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Thromboxane A_2 Synthesis in Human Erythroleukemia Cells

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Human erythroleukemia cells transformed arachidonic acid and prostaglandin endoperoxide H_2 into thromboxane A_2. Stimulation of these cells with A23187 or thrombin, however, produced no thromboxane. Similarly, cells labeled with [^{3}H]-arachidonic acid released no detectable label upon stimulation. Data suggest that human erythroleukemia cells contain the enzymatic capacity for thromboxane formation from exogenous precursors, but lack the endogenous mechanisms for arachidonate release. The presence of thromboxane synthase messenger RNA was verified using the polymerase chain reaction. Amplification and sequence analysis of a 528 bp cDNA demonstrated virtually 100 % identity to a published thromboxane synthase cDNA fragment.

Eicosanoids are oxidative metabolites of arachidonic acid with important functions in thrombosis and hemostasis (1,2). Platelets play a key role in this respect, both producing and responding to thromboxane (Tx)A_2, a potent aggregatory agent. Agonist-dependent formation of TxA_2 depends on three sequential enzymatic reactions: i) phospholipase A_2 (PLA_2) catalyzed liberation of arachidonic acid ii) cyclooxygenase catalyzed conversion of arachidonic acid into prostaglandin endoperoxide H_2 (PGH_2) iii) Tx synthase catalyzed conversion of PGH_2 into TxA_2. This biosynthetic cascade has been studied *in vitro* and *in vivo*, using platelets as the primary cellular model. Platelets have been informative in this respect, shaping our current understanding of the enzymology and pharmacology of thromboxane synthase. However, they lack a nucleus and do not replicate *in vitro*, limiting their value for certain experiments. Consequently, we sought a cellular model which would facilitate investigations on the transcriptional and translational regulation of Tx synthase, particularly those on the relationship between 'suicide' inactivation and genesis of enzyme. Several cell lines which are known to produce TxA_2 have proven unsatisfactory for this purpose. Therefore, we examined human erythroleukemia (HEL) cells, a nucleated, megakaryocyte-derived cell line with platelet-like characteristics. Our data indicate that HEL cells generate appreciable quantities of TxB_2 from

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exogenously supplied arachidonic acid or PGH_2, indicating that they have cyclooxygenase and thromboxane synthase activities. We have also shown the expression of Tx synthase mRNA containing a sequence in agreement with a known partial length cDNA (3). HEL cells have an additional, unusual trait: they do not liberate endogenous arachidonic acid upon stimulation by Ca^{++} ionophore or thrombin. This property is beneficial for their use as a model to examine cellular Ca^{++} regulation without the inconvenience of feedback-activation by constitutive eicosanoid biosynthesis.

MATERIALS AND METHODS

Materials. Reagents for the polymerase chain reaction (PCR) were from Perkin Elmer/Cetus. PCR primers were synthesized by Biosynthesis Inc. Denton, Texas. PGH_2 (Caymen Chemical), U63557a (the Upjohn Co.), arachidonic acid (Nu Chek Prep), aspirin, thrombin, A23187, RPMI-1640 medium, and fetal bovine serum (Sigma), [^{3}H]arachidonic acid (New England Nuclear), and inositol 1,4,5-trisphosphate kits (Amersham) were used. HEL cells were from ATCC.

TxB_2 formation. HEL cells were cultured at a density of 1 x 10^6 cells/ml in RPMI-1640 supplemented with 10 % fetal bovine serum as recomended by ATCC. Cells in logarithmic growth were centrifuged at 100 x *g* for 10 min and resuspended in RPMI at a concentration of 1 x 10^7 cells/ml and held at 37°C. Aliquots (100 ul) were then added to neat PGH_2 or arachidonic acid, mixed briefly and incubated at 37°C for 3 min. In some experiments A23187 or thrombin were used. Reactions were quenched by acidification with 20 ul of 1 M citric acid. TxB_2, the stable hydration product of TxA_2, was quantified by immunoassay (4). In certain experiments cells were preincubated 5 min at 37°C with the thromboxane synthase inhibitor, U63557a, or the cyclooxygenase inhibitor, aspirin.

Labeling and release of [^{3}H]arachidonic acid. HEL cells were resuspended in serum-free RPMI to 3 x 10^6 cells/ml. [^{3}H]arachidonic acid (1 uCi; 240 Ci/mmol) was added and aliquots (200 ul) quenched in 10 ul of 100 mM EDTA at the indicated intervals. The amount of [^{3}H] associated with the cellular fraction was determined after centrifugation (13,000 x *g*, 3 min) and scintillation counting of the pellet. Release of incorporated [^{3}H]arachidonic acid was determined in cells (0.5 ml, resuspended to 3 x 10^6 cells/ml) incubated with agonist for 3 min. The reaction was stopped with 25 ul 1 M citric acid; suspensions were centrifuged at 13,000 x *g*, 3 min, and [^{3}H] in the supernatant was determined by scintillation counting.

Inositol 1,4,5-trisphosphate assay. Washed HEL cells (2 x 10^6 cells) were incubated with thrombin (50 nM) for the indicated times. Reactions were stopped with 0.5 ml 15% trichloroacetic acid, and incubated on ice for 20 min. Proteins were precipitated by centrifugation at 2000 x *g* for 15 min, 4°C. The supernatant was extracted three times with 5 volumes of water-saturated ether. The aqueous phase was assayed with inositol 1,4,5-trisphosphate competitive binding assay kit from Amersham (5).

cDNA Amplification and Sequencing. Poly A(+) mRNA was isolated from HEL cells using a Fast Track mRNA isolation kit from Invitrogen. Single strand cDNA was prepared using 200 units of MLV-reverse transcriptase (BRL) and 1 ug mRNA. Four units of E. coli ribonuclease H was added to the cDNA reaction and incubated at 12°C for 60 min. Following RNA digestion, products were extracted with phenol/chloroform and precipitated with ammonium acetate/ethanol. The precipitate was dissolved in 20 ul of distilled water and 5 ul used as template. An initial amplification was performed using a fully degenerate foward primer from the N- terminal amino acid sequence EVNGPMV (6) and a fully degenerate reverse primer from the proteolytic fragment sequence NGFFNKL (7). Reamplification using 1 ul of the initial reaction was with an upstream primer, designated Tx 24F (5'-

GTGACGGTGGCCCTGTCAGTG-3') used in conjunction with two downstream primers, Tx 191R (5'-GGCAACCACATCTGTGGTGTA-3') and Tx 244R (5'-GGGCAAAATCCGGGCCAGTGGGAC-3') (3). Amplification reactions were conducted in 50 ul of buffer containing template, 20 picomoles of each primer, 50 mM KCl, 10 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.25 mM of each dNTP and 2.5 U Taq polymerase. Targets were amplified according to the following protocol: 30 cycles consisting of 94°C, 1 min; 42°C, 2 min; 72°C, 3 min. Products were analyzed on 1.0% agarose gel and visualized by ethidium bromide staining. Amplified products were cloned into a pCR1000 plasmid using a TA Cloning Kit (Invitrogen) as directed by the manufacturer. Positive colonies were screened by amplifying the inserts (8) with the same primers used for the original amplification. Inserts were sequenced twice, in both directions, with overlapping regions using plasmid specific primers and a Sequenase Kit (United States Biochemical).

RESULTS AND DISCUSSION

We have investigated the thromboxane biosynthetic capacity of human erythroleukemia (HEL) cells as a model system for studying the regulation of thromboxane formation. In these experiments, HEL cells efficiently converted exogenously added PGH_2 into TxB_2 in a dose dependent manner (Figure 1). Conversion of PGH_2 was diminished by the competitive inhibitor, U63557a, verifying the occurence of constitutive Tx synthase activity [Figure 1, upper panel].

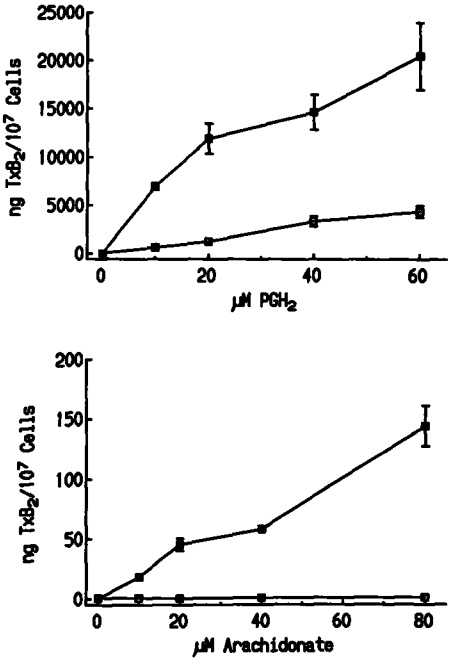


Figure 1. HEL cell TxB_2 production from exogenous PGH_2 and arachidonic acid. HEL cells (1 x 10^7 cells/ml) were incubated with 0 - 80 μM PGH_2 or arachidonic acid for 3 min at 37°C. Reactions were quenched by acidification and TxB_2 quantified by immunoassay. Incubation with 20 μM U63557a, a thromboxane synthase inhibitor, prior to PGH_2 addition decreased TxB_2 levels [upper panel]. Likewise, 100 μM aspirin reduced TxB_2 production from arachidonate [lower panel]. Values are ± SEM, n=3.

TABLE 1
Thromboxane B_2 formation by A23187 and thrombin

Treatment	Basal	10μM A23187	50nM Thrombin
ng/10^7 cells	4.4 ± 1.9	5.1 ± 1.6	3.9 ± 1.5

Values depict mean ± standard error of three or more experiments.

Likewise, HEL cells converted exogenously added arachidonic acid into TxB_2 [Figure 1, lower panel] and this was reduced by 100 uM aspirin, consistent with the occurence of cyclooxygenase, as recently reported (9).

Thrombin, a receptor-dependent agonist (10), and Ca^{++} ionophore A23187, a receptor-independent agonist (11), ordinarily stimulate release and subsequent metabolism of endogenous arachidonic acid. However, neither of these agents stimulated measurable TxB_2 production by HEL cells (Table 1). This contrasts with the transformation of exogenously supplied substrates as noted above. Deficiencies in arachidonic acid uptake or in responsiveness to agonist-activation do not account for this observation. HEL cells readily incorporated [^{3}H]-arachidonic acid. Cellular levels reached 65% of the total arachidonate available within 15 min at 37°C in serum-free medium [Figure 2, upper panel]. Uptake was slower, but equally effective, when serum was present (data not shown). In agreement with the TxB_2 quantitation, we observed no release of [^{3}H]-arachidonic acid from HEL cells stimulated with thrombin or A23187 [Figure 2, lower panel]. Confirming receptor activation by thrombin, we measured a transient, statistically significant accumulation of inositol 1,4,5-trisphosphate which increased from the control value of 0.39 ± 0.20 to 2.95 ± 0.54 pmol/10^7 cells after 15 seconds. Inositol 1,4,5-trisphosphate levels returned to near basal (0.53 ± 0.18 pmol/10^7 cells) within 60 seconds. This increase is in agreement with that seen by Brass et al. (10). Such treatment also leads to a large and transient rise in intracellular Ca^{++} (10). Together, these data indicate an absent or constitutively inhibited PLA_2, similar to that reported with A23187 stimulated monoclonal B cells (12). This property suggests HEL cells as a model system uncomplicated by endogenous arachidonic acid release and potential feedback effects of its metabolites. The lack of detectable PLA_2 activity and its significance warrants further investigation.

HEL cell gene expression of cyclooxygenase and other eicosanoid biosynthetic enzymes has been recently characterized (9,13). We extend these observations by demonstrating that this megakaryocyte-derived cell, also expresses Tx synthase. In addition to detection of constitutive

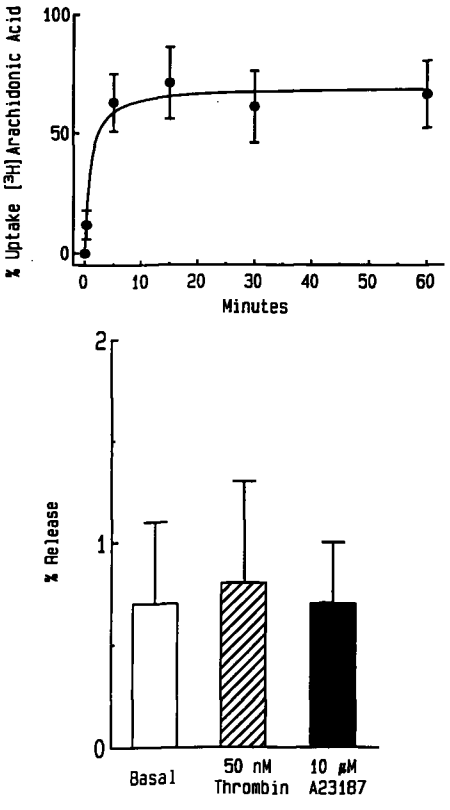
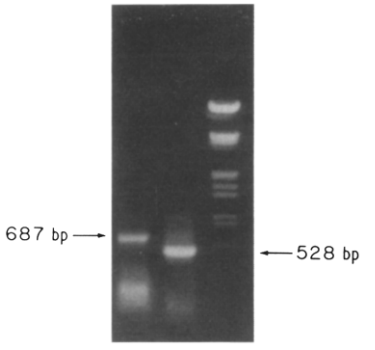


Figure 2. Uptake and stimulated release of [^{3}H]arachidonic acid. HEL cells (3 x 10^6/ml) were incubated with 1 µCi [^{3}H]arachidonic acid in serum free medium. At the indicated time points the amount of radioactivity associated with the cellular fraction was determined as described in Materials and Methods [upper panel]. After resuspension in serum-free medium cells were stimulated with thrombin (50 nM) or A23187 (10µM) for 3 min. Release of label into the supernatant was determined [lower panel]. Values represent the mean ± SEM of 3 or more experiments.

enzymatic activity, we have amplified an HEL Tx synthase cDNA fragment using the polymerase chain reaction. Initially, a fully degenerate forward primer designated Tx16-24F was designed using the N-terminal sequence we determined from human platelet Tx synthase (6). Tx16-24F was used in combination with a fully degenerate reverse primer, Tx IIIR, designed from the sequence reported a Tx synthase proteolytic peptide (7). Amplification of single strand cDNA, generated from HEL messenger RNA, produced 4 DNA fragments (265 - 800 bp in size). To identify which fragment represented Tx synthase, each product was reamplified using specific primers designed from a recently published Tx synthase cDNA fragment (3). Reamplification of a 750 bp product using primers Tx 24F and Tx 191R produced a fragment of 528 bp; Tx 24F and Tx 244R resulted in a fragment of 687 bp, as predicted. These PCR products were cloned into a plasmid vector and the 528 bp insert sequenced to verify



GTGACGGTGG CCCTGTCAGT GGCTCTCTTG GCCCTCCTGA AATGGTACTC
CACATCAGCA TTCTCAAGAC TGGAGAAGTT AGGCCTCAGA CATCCCAAGC
CTTCTCCTTT CATTGGAAAC TTGACATTTT TCCGCCAGGG TTTTTGGGAA
AGCCAAATGG AGCTCAGAAA GCTGTATGGA CCTCTGTGTG GGTACTATCT
TGGTCGTCGG ATGTTTATTG TTATTTCTGA GCCAGACATG ATCAAGCAGG
TGTTGGTTGA GAACTTCAGT AACTTTACCA ACAGAATGGC GTCGGGTTTG
GAGTTCAAGT CGGTAGCCGA CAGCGTTCTG TTTTTACGTG ACAAAAGATG
GGAAGAGGTC AGAGGTGCCC TGATGTCTGC TTTCAGTCCT GAAAAGCTGA
ACGAGATGGT TCCCCTCATC AGCCAAGCCT GCGACCTTCT CCTGGCTCAT
TTAAAACGCT ATGCGGAATC TGGGGACGCA TTTGACATCC AGAGGTGCTA
CTGCAATTAC ACCACAGATG TGGTTGCC

Figure 3. Messenger RNA from HEL cells was converted to single strand cDNA by reverse transcription. Amplification by PCR, using primer designed from Tx synthase amino acid sequences, produced several fragments (data not shown). Reamplification of a 750 base pair fragment using Tx synthase specific primers resulted in fragments of the predicted sizes [upper, panel]. Size standards are Eco RI/Hind III digested lambda DNA. Products were cloned into plasmid vector. The 528 bp fragment was sequenced twice from each end using plasmid specific primers [lower panel]. Comparison to a previously published sequence demonstrated virtual 100% identity. Primer sequences are underlined.

authenticity. Sequence comparison indicated that the 528 bp product was, with the exception of one base, identical to the previously published sequence [Figure 3] (3).

Understanding and intervening in the biochemical mechanism of thromboxane synthesis may be beneficial for certain disorders (14). Few nucleated cell lines have been reported to contain Tx synthase (15,16,17). We have demonstrated the expression of Tx synthase mRNA using PCR, and the occurrence of an appreciable Tx synthase activity in HEL cells. Since, HEL cells display a 'suicide' inactivation profile comparable to platelets (17) they represent a nucleated cell model for investigating transcriptional and translational regulation of its genesis, following 'suicide' inactivation or modulation by other processes. In addition, our data indicate that HEL cells can be used for certain investigations without the complication of endogenous arachidonic acid release and metabolism.

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