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Increased prostacyclin and thromboxane A_2 biosynthesis in atherosclerosis

(arachidonic acid)

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ABSTRACT **It has been proposed that atherosclerotic arteries produce less prostacyclin (PGI_2) than nonatherosclerotic arteries do, thereby predisposing arteries to vasospasm and thrombosis *in vivo*. We reexamined this concept by measuring spontaneous as well as arachidonate-induced PGI_2 biosynthesis in aortic segments from nonatherosclerotic and cholesterol-fed atherosclerotic New Zealand White rabbits. Thromboxane A_2 (TXA_2) generation was also measured. Formation of PGI_2, as well as TXA_2, as measured by radioimmunoassay (RIA) of their metabolites, was increased in atherosclerotic aortic segments relative to nonatherosclerotic segments ($P \leq 0.05$) at 0, 5, 10, 15, and 30 min of incubation with arachidonate. Pretreatment of arterial segments with indomethacin inhibited PGI_2 as well as TXA_2 formation, whereas pretreatment with the selective TXA_2 inhibitor OKY-046 inhibited only TXA_2 release, thus confirming the identity of icosanoids. To confirm the RIA data, aortic segments were incubated with [^{14}C]arachidonate prior to stimulation with unlabeled arachidonate. The uptake of arachidonate was similar, but the release of incorporated [^{14}C]arachidonate was significantly ($P \leq 0.05$) greater in atherosclerotic segments than in nonatherosclerotic ones. Conversions of released [^{14}C]arachidonate to 6-keto[^{14}C]prostaglandin $F_{1\alpha}$ and [^{14}C]thromboxane B_2 were similar in the two types of aortic segments. Thus, synthesis of PGI_2 as well as TXA_2 is increased in atherosclerosis, and this alteration in arachidonate metabolism is related to increased release of arachidonate.**

Decreased prostacyclin (PGI_2) biosynthesis and release by atherosclerotic compared to normal vessels was described by Dembinska-Keic *et al.* in 1977 (1). These initial observations in rabbit vessels were subsequently confirmed in the postmortem examination of human atherosclerotic vessels by D'Angelo *et al.* (2) and Sinzinger *et al.* (3). Larrue *et al.* (4) reported diminished PGI_2 generation by cultured smooth muscle cells from atherosclerotic rabbit aortae.

Thromboxane A_2 (TXA_2) production is also increased in experimental (5) and human atherosclerosis (6–8). Accordingly, it has been proposed that a relative excess of TXA_2 and deficiency of PGI_2 may predispose atherosclerotic vessels to vasospasm and platelet aggregation *in vivo* (9, 10).

Although the plasma concentrations of 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable hydrolysis product of PGI_2, are extremely low in normal plasma (≈4 pg/ml) (11), it has been reported that these concentrations are increased in patients with atherosclerosis (8, 12). Hirsh *et al.* (8) reported mean plasma concentrations in patients with coronary atherosclerosis of 140 pg/ml. In another study (12), mean plasma 6-keto-$PGF_{1\alpha}$ concentrations in similar patients were reported to be 94 pg/ml (vs. 54 pg/ml in control normal subjects). Recently, Fitzgerald *et al.* (13) measured a major PGI_2 metabolite released in the urine of patients with severe atherosclerosis, and they demonstrated increased PGI_2 biosynthesis in these patients relative to normal subjects.

Since many previous studies employed bioassay or only radioimmunoassay (RIA) for measurement of PGI_2, the present studies were designed to reexamine the concept of altered PGI_2 generation in atherosclerosis. We examined the uptake and incorporation of labeled arachidonic acid into the phospholipids of the vessel wall and subsequent arachidonate release and conversion to major metabolites of PGI_2 and TXA_2 in both normal and atherosclerotic vessels.

MATERIALS AND METHODS

Induction of Atherosclerosis. New Zealand White male rabbits weighing 2.26–2.70 kg were fed an atherogenic diet of 1% cholesterol and 5% (wt/wt) lard (Bio-Serve, Frenchtown, NJ) for 2 weeks, which caused an increase of serum cholesterol to 981–1368 mg/dl (mean = 1191 mg/dl). Thereafter, the rabbits were anesthetized (acepromazine at 0.5 mg/kg, Rompun at 3 mg/kg, and ketamine at 50 mg/kg), and their right groins were explored to isolate the right femoral artery. After arteriotomy, a no. 4F Fogarty catheter was inserted and advanced into the abdominal aorta to the level of the diaphragm. The balloon was then inflated and retrieved, resulting in endothelial stripping of the aorta and right iliac artery. The right femoral artery was then ligated, and the incision was closed. The atherogenic diet was thereafter continued for 3 months prior to surgical exposure of the abdominal aorta. As controls, a group of rabbits underwent aortic stripping, but no atherogenic diet was given either before or after stripping.

Preparation of Tissues for PGI_2 and TXA_2 Generation. After 3-months of atherogenic diet, animals were sacrificed with intravenous pentabarbital infusion and the abdominal aorta was rapidly harvested. The vessel was collected in ice-cold Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}, pH 7.4, and carefully cleaned of all loose connective tissue and fat. The aorta was then perfused repeatedly with HBSS to remove blood clots, remaining platelets, and leukocytes until the perfusate contained no leukocytes or platelets visible by light microscopy. The tissues were then cut into 2-mm ring segments, weighed, and immediately placed into 2.0 ml of HBSS containing Ca^{2+} and Mg^{2+}. Approximately 30 mg of tissue (wet weight) was incubated with 10 μM arachidonate (Sigma) alone or with the selective TXA_2 synthetase inhibitor OKY-046 (10 μM) (Ono Pharmaceutical, Osaka, Japan) or the cyclooxygenase inhibitor indomethacin (10 μM) (Merck Sharpe & Dohme) at 37°C for

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Abbreviations: 6-keto-$PGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$; PGI_2, prostacyclin; TXA_2 or TXB_2, thromboxane A_2 or B_2.

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30 min. The supernate was collected at 0, 5, 15, and 30 min for measurement of PGI_2 and TXA_2 metabolites (14).

Incorporation of [^{14}C]Arachidonate. To confirm the results of the PGI_2 and TXA_2 released into the supernates of aortic rings treated with arachidonate, 150-mg samples of aortic rings were incubated with 1.0 μCi of [^{14}C]arachidonate (390 mCi/mmol, New England Nuclear; 1 Ci = 37 GBq) for 5 min at 25°C. The rings were then washed twice in 2.0 ml of HBSS to remove unincorporated radioactivity. Supernate radioactivity was quantified in a liquid scintillation counter (Tracor Analytic, Elk Grove Village, IL) to measure unincorporated [^{14}C]arachidonate. Aortic rings were then resuspended in 2.0 ml of HBSS.

Conversion of [^{14}C]Arachidonate to PGI_2 and TXA_2. To stimulate the conversion of membrane-bound [^{14}C]arachidonate into cyclooxygenase metabolites, 100 μM unlabeled arachidonate was added to the rings, which were incubated at 37°C for 30 min in a shaking water bath. An aliquot of supernate was then assayed for radioactivity to determine the release of [^{14}C]arachidonate and its metabolites. The aortic rings were then isolated and washed and their radioactivities were measured.

To determine the conversion of [^{14}C]arachidonate into 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 (TXB_2), the aortic ring supernates, after stimulation with arachidonate, were extracted and analyzed by thin-layer chromatography (TLC) as follows: To each supernate, unlabeled TXB_2 and 6-keto-$PGF_{1\alpha}$ standards (5 μg each) were added as carriers, the pH was adjusted with 0.5 M formic acid to 3.5, and the supernate was extracted twice with 3.0 ml of ethyl acetate. The resulting organic phase contained 75% of the standards. It was evaporated to dryness under a stream of nitrogen and the residue was dissolved in ethyl acetate and spotted onto TLC plates (Fisher) along with 6-keto-$PGF_{1\alpha}$ and TXB_2 standards (5 μg each). The plates were then developed in a solvent system consisting of ethyl acetate, trimethylpentane, acetic acid, and water (110:50:20:100, vol/vol). 6-Keto-$PGF_{1\alpha}$ and TXB_2 were visualized by spraying the TLC plate with 10% phosphomolybdic acid in ethanol (Sigma). The TLC plates were then analyzed for radioactivity distribution by a radiochromatographic profiler (Packard Instrument, Downers Grove, IL). The resulting radiochromatograms were matched to corresponding R_f values of standards. A 2 × 2 cm area surrounding each 6-keto-$PGF_{1\alpha}$ and TXB_2 spot was then scraped from the plate and resuspended in 5.0 ml of Atomlight liquid scintillation counting fluid (New England Nuclear) and mixed well on a mechanical shaker for 1 hr, and its radioactivity was measured.

RIAs for PGI_2 and TXA_2. 6-Keto-$PGF_{1\alpha}$ and TXB_2, the stable hydrolysis products of PGI_2 and TXA_2, were measured by RIA as described previously (14, 15). Lyophilized standards, tracers, and antisera were obtained from New England Nuclear. Cross-reactivity and anti-TXB_2 antiserum with other prostaglandins was less than 2%. Cross-reactivity of anti-6-keto-$PGF_{1\alpha}$ antiserum with other prostaglandins was also less than 2%. All measurements were performed in duplicate and results are expressed as ng/ml of supernate.

Statistical Analysis. All determinations made in duplicate were averaged. Data are expressed as mean ± SD. Student's *t* test (paired and unpaired data) was used for statistical analysis. A *P* value of 0.05 was considered significant.

RESULTS

PGI_2 and TXA_2 Release. As shown in Fig. 1, PGI_2 (measured as 6-keto-$PGF_{1\alpha}$ by RIA) released from atherosclerotic rabbit aortic rings was greater ($P \leq 0.05$) than that released by normal rabbit aortic rings. This increase was seen at 0, 5, 10, 15, and 30 min of incubation. PGI_2 release continued for 15 min and then stabilized.

TXA_2 (measured as TXB_2 by RIA in aortic ring supernates) was also greater ($P \leq 0.05$) in atherosclerotic rabbit rings. Similar to PGI_2 release, increased TXA_2 release was observed at all time points. However, in contrast to PGI_2 release, TXA_2 release continued for the entire 30 min of incubation.

Effect of Inhibitors on PGI_2 and TXA_2 Release. As shown in Fig. 2 *Upper*, the cyclooxygenase inhibitor indomethacin caused a marked reduction in release of PGI_2 as well as TXA_2. This inhibition was observed at 0 as well as 30 min of incubation, and in both normal and atherosclerotic aortic segments. However, 6-keto-$PGF_{1\alpha}$ concentrations were still higher ($P \leq 0.05$) in the supernates of atherosclerotic rings compared to normal rings. TXB_2 concentrations in the supernates of both sets of rings were similar.

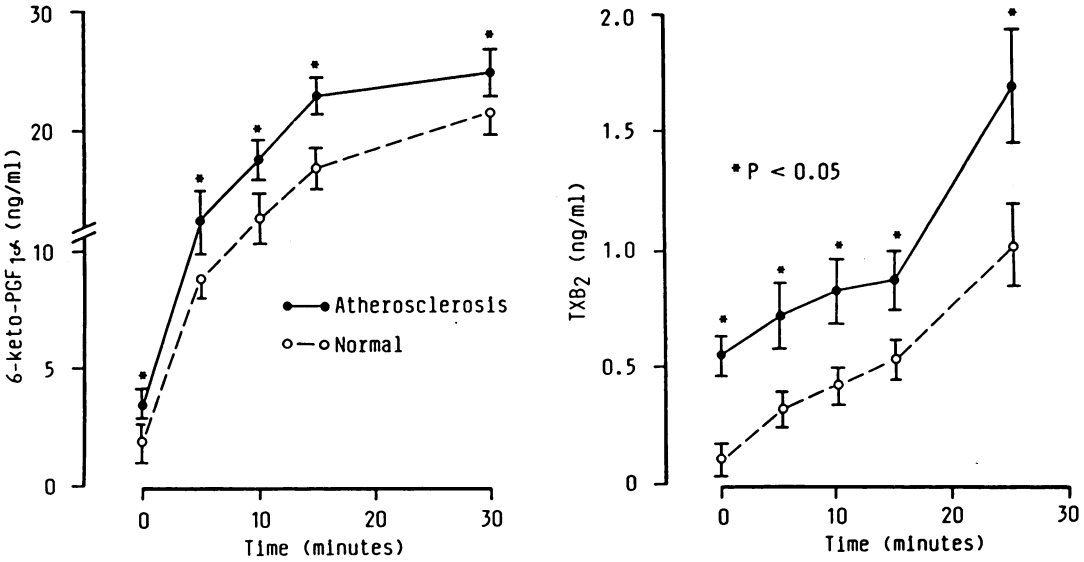

FIG. 1. Increased PGI_2 and TXA_2 biosynthesis by atherosclerotic compared to normal aortic segments. Aortic segments (≈30 mg) were incubated with 10 μM arachidonate for 30 min. Aliquots of supernates from atherosclerotic aortic segments showed greater release of 6-keto-$PGF_{1\alpha}$ and TXB_2, stable hydrolysis products of PGI_2 and TXA_2, respectively, than the normal aortic rings. PGI_2 release stabilized at 15 min, whereas TXA_2 release continued throughout the 30 min of incubation.

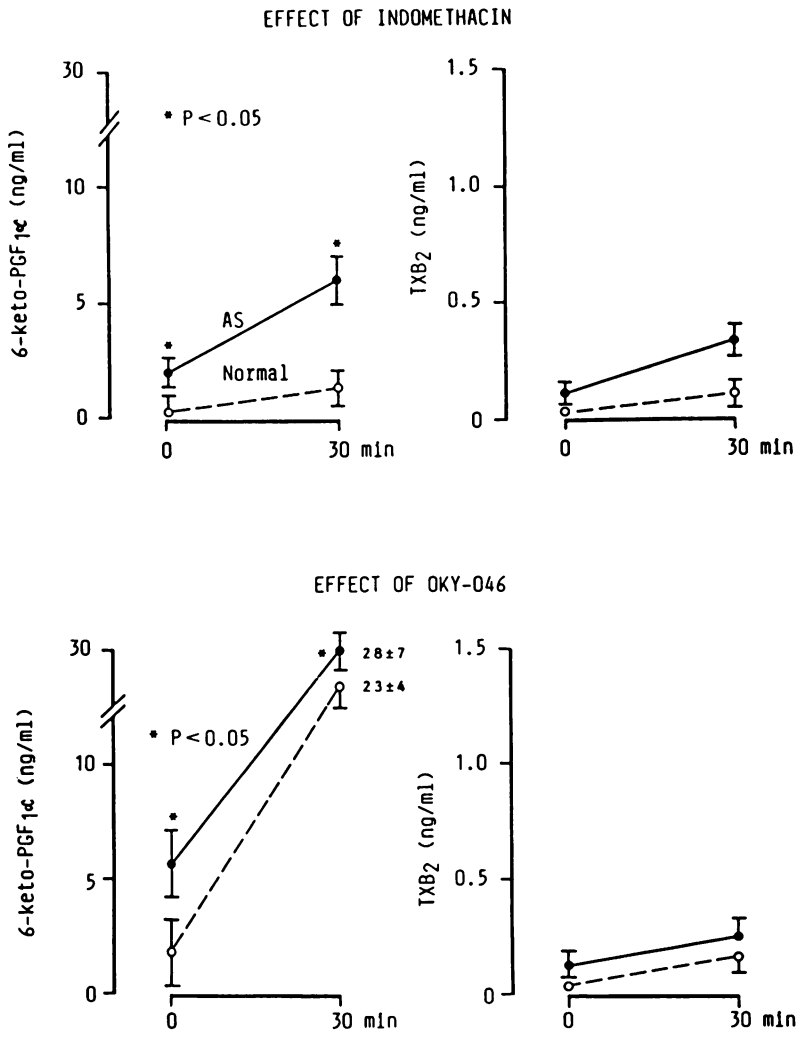

FIG. 2. Effect of prostaglandin inhibitors on PGI_2 and TXA_2 formation. Treatment with the cyclooxygenase inhibitor indomethacin (*Upper*) inhibited both PGI_2 and TXA_2 synthesis in atherosclerotic (AS) and normal rabbit aortic segments. However, PGI_2 release was greater in supernates of atherosclerotic rings than in those of normal rings. Treatment with the selective TXA_2 inhibitor OKY-046 (*Lower*) decreased TXA_2 formation without significant effect on PGI_2 synthesis. 6-Keto-$PGF_{1\alpha}$ concentrations were higher in the supernates of atherosclerotic rings than in those of normal rings.

Treatment of aortic rings with the selective TXA_2 synthetase inhibitor OKY-046 caused a significant ($P \leq 0.01$) reduction in TXA_2 release by both atherosclerotic and normal rabbit aortic rings (Fig. 2, *Lower*). In contrast, PGI_2 release was slightly higher than that in the comparable untreated rings ($P \geq 0.05$), indicating redirection of cyclic endoperoxides to PGI_2 biosynthesis in the presence of OKY-046 (15). PGI_2 release was again greater in the supernates of atherosclerotic rings than in those of normal rings, both at 0 and 30 min of incubation, as observed in the untreated rings.

[^{14}C]Arachidonate Incorporation, Release, and Conversion to PGI_2 and TXA_2. The uptakes of [^{14}C]arachidonate were similar in atherosclerotic rabbit aortic rings (range 15.0–26.7%, mean 22.3 ± 4%, $n = 6$) and normal rings (range 18.7–28.0%, mean 20 ± 1.0%, $n = 3$). The release of [^{14}C]arachidonate by atherosclerotic rings was, however, 20% greater than release by the normal rings (48.4 ± 10.2% vs. 40.5 ± 7.5%, $P \leq 0.05$) (Fig. 3).

Two representative radiochromatograms of TLC plates are shown in Fig. 4. With similar radioactivity counts on the TLC plate, larger 6-keto-[^{14}C]$PGF_{1\alpha}$ and [^{14}C]TXB_2 peaks were identified in the supernates of atherosclerotic aortic rings.

Conversion of released [^{14}C]arachidonate to 6-keto-[^{14}C]$PGF_{1\alpha}$ was similar in atherosclerotic and normal aortic rings (4.18 ± 0.20% and 5.97 ± 3.32%, respectively). However, conversion to [^{14}C]TXB_2 was slightly higher, but not significantly different, in atherosclerotic aortic rings than in the normal rings (2.85 ± 1.19% and 2.34 ± 0.16%, respectively) (Fig. 3).

Because of enhanced release of [^{14}C]arachidonate from atherosclerotic aortic rings, total 6-keto-[^{14}C]$PGF_{1\alpha}$ and [^{14}C]TXB_2 identified by TLC were significantly greater than those from the normal rabbit aortic segments.

DISCUSSION

These studies show that atherosclerotic aortic rings from cholesterol-fed rabbits generate more PGI_2 than do the aortic rings from normal rabbits. Atherosclerotic aortic rings also generate more TXA_2 than do normal aortic rings. Studies with labeled arachidonate demonstrate a significant increase in arachidonate release from the phospholipid pool of the vessel wall cell membranes. Since the conversion of released arachidonate into the major icosanoids PGI_2 and TXA_2 is similar in atherosclerotic and normal aortic rings, it is likely that the enhanced release of arachidonate is responsible for increased biosynthesis of icosanoids.

Similar observations of cholesterol-induced increased release of arachidonate were made by Stuart *et al.* (16) in human platelets *in vitro*. Increased arachidonate release from the phospholipid pool of aortic wall cells could be related to an effect of cholesterol on membrane fluidity (17). Since the diacylglycerol lipase or phospholipase activities are very susceptible to alterations in the lipid–water interphase, it can be hypothesized that hyperlipidemia affects the lipases involved in the release of arachidonate. It has now been established that an increased turnover of the membrane phospholipid phosphatidylinositol is an early event in atherosclerosis (18). This rapid turnover occurs from phospholipase activation and leads to diacylglycerol formation and breakdown, followed by release of free arachidonate (18, 19). Released arachidonate is a substrate for icosanoid formation

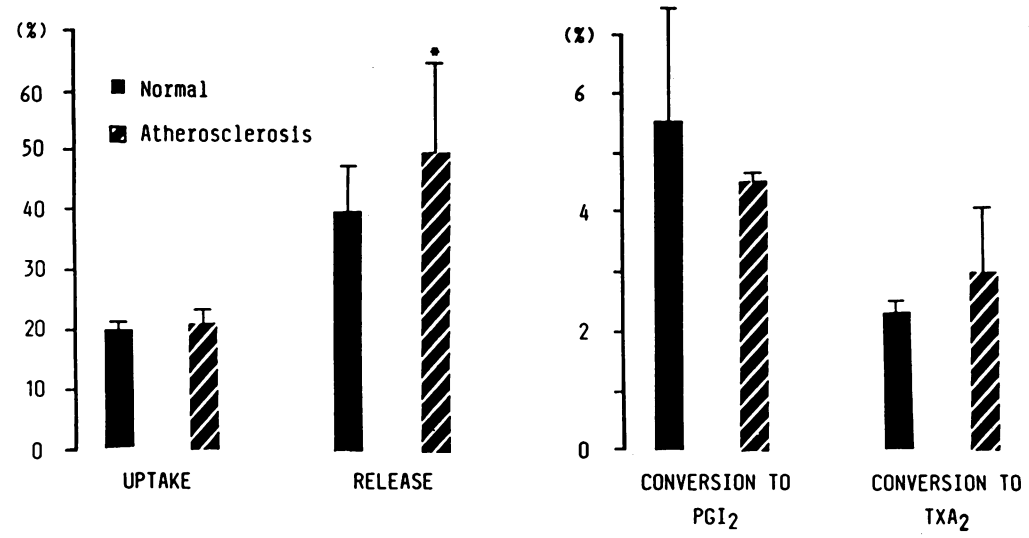

FIG. 3. Uptake of [^{14}C]arachidonate was similar in atherosclerotic and normal aortic segments. However, release of arachidonate was significantly greater in atherosclerotic aortic segments. Conversion of released arachidonate to PGI_2 and TXA_2 was similar in atherosclerotic and normal aortic rings.

by endothelial and smooth muscle cells. Although the precise cellular origin remains uncertain, our studies confirm that arachidonate release is indeed increased in experimental atherosclerosis. However, conversion of released arachidonate to icosanoids is not significantly altered. These observations suggest that increased turnover of phospholipids in lipid-laden vessels results in release of arachidonate. This eventually results in increased total PGI_2 and TXA_2 biosynthesis in atherosclerosis despite similar conversion of arachidonate to its major metabolites.

In support of our studies are the observations of enhanced release of [^{14}C]arachidonate by diabetic rat aorta (20). Fujii *et al.* (21) have also demonstrated increased PGI_2, TXA_2, and prostaglandin formation in diabetic compared to control rat mesenteric vessels; these authors suggested that enhanced production of PGI_2 from vessel wall may be a compensatory mechanism to protect against platelet aggregation.

The results of our studies are different from those of early studies demonstrating reduced PGI_2 synthesis in atherosclerosis (1–4). However, these studies in biopsy specimens of tissues employed bioassays as markers for PGI_2 generation, and bioassays are notoriously inaccurate. In the present study, we used labeled arachidonate to evaluate changes in the pattern of arachidonate metabolism, along with measurement of PGI_2 and TXA_2. The use of [^{14}C]arachidonate confirmed the results of RIA techniques used for measurement of 6-keto-$PGF_{1\alpha}$ and TXB_2. A limitation of the use of radioactive precursors is that the measurement of radioactivity may not correspond to total arachidonate released from the phospholipid and its conversion to icosanoids. In spite of

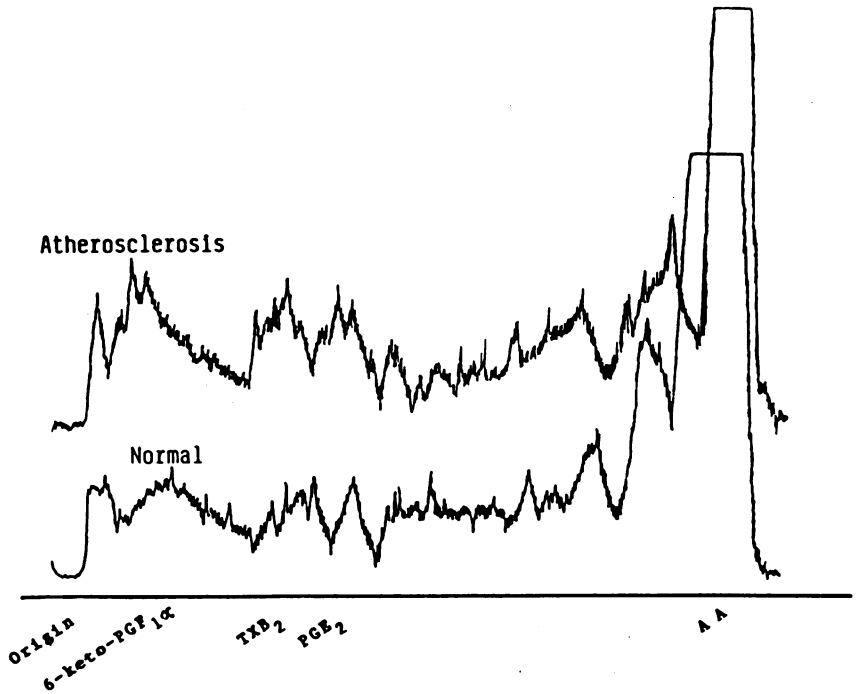

FIG. 4. Radiochromatogram ^{14}C profiles of supernates of atherosclerotic and normal aortic rings. Total radioactivity counts in the TLC plate (after extraction) were similar (59,800 and 59,200 cpm, respectively). Note higher 6-keto-[^{14}C]$PGF_{1\alpha}$ and [^{14}C]TXB_2 peaks in the supernates of atherosclerotic rings. AA, arachidonic acid.

these limitations, these data clearly indicate altered arachidonate metabolism and increased PGI_2 synthesis in atherosclerotic vessels. These data are consistent with the recent observations of increased, rather than decreased, PGI_2 biosynthesis in humans with atherosclerosis (8, 12, 13).

Synthesis of TXA_2 by the blood vessels also needs some comments. We and others have shown TXA_2 generation by human and animal blood vessels (14, 22) as well as by cultured endothelial cells (23). The present studies again demonstrate generation of significant amounts of TXA_2 by atherosclerotic and normal rabbit aortae. The identity of TXA_2 was confirmed by use of selective and nonselective icosanoid synthesis inhibitors and by use of TLC. Conversion of [^{14}C]arachidonate to [^{14}C]TXB_2 was larger than expected. This may relate to the close proximity of PGE_2 to TXB_2 on TLC plate (Fig. 4). In our previous studies, we documented vascular TXA_2 biosynthesis by use of two-dimensional TLC, which separated TXB_2 from PGE_2. Although it is possible that some of TXB_2 may have been derived from platelets adherent to the intimal surface, electron microscopy of the vascular intima in our previous studies (24) did not demonstrate significant numbers of platelets when tissues were prepared in the fashion described in this paper.

TXA_2 biosynthesis becomes relevant to atherosclerosis since the TXA_2/PGI_2 balance may determine vascular tone. It is noteworthy that the magnitude of increase in TXA_2 biosynthesis was greater than the magnitude of increase in PGI_2 biosynthesis. Furthermore, TXA_2 biosynthesis persisted longer than the PGI_2 biosynthesis. Whether those observations have pathophysiologic implications in the consequences of atherosclerosis is not clear, but it can be speculated that enhanced TXA_2 formation by blood vessels and platelets related to increased arachidonate release may be important in vasospasm and thrombosis *in vivo*. Increased PGI_2 synthesis appears to be a consequence of enhanced arachidonate release and could represent a compensatory pathophysiologic response to protect against alterations in vascular tone and platelet aggregation.

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