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Regulation of endothelin-1 production by a thromboxane A_2 mimetic in rat heart smooth muscle cells

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Abstract

Thromboxane A_2 (TXA_2) and ET-1 have been known to play important roles in modulating vascular contraction and growth. The present study was undertaken to examine the effect of TXA_2 on the induction of endothelin-1 (ET-1) mRNA and protein levels in smooth muscle cells derived from rat heart. U-46619, a stable TXA_2 mimetic, superinduced preproET-1 mRNA in the presence of cycloheximide in these cells. This effect could be blocked by SQ-29548, a TXA_2/prostaglandin H_2 receptor antagonist and by actinomycin D, an RNA synthesis inhibitor. In addition, H7, a protein kinase C inhibitor, could abolish the induction. Transient transfection experiment revealed that the elevated ET-1 mRNA level after U-46619 treatment was a result of the activation of ET-1 gene activity. The elevated ET-1 message level was accompanied by increased ET-1 release into the cultured medium. These results show that the short-lived TXA_2 can induce potent and long-lived ET-1. These findings support a potential role for ET-1 in the pathogenesis of coronary atherosclerosis and hypertension evoked by TXA_2.

Keywords: Thromboxane A_2 mimetic; Endothelin-1; Rat heart smooth muscle cells; Protein kinase C

1. Introduction

Thromboxane A_2 (TXA_2) is a cyclooxgenase metabolite of arachidonic acid [1]. It is one of the most potent biological stimuli of platelet aggregation and smooth muscle contraction and has been implicated as an important pathophysiological mediator of various cardiovascular diseases [2,3]. Since thromboxane A_2 has a very short half-life in the blood (less than 30 s), it is possible that part of the vascular activities of TXA_2 may be mediated by ET-1, a long-acting vasoconstrictor.

Endothelin-1 (ET-1) is a very potent vasoconstrictor that has been initially isolated from the supernatant of cultured porcine endothelial cells [4]. The mRNA for preproET-1 is expressed in porcine aortic endothelial cells in vivo [5] and porcine aortic endothelial cells and human umbilical vein endothelial cells in vitro [4,6]. Many physiological stimuli were found to increase ET-1 production and mRNA level, including thrombin [7], calcium ionophore [4], tumor promoter [8], insulin [9], bradykinin [10], cytokines such as transforming growth factor-β [11] and interleukin 1 [12]. More recently, we reported that angiotensin II stimulated ET-1 production in rat heart endothelial cells [13].

The present experiments were carried out in rat heart smooth muscle cells to examine the effect of TXA_2 on the induction of preproET-1 transcripts, which is a key regulatory step in the production of ET-1. Our results show that exposure to the TXA_2 mimetic U-46619 is associated with a rapid rise of preproET-1 mRNA in smooth muscle cells. The increase in the expression and release of ET-1 may augment or prolong the actions of TXA_2.

2. Materials and methods

2.1. Cell culture and materials

Smooth muscle cells derived from rat heart were provided by Diglio et al. [14]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg/ml gentamycin at 37°C in a

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humidified atmosphere of 5% CO_2. Cells were serially subcultured by treatment with trypsin (0.05% trypsin in 0.5 mM Versene) and used for experimentation between passages 12 and 25. The homogeneity of the cell population was confirmed by immunofluorescence staining with smooth muscle α-actin (BioGenex, San Ramon, CA). U-46619 and endothelin ELISA kit were purchased from Cayman Chemical Company (Ann Arbor, MI). SQ-29548 was a gift from Bristol-Myers Squibb. H7 was obtained from Sigma Chemical Company. [^{32}P] dCTP was obtained from ICN. ITS was purchased from Collaborative Biomedical (Bedford, MA). Fetal bovine serum was obtained from Biofluids. All other reagents used were of the highest grade commercially available.

2.2. Northern blot analysis

Cells were seeded into 100 mm dishes containing DMEM and 10% FBS. Near confluent cells were changed to serum-free DMEM supplemented with ITS (1 μM insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenous acid) for 24 h before the addition of test agents. After the designated time total RNA was extracted using the acid guanidinium thiocyanate-phenol-$CHCl_3$ extraction method [15]. Fifteen μg of RNA was denatured and separated by electrophoresis on 1% agarose gel containing 2.2 M formaldehyde. RNA ladder (BRL) was included as size marker. Following transfer, the RNA was covalently bound to Nytran (Schleicher and Schull) by UV-crosslinking. Hybridization and washing conditions were carried out according to the method of Church and Gilbert [16]. A rat ET-1 probe was generously provided by Dr. Tomoh Masaki and Dr. M. Yanagisawa (Kyoto University) [17], and a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe was provided by Dr. Ray Wu (Cornell University). The cDNAs were labeled with [^{32}P] dCTP using a random primer kit from Amersham. The blots were exposed to XAR-5 films with an intensifying screen. The intensity of the bands was quantitated by using the Ambis Image Analysis System. The experiments were repeated three to four times and representative data were shown.

2.3. ET-1 promoter activity

Rat aortic smooth muscle cells (passage 5, provided by Dr. K.S. Ramos, Texas A and M University) [18] in 60 mm plates were transiently transfected with 5 μg of 4.4 Kb ET-1 promoter coupled to chloramphenicol acetyltransferase gene (provided by Dr. Mu-En Lee, Harvard Medical School) [19], pSV2CAT, or pSV0CAT. Transfection was carried out with 40 μg of Lipofectin reagent in the presence of Opti-MEM (Life Technologies) for 16 h. The medium was changed to DMEM containing 10% FBS for 24 h. The cultures were then incubated with serum-free DMEM in the presence or absence of $1 \cdot 10^{-7}$ M U-46619. After 36 h, cell lysates were prepared and aliquots containing the same amount of protein were assayed for CAT activities as described by Gorman et al. [20]. CAT activity was quantitated by counting the radioactivity excised from the spots corresponding to chloramphenicol and acetylated chloramphenicol.

2.4. Secreted levels of ET-1

Rat heart smooth muscle cell cultures were plated in DMEM containing 10% FBS in 35 mm dishes. Near confluent cells were maintained in serum-free DMEM with the addition of ITS (1 μM insulin, 6.25 μg/ml transferrin and 6.25 ng/ml selenous acid) for 24 h before the addition of $1 \cdot 10^{-7}$ M U-46619 for 8 h and 12 h. ET-1 released into the medium was measured using an endothelin ELISA kit.

2.5. Statistical analysis

Statistical analysis was made by the Student's test for comparison of any two sample means. *P* values less than 0.05 were considered statistically significant.

3. Results

Steady-state levels of preproET-1 mRNA were determined in rat heart smooth cell cultures by Northern blot analysis. The basal level of preproET-1 mRNA was low. The mRNA level increased rapidly upon the addition of U-46619, a stable TXA_2 mimetic [21]. Levels of preproET-1 mRNA increased 5-fold at 30 min (Fig. 1, lane 2) and declined over 2 h (lane 4). The same blot was reprobed with a cDNA encoding GAPDH as an internal control to correct for varying loading and/or transfer. As shown in Fig. 2, preproET-1 mRNA responded to U-46619 in a dose-dependent manner.

The next series of experiments were performed to examine the mechanism of preproET-1 RNA induction. Inhibitors of protein and RNA synthesis were included singly or in combination with U-46619. In the presence of actinomycin D, the induction was abolished (Fig. 3, lane 4), suggesting that de novo RNA synthesis is required. Cycloheximide was included to examine whether the induction

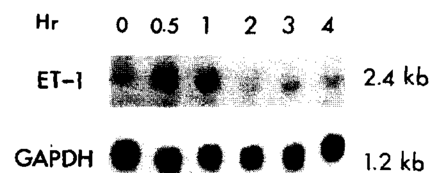

Fig. 1. Time-course of ET-1 mRNA induction in rat heart smooth muscle cells by U-46619. Near confluent cultures were exposed to U-46619 ($1 \cdot 10^{-7}$ M) for the times indicated. 15 μg of total RNA was hybridized with a cDNA probe for rat ET-1. Hybridization patterns of cells treated with U-46619 for 0 min (lane 1); 30 min (lane 2); 1 h (lane 3); 2 h (lane 4); 3 h (lane 5); 4 h (lane 6); are shown.

Fig. 2. Induction of ET-1 mRNA as a function of U-46619 dose. Near confluent cultures of rat heart smooth muscle cells were treated with $1 \cdot 10^{-11}$ M to $1 \cdot 10^{-6}$ M of U-46619 for 1 h. 15 μg of total RNA was hybridized with a cDNA probe for rat ET-1. Hybridization patterns of control cells (lane 1); cells treated with $1 \cdot 10^{-11}$ M U-46619 (lane 2); $1 \cdot 10^{-10}$ M 46619 (lane 3); $1 \cdot 10^{-9}$ M U-46619 (lane 4); $1 \cdot 10^{-8}$ M U-46619 (lane 5); $1 \cdot 10^{-7}$ M U-46619 (lane 6); $1 \cdot 10^{-6}$ M U-46619 (lane 7); are shown.

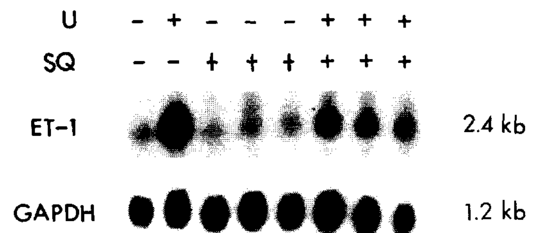

Fig. 4. Effect of SQ-29548, an antagonist of U-46619, on the induction of ET-1 mRNA. Total RNA was isolated from rat heart smooth muscle cell cultures in the absence or presence of SQ-29548 and U-46619. Hybridization patterns of control cells (lane 1); cells treated with $1 \cdot 10^{-7}$ M U-46619 (lane 2); $1 \cdot 10^{-7}$ M SQ-29548 (lane 3); $1 \cdot 10^{-6}$ M SQ-29548 (lane 4); $1 \cdot 10^{-5}$ M SQ-29548 (lane 5); $1 \cdot 10^{-7}$ M SQ-29548 and U-46619 (lane 6); $1 \cdot 10^{-6}$ M SQ-29548 and U-46619 (lane 7); $1 \cdot 10^{-5}$ M SQ-29548 and U-46619 (lane 8); are shown.

of ET-1 gene is a direct effect of U-46619 or whether intermediate protein synthesis is required. Cycloheximide alone induced preproET-1 mRNA (lane 5). In the presence of U-46619 and cycloheximide (lane 6), the induction was higher than U-46619 alone (lane 2), implying that the induction does not require de novo protein synthesis.

SQ-29548, a specific antagonist for TXA_2 receptor [22], was included to examine if the effect of U-46619 is receptor-mediated. A dose curve of SQ-29548 was performed. SQ-29548 alone did not affect the preproET-1 mRNA level (Fig. 4, lanes 3–5). The induction of preproET-1 mRNA by U-46619 was diminished with increasing concentrations of SQ-29548 (lanes 5–8). These results indicate that the induction of preproET-1 mRNA is mediated by the TXA_2 receptor.

To study if the induction of preproET-1 mRNA involves a protein kinase C -dependent pathway, H7, a protein kinase C inhibitor [23], was included. In these experiments, cells were pretreated with 1 μM H7 for 30 min before the addition of U-46619. H7 could block the induction of preproET-1 mRNA (Fig. 5, lane 4), suggesting that the effect of TXA_2 is mediated by a protein kinase C-dependent pathway.

Expression of elevated ET-1 mRNA level after U-46619 treatment may be the result of increased stability of the message and/or activation of ET-1 gene activity. To test if the transcription rate of ET-1 is altered by U-46619, transfection experiments were carried out with ET-1 promoter coupled to the CAT gene in the absence or presence of U-46619. Fig. 6 shows that U-46619 increased CAT

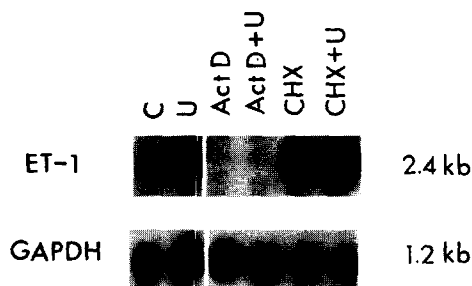

Fig. 3. Effect of cycloheximide (CHX) and actinomycin D (Act D) on the induction of ET-1 mRNA by U-46619. Near confluent cultures of rat heart smooth muscle cells were treated with U-46619 for 1 h in the absence or presence of Act D (1 μg/ml) or CHX (20 μg/ml). 15 μg of total RNA was hybridized with rat ET-1 cDNA probe. Hybridization patterns of control cells (lane 1); cells treated with $1 \cdot 10^{-7}$ M U-46619 (lane 2); Act D (lane 3); U-46619 and Act D (lane 4); CHX (lane 5); U-46619 and CHX (lane 6); are shown.

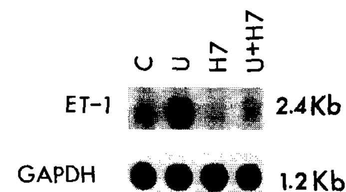

Fig. 5. Effect of protein kinase C inhibitor, H7, on the induction of ET-1 mRNA by U-46619. Near confluent rat heart smooth muscle cells were pretreated with H7 for 30 min before the addition of U-46619 for 1 h. Hybridization patterns of control cells (lane 1); cells treated with $1 \cdot 10^{-7}$ M U-46619 (lane 2); 1 μM H7 (lane 3); U-46619 and H7 (lane 4); are shown.

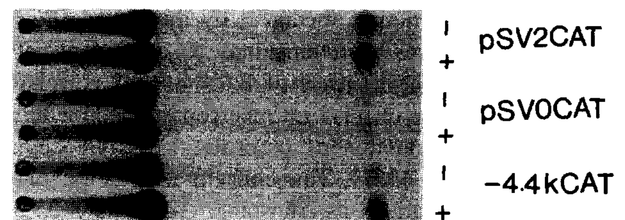

Fig. 6. Stimulation of ET-1 promoter activity by U-46619. Rat aortic smooth muscle cells in 60 mm plates were transfected with 5 μg of either pSV2CAT, pSVOCAT or ET-1 promoter (−4.4kCAT) and 40 μg of Lipofectin for 16 h. The medium was then changed to DMEM in the presence or absence of $1 \cdot 10^{-7}$ M U-46619 for 36 h prior to harvest for CAT assays. CAT activity of cells transfected with pSV2CAT (lanes 1,2), pSVOCAT (lanes 3,4), and ET-1 promoter (lanes 5,6), are shown. Lanes 1,3,5 represent the pattern in the absence of U-46619; lanes 2,4,6 represent the pattern in the presence of U-46619.

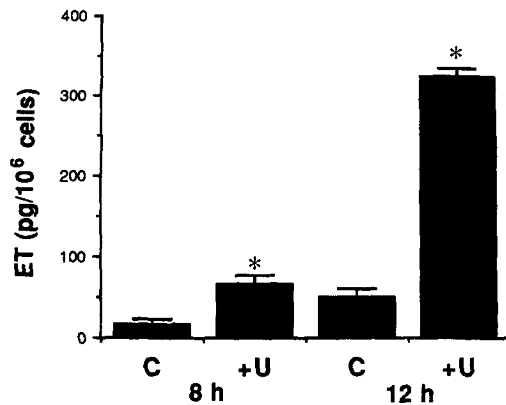

Fig. 7. Release of ET-1 into the medium of rat heart smooth muscle cells. Quiescent rat heart smooth muscle cells were incubated with $1 \cdot 10^{-7}$ M U-46619 for 8 h and 12 h. ET-1 released into the medium was measured by ELISA. Values represent the mean ± S.E.M. of four samples. * $P < 0.05$ vs. control.

activity of ET-1 promoter fourfold (lanes 5,6). U-46619 had no effect on the CAT activities in cells transfected with pSV2CAT (lanes 1,2) or pSV0CAT (lanes 3,4).

The release of ET-1 in response to U-46619 was determined in the conditioned medium by ELISA. After stimulation with $1 \cdot 10^{-7}$ M U-46619 for 8 h, the control cells released 17 ± 6 pg ET-1/well whereas the value for stimulated cells was 66 ± 11 pg/well. After 12 h, the control value was 51 ± 10 pg/well whereas the stimulated cells gave 325 ± 10 pg/well (Fig. 7).

4. Discussion

The present study demonstrated that TXA_2 mimetic U-46619 acts on rat heart-derived smooth muscle cells to induce preproET-1 mRNA in a dose and time dependent manner. The induction is mediated by the TXA_2/PGH_2 receptor and involves a protein kinase C dependent pathway. In addition, the increase of ET-1 mRNA correlated well with the release of ET-1 into the conditioned medium. It is noted that induction of preproET-1 mRNA begins with $1 \cdot 10^{-8}$ M U-46619. This concentration is comparable to the concentrations used in other assays such as the induction of constriction of rat aorta [24] and the mitogenic effect on rat vascular smooth muscle cells [25].

In this study preproET-1 mRNA induction elicited by U-46619 was shown to be diminished with increasing concentrations of SQ-29548. SQ-29548 has been reported to be a specific TXA_2/PGH_2 receptor antagonist [22]. SQ-29548 at 1 μM could block the contraction of rat aortic ring elicited by $1 \cdot 10^{-7}$ M U-46619 [26] and suppress its mitogenic effect in rat vascular smooth muscle cells [25].

Our data demonstrated that the induction of preproET-1 mRNA by U-46619 could be attributed partially to transcriptional activation. Although the elements responsible for this activation were not characterized in the present study, the promoter of the ET-1 gene has been shown to contain AP-1 sites [19,27]. Whether the effect of U-46619 is mediated by the AP-1 element in our cell system is under investigation. It is interesting to note that preproET-1 mRNA is 'superinduced' in the presence of cycloheximide and U-46619. The level of preproET-1 mRNA in the presence of cycloheximide alone is higher than the basal level. Yanagisawa et al. [1] reported the 3′-end of preproET-1 mRNA contains several 'AUUUA' motifs that confer mRNA instability. Cycloheximide might inhibit the synthesis of labile proteins with RNase activities or short-lived repressors.

Our results showed that H7, a protein kinase C inhibitor, could block the induction of preproET-1 mRNA by U-46619. This observation concurred with the previous report that TXA_2 activates phospholipase C in vascular smooth cells leading to the production of IP_3, increased Ca^{2+} mobilization from the intracellular store and increased protein kinase C activity [28].

It is well established that TXA_2 and prostacyclin (PGI_2) are involved in the control of vascular tone and hemostasis via a reciprocal function in blood-endothelium interactions. There is compelling evidence to support the possible involvement of TXA_2 in the pathogenesis of severe hypertension. The biosynthesis of TXA_2 is increased in several models of experimental hypertension [29–34]. The development of hypertension could be attenuated by administration of thromboxane synthase inhibitors or TXA_2 antagonists [29,30,35,36].

TXA_2 has also been suggested to participate in the promotion of atherosclerosis. TXA_2 is secreted in large amounts by activated platelets during the development of atherosclerosis and acute myocardial ischemia [37,38]. Besides its prominent action on platelet activation, TXA_2 has been shown to have a mitogenic effect on rat aortic smooth muscle cells [39,40]. Our preliminary experiments showed that in rat heart smooth cells, both TXA_2 and ET-1 could increase the incorporation of [^{3}H] thymidine into DNA by two- and three-fold, respectively (results not shown). The proliferative activities of rat heart smooth muscle cells in response to TXA_2 may be one of the underlying mechanisms leading to coronary artery disease.

In conclusion, we present evidence that ET-1 is rapidly induced by TXA_2 in rat heart smooth muscle cells. ET-1 has been shown to stimulate TXA_2 biosynthesis in smooth muscle cells [41]. These interesting interactions between TXA_2 and ET-1 might serve to amplify and perpetuate the effect of TXA_2 on both coronary constriction and the development of atherosclerosis under pathophysiological conditions.

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