydroxy mercuribenzoate (PHMB) was obtained from E. Merck (Germany). Iodoacetamide was a product of Lancaster Synthesis. Access Quick RT-PCR system was procured from Quigen. TRI-

zol was obtained from Invitrogen. DAB system was procured from Bangalore Genei, India. Anti-acetyl lysine primary antibody was obtained from Cell Signalling, USA. All other chemicals used were of high purity and were obtained from local suppliers.

4.1.3. Isolation of platelet-rich plasma (PRP). Nine milliliters of venous blood was collected from healthy human volunteers and was mixed with 1.0 ml of 3.8% trisodium citrate (anticoagulant). The citrated blood was centrifuged at 180*g* for 10 min at room temperature. The upper two-third fraction of plasma (PRP) was transferred to another centrifuge tube leaving behind lower one-third layer to avoid contamination with WBCs and RBCs. PRP was then further centrifuged at 4000*g* for 5 min to produce a platelet button. The platelet button was lysed using a hand homogenizer and protein content of the homogenate was determined by Lowry's method.[12] This homogenate was then accordingly used for the various spectrophotometric assays mentioned below.

4.1.4. Purification of rat liver TAase. TAase was purified from rat liver following the published procedure[8] for demonstrating TAase catalyzed acetylation of eNOS by (**1**).

4.1.5. RNA isolation and RT-PCR of human platelet NOS. 4.1.5.1. (a) RNA isolation. Total RNA was isolated from human platelet by the TRIzol method of Zhang et al.[13] Total RNA was purified using chloroform and precipitated by the addition of isopropanol. Pellet obtained was washed twice with 75% chilled ethanol and stored at −80 °C. The RNA was redissolved in 30 μl RNAase free water and used for RT-PCR.

4.1.5.2. (b) RT-PCR. RT-PCR was carried out using total RNA extracted from blood platelet using Gradient PCR Bio-Rad I cycler thermal cycler.

For eNOS the primers used were:

FP: 5′ CAG TGT CCA ACA TGC TGC TGG AAA TTG-3′
RP: 5′ TAA AGG TCT TCT TCC TGG TGA TGC C 3′

RT-PCR was carried out in a total volume of 50 μl containing 1× Access quick master mix, 1 μM forward primer, 1 μM reverse primer, 2 μg RNA, and 5 U AMV reverse transcriptase. The reaction mixture was incubated at 48 °C for 45 min followed by PCR cycling. The conditions for amplification were: an initial denaturation step at 94 °C for 5 min followed by 36 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and extension of 1 min at 72 °C. The final cycle was followed by strand extension at 72 °C for 5 min.

For iNOS primers used were:

FP: 5′ TGG AAT TCA CTC AGC TGT GC 3′
RP: 5′ GAT GTA GCG CTG GAG G 3′

All conditions for iNOS amplification were similar to those for eNOS (as mentioned above) except that the annealing temperature was 57 °C. The amplified products were analyzed by electrophoresis using 1% agarose gel and visualized by ethidium bromide staining.

4.1.6. Glutathione *S*-transferase (GST) assay. The method of Habig et al[14] was followed using GSH and CDNB as the substrates and the assay was carried out in 1.0 ml spectrophotometric cuvette (1 cm light path). Briefly, the reaction mixture consisting of 0.25 M phosphate buffer (pH 6.5), rat liver cytosol (10–12 μg protein), 1 mM CDNB (added in 50 μl ethanol), and 1 mM GSH was mixed and progress of the reaction was followed by monitoring the absorbance at 340 nm using a Cary Spectrophotometer (Cary Bio 100). The kinetic software was used to ensure that the analysis was restricted to the linear range with respect to incubation time and enzyme concentration.

4.1.7. Assay of platelet TAase. TAase in platelet was assayed using PA as the first substrate and cytosolic GST as the second substrate as described earlier.[5] The assay mixture in a total volume of 0.8 ml consisted of 0.25 M phosphate buffer (pH 6.5), platelet lysate (50 μg protein), cytosol (10–12 μg protein), and PA (50 μM in 50 μl DMSO), and preincubated for 10 min, followed by the addition of GSH and CDNB for the assay of GST as described above. The unit of TAase catalytic activity was expressed in terms of % inhibition of GST under the conditions of the assay.

4.1.8. Measurement of kinetic parameters of platelet TAase. Platelet lysate (50 μg protein) was mixed separately with varying concentrations of DAMC (25–400 μM, in 50 μl DMSO), 0.25 M phosphate buffer (pH 6.5), and cytosol (10–12 μg) in a volume of 0.8 ml, and preincubated for 10 min followed by the addition of GSH and CDNB in order to assay GST as described above. The % inhibition of GST is plotted against the DAMC concentration in order to calculate the kinetic constants.

4.1.9. Effect of PHMB on platelet TAase activity. PHMB at a concentration of 10^{-2} M was included in the preincubation reaction mixture and the assay of TAase was carried out as described above.

4.1.10. TAase catalyzed modulation of NADPH cytochrome *c* reductase by PA. For the reductase assay,[15] the platelet lysate (50 μg protein) was mixed with PA (25 μM), 0.05 M phosphate buffer (pH 7.7), and water to make 0.5 ml volume. The contents were preincubated at 37 °C for 10 min. The aliquots were removed periodically into a spectrophotometer cuvette containing 0.1 mM EDTA, 36 mM cytochrome *c*, and 1 mM NADPH in a total volume of 1 ml. The progress of the reaction was monitored by following the absorption at 550 nm. In the control, PA was replaced by DMSO. The increment in reductase activity due to PA over the control was expressed as percent activation.[16]

4.1.11. Assay of NOS by flow cytometry. The method outlined by Imrich and Koobzik[17] was followed for the assay of NOS by flow cytometry. Measurements were made with a 488 nm laser based flow cytometer (FACScalibur, Becton Dickinson, USA) and data (light

scatter and green fluorescence) were acquired using the Cell Quest software (Becton Dickinson, USA). Analysis was performed by applying appropriate gates with reference to the autofluorescence measured under similar conditions. Platelet isolated as described earlier was suspended in PBS and platelet counts were adjusted to 10^6/ml using electronic particle counter (SYSMEX, Model No. FA20). DCFH-DA (20 μM) and L-arginine at various concentrations were then added to the cell suspension and preincubated for 37 °C for 30 min while rotating (10 rpm) to prevent adherence during assay. To stop the assay, samples were placed on ice for 10 min in dark. Relative green DCF fluorescence was measured. Similarly, various PA (**1**–**5**) were preincubated separately with L-arginine and their relative fluorescence was measured thereafter.

4.1.12. Demonstration of TAase catalyzed acetylation of NOS by 7,8-diacetoxy-4-methylcoumarin. Anti-acetyl lysine as primary antibody was used to show the acetylation of NOS. For this purpose purified TAase (50 μg) was incubated with eNOS (50 μg), compound (**1**) (200 μM), and 10 mM phosphate buffer (pH 7.2), and incubated for 30 min at 37 °C. After completion of the reaction, sample buffer (loading dye) was added to the reaction mixture to stop the reaction. This reaction mixture was used to detect the acetylated NOS by Western blot.

For Western blot, electrophoretically separated proteins were transferred onto nitrocellulose membrane at 300 mA for 3 h at 4 °C. Non-specific sites on the nitrocellulose sheet were blocked with 5% blocking buffer. Primary antibody dilution (1:1000) was prepared in TBST containing 1% BSA and incubated overnight at 4 °C. The nitrocellulose membrane was extensively washed with TBST (TBS with 0.05% Tween). Goat anti-rabbit–HRPO (horseradish peroxidase)-conjugated secondary antibody, appropriately diluted (1:1000) in TBST, was then added and an incubation of 1 h at room temperature was carried out. The membrane was washed extensively and antibody-reactive moieties were visualized with DAB (diaminobenzidine) system.

4.1.13. Aggregation studies. PRP was prepared by the above-mentioned technique. Platelet count was adjusted to 250,000/μl with homologous platelet-poor plasma (PPP). PPP was prepared by centrifugation of the remainder of blood at 2500*g* for 10 min. Platelet counts (PC) were adjusted according to the following formula:

$$\text{PC (PRP)} \times \text{ml PRP} \div 250,000 = \text{ml PRP}(250,000)$$

Various PA (in appropriate concentrations) were preincubated with PRP to make the final volume of 0.18 ml. Platelet aggregation was induced by the addition of 5 μM ADP. Platelet aggregation studies were performed using BIO-DATA Corporation, Platelet Aggregation Profiler, Model No. PAP-4. Platelet aggregation was expressed as the maximum percentage of light transmittance change (% max) from the baseline at the end of the recording time, using PPP as a reference. Platelet aggregation curves were recorded for 6 min and analyzed according to internationally established standards.[18]

4.1.14. Confocal microscopy. To 1 ml of PRP, 20 μl compound (**1**) (100 μM), 20 μl arginine (50 μM), and 10 μl DCFH-DA (20 μM) were added. The contents were incubated for 30 min at 37 °C and centrifuged at 1000*g* for 15 min at 4 °C. The pellet obtained was washed and finally suspended in 0.5 ml PBS. Hundred microliters of the cell suspension was placed on microscopic slide with coverslip for observation under the confocal microscope (Model LSM 510, Carl, Zeis, Germany). The images were visualized using 40× oil objective and the green DCF fluorescence was observed following excitation at 488 nm from argon laser with the help of long pass filter LP 505. The fluorescence was confirmed from intensity profile measurement with the built in software provided by the manufacturer.

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