Calreticulin Transacetylase Catalyzed Activation of Rat Tracheal Smooth Muscle Cell Nitric Oxide Synthase by Acetoxycoumarins

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The Transacetylase function of Calreticulin (CR) catalyzing the transfer of acetyl groups from acetoxycoumarins (AC) to certain proteins was identified for the first time in our laboratory. Protein acetyltransferase action of CR was termed Calreticulin Transacetylase (CRTAase). In the present work, CRTAase of rat tracheal smooth muscle cells (TSMC) was characterized with respect to the specificity for various AC and its role in the activation of nitric oxide synthase (NOS). 7,8-Diacetoxy-4-methylcoumarin (DAMC), a model AC, when incubated with TSMC along with L-arginine caused profound activation of NOS as compared to that with L-arginine alone. Further, the inclusion of *N*-ω-nitro-L-arginine methyl ester (L-NAME) along with DAMC resulted in the reduction of NO levels of TSMC to that of control, there by confirming the activation of TSMC NOS. Also, several AC were found to activate TSMC NOS in tune with their specificities to CRTAase. The results presented in this paper bear evidence for the activation of TSMC NOS by AC and their effectiveness to enhance NO of airway cells may be expected to find useful applications in respiratory diseases.

Key words calreticulin transacetylase; nitric oxide synthase; acetoxycoumarin

Our laboratory documented progressive developments in our understanding of the unknown biological action of several classes of acetoxycoumarins (AC). These studies could be considered as significant from the point of view of unraveling the biological activity of AC for the first time when there was explosion of knowledge on the parent polyphenols alone.[1)] Firstly, 7,8-diacetoxy-4-methylcoumarin (DAMC) was found to interact with free radical resulting in the removal of acetyl group as the acetyl radical (CH_3CO^+) and the formation of phenoxyl radical as confirmed by pulse radiolysis.[2)] This finding accounted for the antioxidant action of AC independent of the formation of parent polyphenol 7,8-dihydroxy-4-methylcoumarin (DHMC). The observation that microsomes acted upon AC and caused the irreversible inhibition of several enzymes such as cytochrome P-450-linked mixed function oxidases (MFO) and the activation of nicotinamide adenine dinucleotide reduced (NADPH) cytochrome *c* reductase, prompted us to investigate the mode of action of AC.[3)] Further, detailed studies revealed the role of a unique enzyme (Fig. 1) catalyzing the transfer of acetyl group from AC to receptor proteins.[4,5)] This enzyme was termed Acetoxy Drug: Protein Transacetylase (TAase), since the acetoxy derivatives of several classes of polyphenols were found to be substrates for TAase.[5—8)] TAase from microsomes of rat and bovine liver as well as from human placenta were purified to homogeneity and its identity with the endoplasmic reticulum luminal protein Calreticulin (CR) was established and TAase was termed Calreticulin Transacetylase (CRTAase).[9,10)] In our earlier work, we have focused our attention on the role of CRTAase mediated acetylation of certain protein targets by acetoxycoumarins in the blood lymphocytes of asthmatic patients. We have demonstrated the irreversible inhibition of human blood lymphocyte Protein Kinase C (PKC) by way of acetylation. These studies revealed that several AC inhibited PKC of asthmatic patients.[11)] Several respiratory diseases such as coronary obstructive pulmonary diseases (COPD) and asthma are associated with hyperplasia and hypertrophy of airway smooth muscle. NO is strongly implicated in the amelioration of pathologies associated with the proliferation of airway smooth muscle cells.[12)] Hence, it was thought interesting to investigate the influence of AC on tracheal smooth muscle cell (TSMC) NOS, with a view to highlight the possible application of AC in the management of airway diseases.

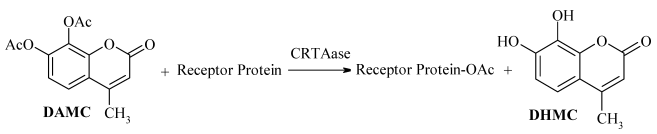

Fig. 1. CRTAase Catalyzed Reaction

Receptor proteins: glutathione-*S*-transferase, NADPH cytochrome *c* reductase and nitric oxide synthase have been shown to be the substrate for CRTAase catalyzed reaction. DAMC: A model AC (Raj *et al.*[4,5)] Khurana *et al.*[15)]).

RESULTS

Characterization of CRTAase in TSMC AC was separately preincubated with TSMC lysate for 10 min at 37 °C

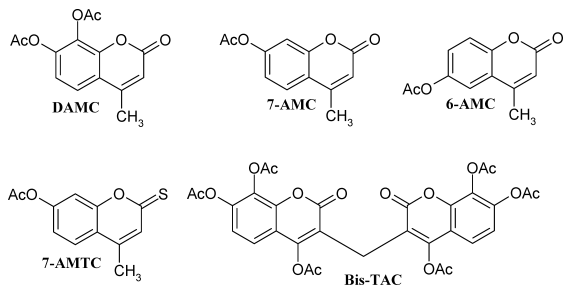

Fig. 2. Structure of Various Acetoxycoumarins

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followed by the addition of substrate of glutathione *S*-transferase (GST) *i.e.* glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) under condition of the assay. Inhibition of GST by AC was considered proportional to CRTAase activity in TSMC. The K_m and V_{max} values of CRTAase of TSMC for several AC are listed in Table 1. CRTAase exhibited lowest K_m for DAMC and the highest for 6-acetoxy-4-methylcoumarin (6-AMC).

CRTAase Mediated Biological Effects Activation of NADPH cytochrome c reductase and NOS by AC were considered as CRTAase catalyzed biological effects.

a) Activation of NADPH Cytochrome c Reductase: The irreversible activation of TSMC NADPH cytochrome c reductase by AC catalyzed by CRTAase is depicted in Fig. 3. The AC were separately preincubated with the cell free extract of TSMC followed by the addition of EDTA, Cytochrome c and NADPH for the assay of the reductase resulted in profound activation of TSMC reductase. 6-AMC which is a poor substrate for CRTAase could activate reductase to the least extent compared to others (Fig. 3).

Table 1. Specificity of Tracheal Smooth Muscle Cells CRTAase to Various Acetoxycoumarins

Test compound	K_m (μM)	V_{max} (units)
DAMC	1077	355
7-AMC	1602	198
6-AMC	1988	152
7-AMTC	1286	298
Bis-TAC	1476	220

Units of CRTAase were expressed in terms of percent inhibition of glutathione *S*-transferase under condition of the assay.

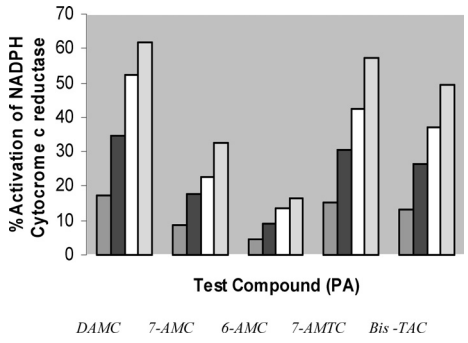

Fig. 3. TSMC CRTAase Catalyzed Activation of NADPH Cytochrome *c* Reductase by AC

Test compound was separately preincubated for different periods of time (5, 10, 15, 20 min) followed by the addition of the substrates, NADPH and cytochrome *c* for the assay of NADPH cytochrome *c* reductase.[23] Values are an average of four observations. In the control samples, DMSO replaced test compound. The increment in reductase activity due to test compound over the control was expressed as percent activation. Concentration of the test compound was 100 μM.

b) CRTAase Catalyzed Activation of TSMC NOS by DAMC: The Effect of *N*-ω-nitro-L-arginine methyl ester (L-NAME): The activity of NOS in TSMC was measured by incubation of TSMC with L-arginine and the fluorochrome dichloro fluoresein-diacelate (DCFH-DA) followed by the flowcytometric measurement of DCF fluorescence which was proportional to the extent of NO formation. The pre-incubation of DAMC with L-arginine resulted in the enhanced formation of NO as compared to with L-arginine alone. The results shown in Fig. 4 reveal that DAMC at concentration of 100 μM when pre-incubated with TSMC was found to enhance NO level by nearly two folds compared to DAMC at 50 μM. The inclusion of an inhibitor of NOS *i.e.*, L-NAME in the pre-incubation reaction mixture caused reduction of NO level to that observed in the case of control (Fig. 4).

Specificities of CRTAase to Various AC for the Activation of NOS Studies were extended to several other AC with a view to examine their influence on rat TSMC. The monoacetoxycoumarin (7-AMC) under the aforementioned conditions was 50% effective compared to the diacetoxycoumarin (DAMC) in the activation of NOS. 6-AMC was least effective in causing the activation of NOS in TSMC. On the contrary 7-acetoxy-4-methylthiocoumarin (7-AMTC)

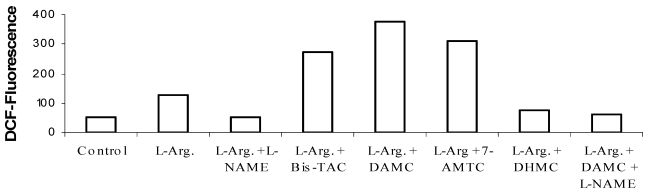

Fig. 5. The Effect of Various Acetoxycoumarins and L-NAME on the CRTAase Catalyzed Activation of TSMC Nitric Oxide Synthase

Platelets were incubated separately with various acetoxycoumarins (100 μM) along with arginine, the DCF fluorescence due to the formation of NO was measured. Values are average of four observations with variations <5%.

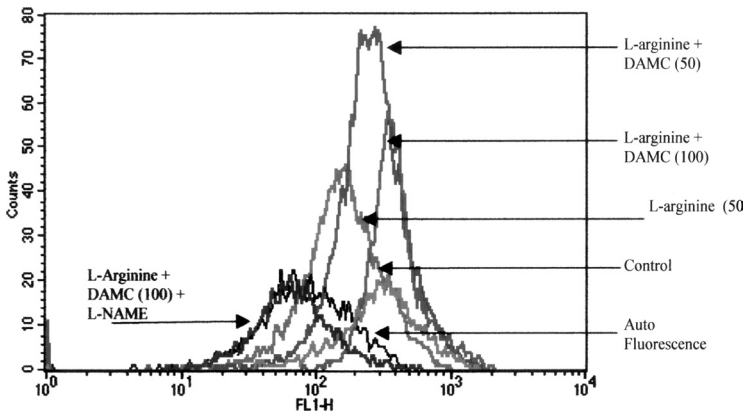

Fig. 4. Rat TSMC CRTAase Catalyzed Activation of NOS by DAMC: the Effect of L-NAME

Rat TSMC were preincubated with DAMC along with arginine for the measurement of NO levels catalyzed by CRTAase (Fig. 5). The effect of various acetoxycoumarins and L-NAME on the CRTAase catalyzed activation of TSMC nitric oxide synthase. Platelets were incubated separately with various acetoxycoumarins (100 μM) along with arginine, the DCF fluorescence due to the formation of NO was measured. Values are average of four observations with variations <5%.

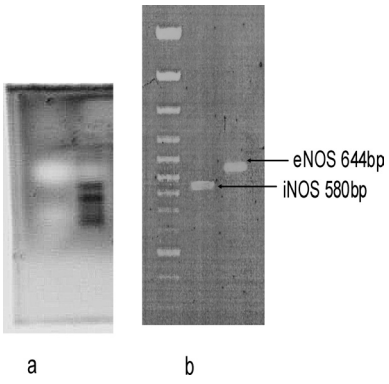

Fig. 6. (a) RNA Isolated from TSMC and (b) Expression of eNOS and iNOS

was found more superior as compared to 7-AMC in enhancing the levels of NO in TSMC. The extent of activation of NOS by 7-AMTC a monoacetoxycoumarin (Fig. 5) was found comparable to that caused by DAMC a diacetoxycoumarin. The results shown in Fig. 5 reveal that 4,7,8-triacetoxy-3′,3′-biscoumarin (Bis-TAC) was effective in enhancing the NO levels in rat TSMC but to a much lesser extent compared to DAMC. 7,8-Dihydroxy-4-methylcoumarin (DHMC) unlike DAMC failed to enhance NO levels in TSMC.

Identification of Isoform of NOS by RT-PCR An attempt was made to examine the expression of NOS in rat TSMC. For this purpose, RNA was isolated from rat TSMC (Fig. 6a) and RT-PCR was performed. Results shown in (Fig. 6b) indicate the activation of TSMC iNOS and eNOS by DAMC.

DISCUSSION

Protein acetylation is known to bring about alteration in several functions of the protein such as catalytic activity, flexibility, DNA binding, protein interaction and peptide receptor recognition. A wide range of acetyl transferases catalyze the transfer of acetyl group from acetyl CoA to largely ε-amino group of lysine residues located at different position. The enzymatic acetylation of protein independent of acetyl CoA was established for the first time by our identification of a unique enzyme termed Acetoxy Drug: Protein Transacetylase (TAase) in the endoplasmic reticulum of mammalian tissues using several classes of polyphenolic acetates as the donors of acetyl group.[5—8] The extensive investigation carried out in our laboratory deciphered the identity of TAase with Calreticulin (CR), a resident protein of endoplasmic reticulum. Consequently TAase was given the name Calreticulin transacetylase (CRTAase). We provided the mass spectrometric evidence[13,14] for the acetylation of glutathione *S*-transferase by DAMC, mediated by TAase purified from buffalo liver. Our earlier investigations strongly indicated the TAase catalyzed acetylation of NADPH cytochrome *c* reductase by DAMC culminating in the hyperbolic activation of the reductase[4] and several classes of polyphenolic acetates (PA) were found effective in causing the activation of reductase.[6—8] These observations prompted us to examine whether NOS bearing a domain of reductase similar to NADPH cytochrome c reductase could similarly be activated leading to the enhancement of intracellular levels of NO. Present investigation was carried out with a view to characterize CRTAase catalyzed activation of reductase in rat (TSMC) by AC and its implications on NOS. TSMC exhibited substantial activity of CRTAase and various AC were found to be substrates for CRTAase. V_{max} of CRTAase was found in the following order: DAMC>7-AMTC>Bis-TAC>7-AMC>6-AMC. Several AC were found to activate the reductase of TSMC in tune with their specificities for CRTAase in the following order: DAMC>7-AMTC>Bis-TAC>7-AMC>6-AMC. We then set forth to examine the influence of AC on NOS of TSMC. TSMC upon incubation with DAMC (100 μM) and arginine was found to produce NO twice higher than cells that were incubated with arginine alone. The enhanced formation of NO by DAMC as described above was diminished to the control level by the inclusion of the well known inhibitor of NOS *i.e.*, L-NAME in pre-incubation reaction mixture indicating that the enhanced formation of NO in TSMC by DAMC was due to the activation of NOS. DHMC, the deacetylated product of DAMC failed to enhance the level of NO in the cells. These results strongly indicated the possible acetylation of the reductase domain of NOS leading to its activation. Earlier, we reported that purified eNOS upon incubation with DAMC and purified CRTAase from rat liver microsomes avidly interacted with the anti acetyl lysine antibody.[15] This observation substantiated CRTAase mediated acetylation of NOS by AC. A number of AC were examined for their abilities to activate smooth muscle cell NOS. AC such as DAMC, 7-AMTC, 7-AMC, Bis-TAC and 6-AMC were found to activate NADPH cytochrome c reductase of smooth muscle cells to varying degrees. The relative ability of these AC to activate NOS was in tune with the specificities of TSMC CRTAase. The activation of eNOS and iNOS in TSMC is confirmed (Fig. 6). Earlier work highlighted the acetylation of eNOS by PA.[15] iNOS is reported to be required for mediating pathophysiological events. The results presented here demonstrate for the first time the activation of TSMC iNOS and eNOS, possibly by way of acetylation. The findings of these studies have relevance from the point of view of enhancement of NO in airway cells by AC. Since NO is known to relieve the exacerbation of airways in diseases such as asthma and COPD,[16] AC may find therapeutic applications.

CONCLUSION

In the present work, the role of CRTAase in the activation of TSMC NOS by various AC is highlighted. TSMC were found to express iNOS and eNOS. The activation of NOS by AC can assume importance in the management of inflammatory diseases of the lung.

MATERIALS AND METHODS

Synthesis and Characterization of Various Acetoxycoumarins Synthesis of 7,8-dihydroxy-4-methylcoumarin (DHMC); 7,8-diacetoxy-4-methylcoumarin (DAMC) and 7-acetoxy-4-methylcoumarin (7-AMC) as reported in our earlier paper.[17] 6-Acetoxy-4-methylcoumarin (6-AMC) was synthesized and characterized according to the method of Dixit and Padukone.[18] Synthesis of 3′-methylene-bis-(4,7,8-trihydroxycoumarin) was prepared according to the

method of Desai and Sethna[19] and its acetylated product 3′-methylene-bis-(4,7,8-triacetoxycoumarin; Bis-TAC) reported in our earlier communication.[8] Synthesis of 7-acetoxy-4-methylthiocoumarin (7-AMTC) was reported in our earlier communication.[20]

Chemicals Reagents Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), NADPH, cytochrome *c*, dichloro fluorescein-diacetate (DCFH-DA), *N*-ω-nitro-L-arginine methyl ester (L-NAME), L-arginine, agarose, chloroform and isopropanol and ethanol were purchased from Sigma Chemical Co., St. Louis, MO (U.S.A.). Trizol (Invitrogen life technologies, Carlsbad, CA, U.S.A.), RT-PCR Kit, RNAase inhibitor (Promega Corporation Madison U.S.A.), Tripling (Hyclone Utah U.S.A.) and all other chemicals used were of high purity and were obtained from local suppliers.

Animals Male albino rats of Wistar strain around (150—200 g) were fed on rats chow supplied by Hindustan Lever Ltd., Mumbai (India) and water was freely available. The animal experiments were performed at the Animal Experiment Center at V. P. Chest Institute, (Delhi, India). Ethical permission No. 00170 was provided by the Animal Research Committee of V. P. Chest Institute, University of Delhi (India).

Preparation of Cytosol Cytosol was prepared as described in Kohli *et al.*[13, 14]

Preparation of Rat Tracheal Smooth Muscle Cells (TSMC) The animals were sacrificed by cervical dislocation and their chest cavity was opened by cutting diaphragm. The trachea was clamped on both ends and then removed. The Rat tracheal smooth muscle (TSM) was stripped off the trachea using forceps. TSM isolated was kept in isotonic saline solution (0.9% NaCl). To isolate TSMC, TSM was treated with trypsin (0.1 μM for 1 h). The undigested tissue was discarded and the released cells are counted and cells are sonicated using Misonix ultrasonic processor where 4 burst are given to lyse the cells. The sonicated sample was then centrifuged at 10000 rpm for 30 min in the ultracentrifuge (Beckman Model L) and the supernatant represents the cells free extract.

Protein Estimation The protein estimation was carried out by using the method of Lowry *et al.*[21]

Assay of Calreticulin Transacetylase (CRTAase) The CRTAase assay using PA and cytosolic glutathione *S*-transferase (GST) as the substrates was described in detail by Raj *et al*.[5] and Habig *et al.*[22]

CRTAase-Mediated Biological Action of Acetoxycoumarins Activation of NADPH cytochrome *c* reductase: The modulation of rat TSMC NADPH cytochrome *c* reductase was carried out by the method of Masters *et al.*[23]

Assay of NOS by Flow Cytometry The method outlined by Imrich and Koobzik was followed for the assay of NOS by flow cytometry.[24]

Isolation of RNA from Rat Tracheal Smooth Muscle Cells (TSMC) RNA was isolated from TSMC as described in Khurana *et al.*[15]

RT-PCR RT-PCR was carried out using total RNA extracted from Rat TSMC. Promega (Cat No. A1702) Access Quick RT-PCR system was used. For eNOS (Gene ID NO. 24600) the primers used were: FP: 5′-CTGCGCTGGTATGCCCTCC-3′; RP: 5′-AAGAGCCTCCCCAGCTGCTG-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 units of AMV reverse transcriptase.

The reaction mix was incubated at 42 °C for 15 min followed heating at 95 °C for 5 min and final incubation at 2 °C. The cDNA synthesized was used for 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 61 °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 (Gene ID NO. 24599) primers used were: FP: 5′-TACATGGGCACCGAGATTGG-3′; RP: 5′-TGAAGGCGTAGCTGAACAAGG-3′. All the conditions for iNOS was the same as that of eNOS (mentioned above). The only difference was in annealing temperature which was 54 °C.

Amplified products were analysed by electrophoresis using 1% agarose gels and visualized by ethidium bromide staining.

Statistical Analysis Each set of experiments was repeated at least three times. Student's *t*-test was used using 95% confidence levels.

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