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AUGMENTATION OF LEUKOTRIENE C_4 AND D_4 RELEASE DUE TO SEVERE STENOSIS IN THE CANINE CORONARY ARTERY STIMULATED BY THE CALCIUM IONOPHORE A23187*

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Abstract—In dogs undergoing 24- or 72-hr severe narrowing of the left anterior descending coronary artery (LAD), the *in vitro* formation of immunoreactive leukotriene C_4 (LTC_4) by the stenosed LAD was greatly augmented by 1 μM A23187 in a 10-min incubation at 37°. This stimulated LTC_4 formation was abolished by 30 μM nordihydroguaiaretic acid (NDGA). The incubation products were identified by high performance liquid chromatography and radioimmunoassay to be largely composed of LTC_4 and LTD_4 in similar proportion. In contrast to the stenosed LAD, the non-stenotic left circumflex coronary artery, apex of the heart, and renal artery of the same experimental animals failed to respond to the calcium ionophore up to 10 μM. The vascular and cardiac tissues from sham-operated animals also remained quiescent in the presence of A23187. The normal coronary artery showed low levels of leukotriene formation and was resistant to the ionophore. It is proposed that a latent portion of leukotriene synthesis, which can be triggered by the calcium ionophore, may play a significant role in the pathogenesis of coronary artery spasm associated with acute myocardial infarction and angina pectoris in patients with obstructive coronary artery disease.

The peptido-leukotrienes (LTs) are arachidonic acid metabolites of the lipoxygenase pathway that constitute the major components of slow reacting substance of anaphylaxis (SRS-A) [1]. Polymorphonuclear leukocytes, macrophages and other inflammatory cells are believed to be the primary source of leukotriene formation; however, it has been reported recently that the coronary artery, in addition to the pulmonary artery and parenchyma, produces a significant amount of LTs [2, 3]. Because of the potent vasoconstrictor effects of LTC_4 and LTD_4 on the coronary vascular bed in various species including humans [4–8], it has been proposed that the leukotrienes may be involved in coronary vasospasm associated with acute myocardial infarction and angina pectoris [3, 9, 10].

Thromboxane A_2, a principal arachidonic acid metabolite of the cyclooxygenase pathway in platelets, has also been implicated in ischemic heart disease due to its potent vasoconstrictor and proaggregatory effects [11]. Radioimmunoassay measurements demonstrated significant elevation in thromboxane concentrations across the coronary circulation of patients with angina pectoris [12, 13] and in the distal coronary arteries of open-chest dogs with experimentally-induced coronary artery stenosis [14]. In comparison, alterations in coronary arterial leukotriene concentrations under similar conditions have not yet been studied. Consequently, we have measured the *in vitro* production of leukotrienes by radioimmunoassay in the coronary artery from dogs with severe stenosis in an attempt to ascertain the possible role of LTs in the pathogenesis of coronary artery spasm [10].

MATERIALS AND METHODS

Surgical preparation for narrowing the left anterior descending coronary artery (LAD)

Mongrel dogs of either sex weighing 13–17 kg were anesthetized with sodium pentobarbital, 30 mg/kg, i.v. (Nembutal, Abbot Laboratories). The trachea was intubated, and the animals were artificially ventilated with a Harvard respirator (stroke volume, 15 ml/kg; rate, 12 strokes/min). A lead II electrocardiogram (ECG) was monitored, starting before the surgery. Under aseptic conditions, a left thoracotomy was performed at the fourth intercostal space and the heart was suspended in a pericardial cradle. The LAD coronary artery was carefully isolated between the first and second diagonal branches and tied by means of a 2-0 silk together with a stylus (0.4 mm in diameter) alongside the vessel (inner diameter: ~2 mm); the stylus was then removed immediately. In this way the lumen of the artery was narrowed by approximately 80%. A sham procedure, which included all of the surgical steps, except for LAD constriction, was performed in fifteen animals. Pneumothorax was minimized by aspiration after the pericardium and the chest were closed. The animals were allowed to recover from

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anesthesia; this took several hours. ECG monitoring was resumed on completion of the surgery and at 24, 48 or 72 hr after surgery. One to three days following the LAD narrowing, the dogs were killed by intravenous injection of 7 ml of Euthanasia solution (Taylor Pharmacal Co., Decatur, IL). The LAD coronary artery distal to the stenosis, left circumflex coronary artery (LCX), renal artery, and apex of the heart were removed for analysis of peptidoleukotriene generating capacity.

Preparation of tissues for radioimmunoassay

The blood vessels were cleared of adhering and connective tissues and cut open during immersion in ice-cold Krebs–Henseleit buffer (composition in mM: NaCl, 137; KCl, 2.7; $CaCl_2$, 2.0; $MgCl_2$, 0.88; KH_2PO_4, 0.25; $NaHCO_3$, 24; dextrose, 10). All samples were blotted with a filter paper to remove excess fluid, and the minced tissues were then divided into several aliquots and weighed (30–40 mg/aliquot). One milliliter of Krebs–Henseleit buffer, aerated with a mixture of 95% O_2 and 5% CO_2, was added to individual samples. Unless specified otherwise, a 10-min incubation at 37° was performed on all aliquots from the same tissue sample with and without 1 μM A23187, added in 100 μl of 1% dimethyl sulfoxide (DMSO). The incubation was stopped with 4 ml of 95% ethanol, and the mixture was then kept at −15° for 1 hr to precipitate proteins. The samples were centrifuged at 3000 *g* for 10 min in a refrigerated Sorvall centrifuge (model RC-313) at 5°. The ethanolic supernatant fraction was removed and dried under N_2 at 50°. The samples were kept at −15° until measurements of LTC_4, which were performed within 2 weeks.

Normal (LCX) and post-stenotic (LAD) arterial tissues from five dogs were divided into three aliquots each and bathed in 1 ml of Krebs–Henseleit buffer. DMSO (1 μl) was added to two aliquots from each animal, whereas 1 μl of 30 mM nordihydroguaiaretic acid (NDGA; Sigma) (final concn: 30 μM) was added to the third aliquot. All samples were incubated for 5 min at 37°. This was followed by challenge with 1 μM A23187 with or without NDGA pretreatment in two aliquots from each dog.

In a separate study, the normal and stenotic arterial tissues were incubated with A23187 (0.1, 1 and 10 μM) to compare their sensitivity to the ionophore in terms of immunoreactive LTC_4 release. In addition, LTC_4 generation by both tissues in response to 1 μM A23187 was compared by varying the length of incubation time (0, 10, 30 and 60 min).

Radioimmunoassay of LTC_4

Radioimmunoassay (RIA) of LTC_4 and LTD_4 was carried out with a leukotriene C_4 RIA kit (New England Nuclear). The cross-reactivity reported by the supplier is 55% for LTD_4 and 9% for LTE_4 respectively. The N_2-dried samples were dissolved in 150 μl assay buffer and were assayed in duplicate over a range of 20–800 pg of LTC_4 equivalents.

High performance liquid chromatography (HPLC) identification of immunoreactive LTC_4 and LTD_4 from ionophore-challenged coronary artery

HPLC analysis was performed on a Waters (Milford, MA) trimodule system consisting of a model 720 controller, a model 730 data module, and a model 710B WISP automatic injector connected to two M 6000 pumps. A 10 μm Bondapak C_{18} Radialpak column (8 mm internal diameter) and pre-column were used and connected to a model 480 variable wavelength detector. For separation of leukotrienes, an isocratic solvent system of methanol–water–acetic acid (67:33:0.08, by vol.) which also contained 0.03% EDTA (w/v) buffered to pH 6.0 with NH_4OH was used. Regeneration of the column with 0.3% EDTA maintained the recovery of LTC_4 standards, as measured by u.v. absorbance at 280 nm [15].

The column was calibrated with authentic leukotriene C_4, D_4, and E_4 standards prior to analysis of biological samples. LTC_4 was obtained from Professor E. J. Corey of Harvard University, while LTD_4 and E_4 were generated with crude γ-glutamyltranspeptidase [16].

Samples of sham and stenotic arteries were stimulated with 1 μM A23187 as described above. The final N_2-dried samples were redissolved in 2 ml of water–methanol (19:1, v/v). This solution was passed over a C_{18}-Sep Pak column (Waters Associates, Milford, MA) which was then washed with 5 ml of water and 5 ml of methanol. The methanol eluate containing leukotrienes and other lipids was reduced in volume to about 0.25 ml, filtered (0.45 μm), and injected onto the high performance liquid chromatograph. Fractions (0.5 min) were collected, evaporated to dryness under N_2, and redissolved in RIA buffer and assayed for LTC_4/LTD_4 immunoreactivity as described above. Recovery of [^{3}H]LTC_4 standards through Sep-Pak isolation and HPLC for these samples was *ca.* 60%.

Statistical analyses

Student's *t*-test and Duncan's multiple range test were used to determine significant differences ($P < 0.05$). All values represent mean ± standard error of the mean.

RESULTS

LTC_4 formation in vascular and cardiac tissues following a 24- or 72-hr severe stenosis of the left anterior descending coronary artery

Twenty-four hour. As shown in Fig. 1, 1 μM A23187 significantly ($P < 0.05$, vs aliquots without A23187; Student's *t*-test for paired comparison) stimulated formation of immunoreactive (*ir*)-LTC_4 by 2.6-fold in the post-stenotic left anterior descending coronary artery (LAD). Similar increases did not occur in the non-stenotic left circumflex coronary artery (LCX), apex of the heart, and renal artery of the same animals undergoing a 24-hr LAD stenosis. In addition, none of the tissues including LAD in the sham-operated animals showed enhanced production of *ir*-LTC_4 when challenged with A23187 (Table 1 and Fig. 2). Hence, the results reveal that there is a latent component of leukotriene synthesis which can be activated by the calcium ionophore in the stenosed LAD coronary artery but not in normal vascular tissues nor in the apex that is perfused by the stenosed LAD.

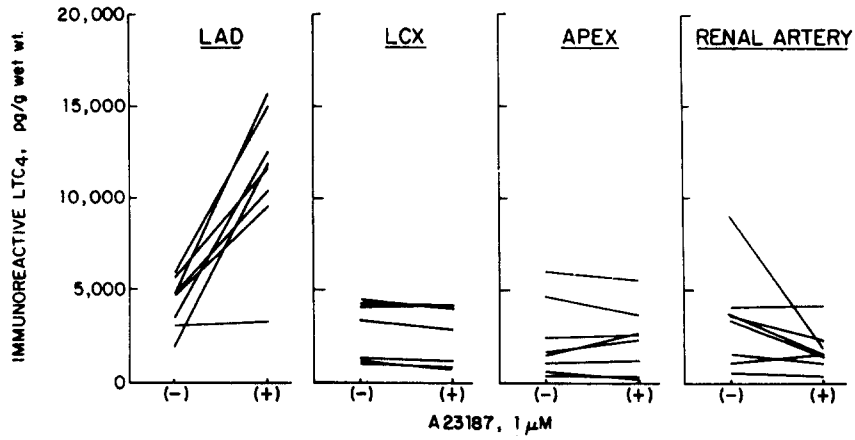

Fig. 1. Vascular and cardiac tissue concentrations of immunoreactive LTC_4 in eight dogs 24 hr following severe stenosis of the left anterior descending coronary artery (LAD). The LAD segment distal to the stenosis, left circumflex coronary artery (LCX), apex of the heart and renal artery were removed and processed for immunoreactive LTC_4 production as measured by the RIA kit (cf. Materials and Methods). Aliquots of the same tissue specimens were challenged with (+) or without (−) 1 μM A23187, and each pair of values represents individual animals. LCX was not studied in one of the eight dogs. The immunoreactive (*ir*)-LTC_4 levels are expressed in pg/g wet wt of tissue (in Table 1 they are expressed in ng/g). Only the post-stenotic LAD responded to A23187 with a significant increase in *ir*-LTC_4 formation ($P < 0.05$, Student's *t*-test for paired comparison).

A comparison between the animals undergoing sham operation and 24-hr LAD stenosis indicated that the latter had significantly greater *ir*-LTC_4 contents in the LAD and LCX in the absence of A23187 challenge (Table 1). The reason for this discrepancy is not clear.

NDGA at 30 μM effectively inhibited the augmented LTC_4 formation by the stenosed LAD in response to A23187 (Table 2).

Seventy-two hour. In the animals following a 72-hr LAD stenosis, the post-stenotic LAD still responded to 1 μM A23187 with a 2.7-fold increase in *ir*-LTC_4 formation (5.78 ± 1.39 ng/g wet wt, N = 5), in comparison to their own control not challenged with the ionophore (2.11 ± 0.35 ng/g wet wt, N = 5; $P < 0.05$, Student's *t*-test for paired comparison). The stimulated 72-hr *ir*-LTC_4 value appeared to be lower than the stimulated 24-hr value in the stenosed LAD ($P < 0.05$, Student's *t*-test) (cf. Table 1).

As in the 24-hr results, the other tissues in the

Table 1. Vascular and cardiac tissue levels of immunoreactive (*ir*) LTC_4 in dogs 24 hr following sham operation or severe stenosis of the LAD coronary artery

	A23187 challenge	N	Immunoreactive LTC_4 (ng/g wet wt) Sham	N	LAD stenosis
LAD	−	5	1.70 ± 0.22	8	4.27 ± 0.48*
	+		1.33 ± 0.33		11.23 ± 1.36*†
LCX	−	5	0.99 ± 0.16	7	2.85 ± 0.59*
	+		0.97 ± 0.24		2.62 ± 0.61*
Apex	−	5	0.80 ± 0.32	8	2.36 ± 0.71
	+		0.77 ± 0.26		2.33 ± 0.63*
Renal artery	−	5	1.65 ± 0.57	8	3.44 ± 0.94
	+		0.98 ± 0.10		1.83 ± 0.39

Dogs underwent either sham operation or severe stenosis of the left anterior descending coronary artery (LAD) and were killed after 24 hr. The LAD distal to the stenosis, left circumflex coronary artery (LCX), apex of the heart and renal artery were removed and processed for *ir*-LTC_4 production as measured by RIA (cf. Materials and Methods). Aliquots (30–40 mg) of the same tissue specimens were challenged with (+) or without (−) 1 μM A23187, the Ca^{2+} ionphore. All values represent mean ± S.E. The immunoreactive LTC_4 levels are expressed in ng/g wet wt of tissue (in Fig. 1 levels are expressed in pg/g wet wt).

* $P < 0.05$, vs corresponding sham control (Duncan's multiple range test).

† $P < 0.05$, vs aliquots of the same animal without A23187 (Student's *t*-test for paired comparison).

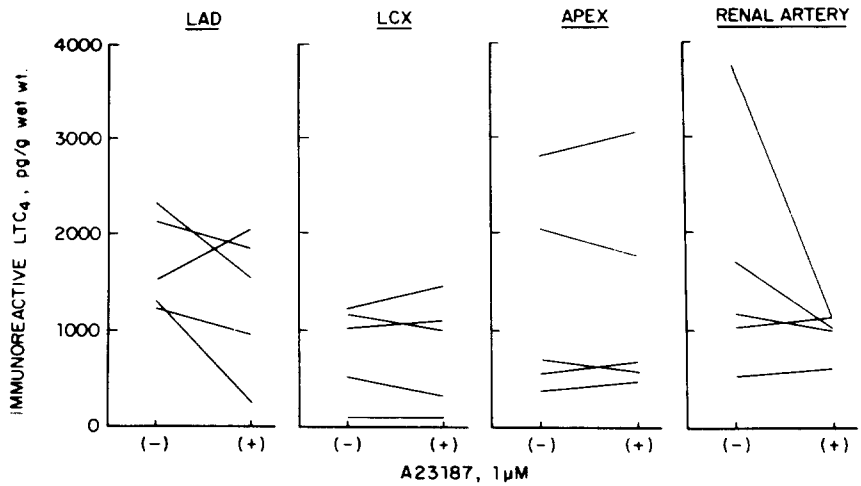

Fig. 2. Vascular and cardiac concentrations of immunoreactive LTC_4 in five dogs 24 hr following sham stenosis of the LAD coronary artery (cf. Table 1 and Fig. 1). None of the tissues including the sham-operated LAD showed a consistent rise in *ir*-LTC_4 formation in response to A23187.

Table 2. Inhibition with NDGA of the A23187-stimulated LTC_4 formation in post-stenotic LAD coronary artery

		LTC_4 (ng/g net wt)		
	N	Without A23187	1 μM A23187	30 μM NDGA +1 μM A23187
LAD	5	6.62 ± 0.60	12.42 ± 1.97*	5.37 ± 0.59†
LCX	5	8.35 ± 1.51	6.23 ± 0.88	8.04 ± 1.59

Five dogs underwent severe stenosis of the left anterior descending coronary artery (LAD) and were killed after 24 hr. The LAD distal of the stenosis and the left circumflex coronary artery were removed and divided into three aliquots. Following a 5-min incubation at 37° with DMSO or NDGA, two aliquots from each animal underwent challenge with A23187 to determine the effect of NDGA on LTC_4 formation (cf. Materials and Methods). All values represent mean ± S.E.

* $P < 0.05$, vs corresponding LAD without A23187 (Duncan's multiple range test).

† $P < 0.05$, vs corresponding LAD with A23187 (Duncan's multiple range test).

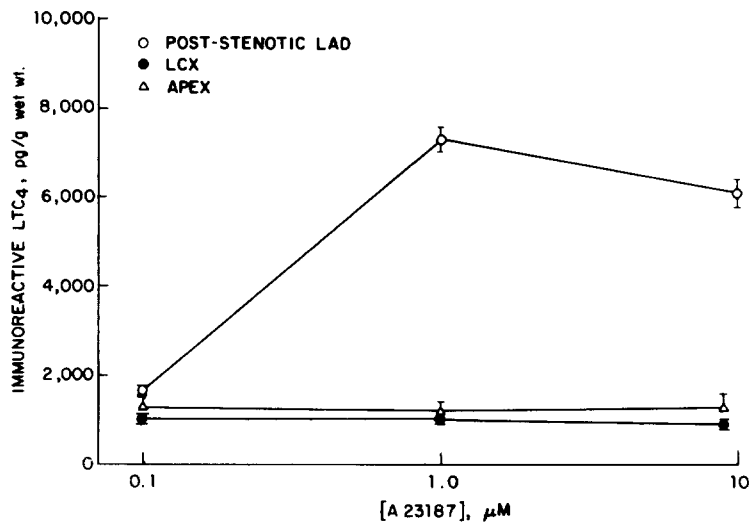

Fig. 3. Effects of A23187 concentration on *ir*-LTC_4 production by post-stenotic left anterior descending coronary artery (LAD) (—○—), non-stenotic left circumflex coronary artery (LCX) (—●—), and apex of the heart (—△—). All values represent mean ± S.E. of the same five dogs undergoing 24-hr severe LAD stenosis. The immunoreactive LTC_4, obtained after a 10-min incubation, was expressed in pg/g wet wt of tissue.

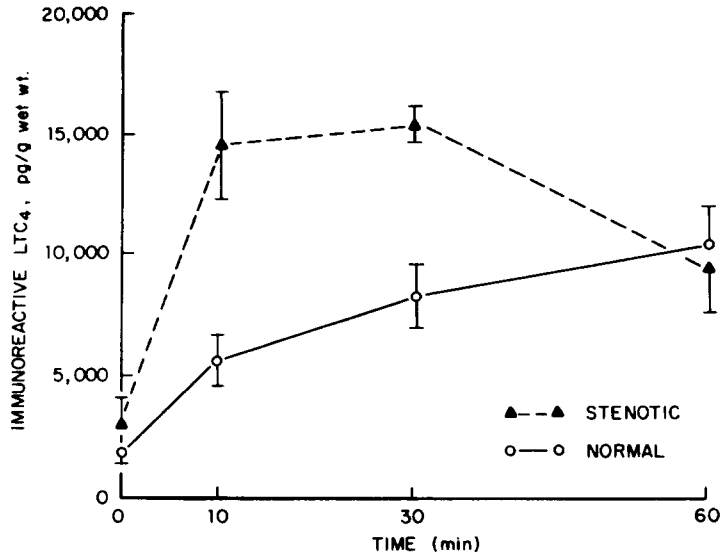

Fig. 4. Effect of incubation time on LTC_4 generation in response to 1 μM A23187 by post-stenotic (LAD) vs normal (LCX) coronary artery. Data were derived from nine dogs undergoing 24-hr severe LAD stenosis. Each point represents mean ± standard errors of the mean for three samples. The stenotic tissue values were significantly greater than the corresponding normal tissue values at 10 and 30 min ($P < 0.05$, Duncan's multiple range test).

experimental group and all tissues including LAD in the sham control failed to respond to A23187 with regard to *ir*-LTC_4 production (data not shown).

Comparative sensitivity for LTC_4 formation by the post-stenotic LAD and other tissues in response to A23187

As illustrated in Fig. 3, A23187 at 1 μM produced a maximal stimulation of *ir*-LTC_4 formation by the post-stenotic LAD; 10 μM A23187 had no further effect. By contrast, *ir*-LTC_4 formation by the non-stenotic LCX and the apex of the same animals was not affected by A23187 in concentrations of 0.1, 1 and 10 μM.

The post-stenotic LAD did not demonstrate a further increase in LTC_4 formation from a 10- to 30-min incubation with 1 μM A23187. Further prolongation of incubation to 60 min actually led to diminished formation (Fig. 4). The LTC_4 generation by the non-stenotic tissue (LCX) showed a gradual increase with time in the presence of A23187 but was lower in magnitude than stenotic tissue at 10 and 30 min. Overall, the disparity between stenotic and non-stenotic tissues in ionophore-stimulated LTC_4 generation disappeared after 60 min of incubation.

HPLC identification of LTC_4 and LTD_4 from the LAD challenged with A23187

The LTC_4 antibody used in this study has a cross-reactivity of 55% for LTD_4 and 9% for LTE_4. Consequently, it was necessary to identify and evaluate the proportions of peptido-leukotrienes in the incubation products from the stenosed LAD challenged with 1 μM A23187.

Figure 5 illustrates the identification of LTC_4 and LTD_4 by combined HPLC–RIA (Fig. 5) methodology from the post-stenotic LAD samples challenged with 1 μM A23187. After corrections were made for 55% cross-reactivity of the antiserum for LTD_4, a 60% overall recovery of [^{3}H]leukotrienes and tissue weight, it was estimated that production of LTC_4 of 2.00 ng (3.2 pmoles) per g tissue, and LTD_4 of 2.04 ng (4.1 pmoles) per g tissue occurred in the stenosed LAD. A small peak of LTC_4 immunoreactive material (0.86 ng/g tissue) also eluted before LTC_4. The nature of this more polar material

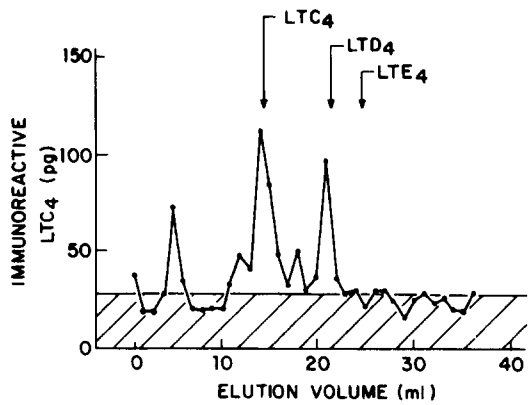

Fig. 5. HPLC–RIA identification of immunoreactive LTC_4 and LTC_4 formed in the post-stenotic left anterior descending coronary artery (LAD) in two dogs undergoing a 72-hr severe LAD stenosis. The vascular tissue was incubated with 1 μM A23187 for 10 min, and the leukotrienes produced were separated by HPLC as described in Materials and Methods. Fractions of the HPLC effluent were then measured for immunoreactive LTC_4 and LTD_4 contents. The hashed area shows the approximate noise level of the radioimmunoassay. The elution pattern of authentic leukotriene standards is shown by arrows.

is not known but represents less than 20% of the immunoreactive leukotrienes isolated. Based upon the HPLC–RIA data and the 9% cross-reactivity of the antiserum for LTE_4, the amount of LTE_4 if present must be at least several-fold less than the amount of LTC_4 or LTD_4 shown.

Changes in lead II electrocardiogram (ECG) after LAD stenosis

Electrocardiographic monitoring was performed to detect any ECG abnormalities characteristic of ischemic damage [17] which may occur as a result of severe narrowing of a major coronary artery.

Changes in ECG were not detected immediately after narrowing of the LAD coronary artery was performed. However, 24 hr after LAD stenosis, the T wave became inverted and elevation of the ST segment occurred. In addition, the R wave disappeared partially or completely and was replaced by wider Q waves, suggesting transmural infarction. These abnormalities persisted up to 72 hr when the observation was ended. In the sham-operated animals no overt ECG changes were observed.

Out of thirty dogs with severe LAD stenosis, two dogs died in 1 hr and two died overnight after surgery. One sham control also died overnight. No tissue specimens were taken from these animals.

DISCUSSION

A23187 as a calcium ionophore promotes the accumulation and mobilization of Ca^{2+} intracellularly and is a very effective agent for the activation of 5-lipoxygenase enzyme leading to the production of leukotrienes [18].

With a 10-min *in vitro* incubation at 37°, the left anterior descending coronary artery (LAD) distal to a severe stenosis responded to 1 μM A23187 with a marked increase in production of LTC_4 and LTD_4. By means of combined HPLC–RIA analysis, LTC_4 and LTD_4, which exist in similar amounts, account for the bulk of the peptido-leukotriene products in the coronary artery. The antioxidant NDGA, which inhibits lipoxygenase selectively [19], abolished the augmented response to A23187 by the stenotic LAD in LT formation.

In comparison, A23187 did not augment release of LTs in the non-stenotic coronary artery, renal artery, and apex of the heart after the same 10-min incubation. The LT formation was similar in magnitude in these tissues. They all had very low yield of LTs even in the presence of the calcium ionophore. In fact, the amount of LTs in a few samples had approached the limit of detection of the radioimmunoassay method used. By comparison, leukotriene-like activity in the porcine coronary artery estimated by guinea pig ileum assay [2] was about an order of magnitude higher than the values we obtained with canine coronary artery. In that study, tissue incubation with 9.5 μM A23187 continued up to 90 min. However, our LT values are similar to those of the cat coronary artery as reported by Lefer *et al.* using both RIA and bioassay measurements [3]; the tissue was incubated for 15–90 min in the presence of about 4 μM A23187. Since our data indicated that the production of LTs by normal tissues was not affected by A23187 up to 10 μM, it is more likely that the cause of discrepancy in the reported LT values is due to the duration of tissue incubation. But, our time–response study showed that in a 10-min incubation the stenotic tissue had practically attained a maximal increase in LTC_4 generation. By comparison, the non-stenotic coronary arteries showed a gradual rise in LT formation at a greatly lower level and reached the stenotic tissue levels only when the incubation time was prolonged to 60 min. Consequently, the differences in vascular LT values may well be due to species difference.

In the dogs undergoing a 24- or 72-hr LAD stenosis, the electrocardiographic recordings indicated the presence of transmural infarct. It has been demonstrated that myocardial infarction following coronary artery occlusion invokes acute inflammatory response and is characterized by encroachment of polymorphonuclear leukocytes (PMN) upon regions with ischemic injury [20–22]. Therefore, it is possible that PMN may have infiltrated the post-stenotic coronary artery and become a source of increased leukotriene production in the presence of A23187. In this regard, washed peripheral canine PMNs produced 1.65 ng of LTC_4 per 10^6 cells per 10 min in response to A23187. The cellular source of the enhanced peptido-leukotriene production will therefore require further investigation.

The apex of the heart, which is almost entirely supplied by the stenosed LAD, should be similarly affected with ischemic injury but the ionophore-stimulated LT release never occurred. This appears to speak against the PMN as the source of increased LTs in the post-stenotic coronary artery. A study in neutrophil-depleted animals [21, 22] ought to shed light on the role of PMN in the augmented formation of LTs. Similarly, the non-responsiveness of the ischemic apex to A23187 also argues against the possibility that the markedly enhanced LT formation by the stenotic LAD is consequent to tissue damage which might then facilitate entry of the calcium ionophore into the muscle cells.

Meerson *et al.* [23] have proposed that the "lipid triad" (activation of lipid peroxidation, activation of phospholipases and the detergent-like action of excess free fatty acid and lysophospholipids) plays a crucial role in the pathogenesis of ischemic heart damage. Defective membrane phospholipid metabolism, accumulation of arachidonic acid, and membrane Ca^{2+} permeability defect have been demonstrated in canine ischemic myocardium [24, 25]. Presumably these complex events are also applicable to the stenosed coronary artery, rendering it more responsive to various stimuli (esp. Ca^{2+}) that lead to production of excess LTs in a hypoxic environment. Also, calcium has been implicated in coronary vasconstriction during hypoxia in dogs [26] and rats [27]. This coronary vasoconstriction is effectively ameliorated by the use of calcium channel blocking agents. The observation that the potent vasoconstrictor effect of LTs (in the nanomolar range) depends on Ca^{2+} [7] is consistent with the hypothesis that the peptido-leukotrienes mediate coronary vasoconstriction in ischemic or hypoxic myocardium.

The injury-spasm concept enunciated by Hellstrom [28] assumes that severe myocardial

ischemia secondary to stenotic coronary artery disease causes vascular spasm as a hemostatic response to tissue injury. Indeed, patients with obstructive coronary artery disease and exertional angina respond to the alpha-adrenergic stimulus of the cold pressor test with an inappropriate increase in coronary artery resistance suggesting vascular hyperactivity [29]. In recent years more attention has been paid to the role of coronary artery spasm in acute myocardial infarction and various forms of angina pectoris [30, 31]. Our finding that the stenosed coronary artery is more responsive to the calcium ionophore with regard to the release of leukotriene C_4 and D_4 gives support to the hypothesis that the leukotrienes in conjuction with other factors are involved in coronary vasospasm associated with ischemic heart disease [3, 9, 10, 32]. Since leukotrienes may also play an important role in ischemic myocardial damage [22, 33–35], agents that are capable of interfering with the 5-lipoxygenase pathway and/or leukotriene receptors may be useful in the management of ischemic heart disease.

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