Regulation of Vascular Prostaglandin Synthesis by Metabolites of Arachidonic Acid in Perfused Rabbit Aorta

RICHARD S. KENT, SUSANNE L. DIEDRICH, and A. RICHARD WHORTON,
Divisions of Cardiology and Clinical Pharmacology, Departments of Medicine and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT To address the hypothesis that metabolites of arachidonic acid are important regulators of prostaglandin (PG) synthesis in intact vascular tissue, we studied arachidonate metabolism in rabbit aortas in response to a continuous infusion of arachidonic acid, 10 μg/ml. Prostacyclin (PGI_2; measured as 6-keto-$PGF_{1\alpha}$) production rate accelerated during the first 2 min, reached peak velocity at 2 min, and then progressively decelerated. The velocity profile of PGI_2 production was similar to that previously reported for cyclooxygenase holoenzyme assayed in vitro, and was consistent with progressive inactivation of the enzymes leading to PGI_2 synthesis. We determined the specific inhibition of cyclooxygenase and prostacyclin synthetase by measuring PGI_2 and PGE_2 production rates and by infusing cyclic endoperoxides. Our results indicate preferential inactivation of cyclooxygenase during arachidonate metabolism, most likely due to cyclooxygenase-derived oxidative intermediates. This was a dose-dependent response and resulted in a progressive decrease in the 6-keto-$PGF_{1\alpha}/PGE_2$ ratio. Exogenously added 15-hydroperoxy eicosatetraenoic acid, on the other hand, actually stimulated cyclooxygenase activity at low doses, while markedly inhibiting prostacyclin synthetase. This finding, along with the accelerating nature of arachidonate metabolism, is consistent with the concept of "peroxide tone" as a mediator of cyclooxygenase activity in this system.

These results demonstrate that arachidonate metabolites regulate PG synthesis in intact blood vessels. The progressive enzymatic inhibition intrinsic to arachidonate metabolism may be a model for similar changes occurring in states of enhanced lipid peroxidation. These metabolic alterations might greatly influence the numerous vascular functions known to involve arachidonic acid metabolism.

INTRODUCTION

Vascular endothelium synthesizes prostacyclin (PGI_2),[1] an extremely potent vasodilator and platelet antiaggregatory agent. Synthesis of PGI_2 by blood vessels is an important component in a number of vascular functions including the thromboresistance of the intact vascular surface (1), the recovery of thromboresistance and limitation of platelet aggregation after vascular injury (2), the inhibition of vascular smooth muscle proliferation (3), and the modulation of cholesterol ester hydrolase activity and cholesterol egress from cultured smooth muscle cells (4). In addition, prostaglandin (PG) (probably PGI_2) synthesis by the vascular endothelium plays an important role in the full hemodynamic effect of many clinically used vasoactive drugs (5–8), and is involved in the vascular response to endogenous vasoactive agents (9–11). Therefore, although circulating levels of PGI_2 are unlikely to have significant antiplatelet or hemodynamic effects in the basal state (12), the metabolism of arachidonic acid to PG in blood vessel walls appears to be a key process in the response of the vasculature to a number of pathological and pharmacological stimuli. Despite much evidence, however, of the role of arachidonic

Portions of this work were presented at the Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, LA, 22 April 1982, and appeared in abstract form in 1982. *Fed. Proc.* 41:1642*a*. (Abstr.)

Received for publication 23 December 1982 and in revised form 12 April 1983.

[1] *Abbreviations used in this paper:* 15-HPETE, 15-hydroperoxy eicosatetraenoic acid; PG, prostaglandin (E_2, $F_{2\alpha}$, etc.); PGI_2, prostacyclin.

acid metabolites in overall vascular function, there exist few data on the factors regulating the production of these metabolites.

It is clear from in vitro studies that the arachidonic acid cascade is modulated by metabolites of arachidonic acid itself. In preparations of ram seminal vesicle microsomes, for example, the oxidation of arachidonic acid leads to a rapid, irreversible inactivation of both cyclooxygenase and prostacyclin synthetase (13, 14). The cyclooxygenase of intact platelets undergoes a similar substrate-induced autodeactivation (15). It has been postulated that a highly reactive free radical, probably generated in the reduction of PGG_2 to PGH_2, is responsible for the irreversible inactivation of these enzyme systems (14, 16). Indeed, in seminal vesicle microsomes, arachidonate metabolism at increasing substrate/enzyme ratios results in a progressive decrease in the enzymatic production of PGI_2 relative to the nonenzymatic production of PGE_2 (17). This altered pattern of arachidonate metabolism is attenuated by antioxidants such as ascorbic acid and propylgallate (18). The effects of oxidizing radicals, however, generated either in the metabolism of arachidonic acid, or in the reduction of a variety of lipid peroxides, are quite complex. Thus, it appears that a certain ambient level of hydroperoxides is required for cyclooxygenase activity, and that PGG_2, once formed, may also stimulate cyclooxygenase activity in an accelerating fashion (19, 20). This concept of "peroxide tone" suggests an intricate interplay between a cellular oxidative state sufficient to activate the cyclooxygenase enzyme system and the oxidative mechanisms intrinsic to the enzyme, which result in progressive self-inactivation.

The relevance of these findings to intact vascular tissue is unknown. Although states of enhanced lipid peroxidation, such as vitamin E deficiency, atherosclerosis, and aging, are associated with reduced vascular PGI_2 production (21–23), the mechanisms involved have not been fully explained, and in particular, the potential role of arachidonate metabolite-related enzymatic inactivation remains unexplored. Accordingly, we have investigated the regulation of the arachidonic acid cascade enzymes by oxidative products of arachidonic acid metabolism in perfused rabbit aorta. Our results indicate that these enzymes are highly sensitive to arachidonate metabolites, undergo autodeactivation in response to metabolism of both exogenous and endogenous arachidonic acid, and show differential sensitivity to the inhibitory effects of endogenous and exogenous oxidizing intermediates.

METHODS

Materials. Arachidonic acid was obtained from Sigma Chemical Co., St. Louis, MO, and ionophore A23187 from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. Deuterated, tritiated PG internal standards were prepared from $[^3H_7]PGE_2$, $[^3H_7]PGF_{2\alpha}$, $[^3H_7]$6-keto-$PGF_{1\alpha}$, 80–100 Ci/mmol, (New England Nuclear, Boston, MA), $[3,3,4,4\text{-}^2H_4]PGE_2$, $[3,3,4,4\text{-}^2H_4]PGF_{2\alpha}$ (Merck, Sharp, and Dohme, Montreal, Que.), and $[3,3,4,4\text{-}^2H_4]$6-keto-$PGF_{1\alpha}$ (a kind gift of Dr. U. Axen of the Upjohn Co., Kalamazoo, MI). All solvents were high-pressure liquid chromatography grade and all derivatizing reagents were of the highest quality available.

Rabbit aorta perfusions. New Zealand White rabbits of either sex weighing 2.5–3.5 kg were maintained on standard chow. Rabbits were killed by cervical dislocation. After opening the thorax, a 4–6-cm segment of thoracic aorta was isolated and cannulated with polyethylene tubing (i.d., 1.67 mm; o.d., 2.42 mm). To minimize trauma to the aorta we did not ligate the small intercostal arteries emerging from the cannulated segment. Perfusate loss through these arteries was generally <10%. The aorta was perfused in situ with Hanks' balanced salt solution (HBSS, Gibco Laboratories, Grand Island, NY), 37°C, pH 7.4. The thoracic cavity was filled with perfusate and maintained at 37°C by a heat lamp above and a heating pad below the animal. In some experiments, as noted, the aortic segments were perfused at room temperature instead of 37°C. Perfusions were performed with a peristaltic pump (Masterflex, Cole-Parmer Instrument Co., Chicago, IL). Aortas were perfused and washed with HBSS at a rate of 10 ml/min for 10 min before the initiation of an experiment. To assess the effects of arachidonate metabolism on cyclooxygenase and prostacyclin synthetase, we perfused the aortas in various experiments with arachidonic acid (as the sodium salt), ionophore A23187, prostaglandin cyclic endoperoxides (a mixture of PGG_2 and PGH_2, prepared by the reaction of arachidonic acid with sheep seminal vesicle microsomes using standard techniques [24]), and 15-hydroperoxy arachidonic acid (prepared by the reaction of arachidonic acid with soybean lipoxygenase (Lipoxidase, Sigma Chemical Co.) using standard techniques [25]). At various time points during an experiment aliquots of perfusate were taken for PG analysis. After each experiment the aorta was measured and the endothelial surface area of the segment determined. Similar to the findings of other investigators (26), scanning electron microscopy of aortic segments excised after perfusion studies revealed preservation of the normal endothelial architecture in areas distal to the catheter tips. Loss of endothelium was demonstrated in areas around the catheters but did not extend into the perfused portion of the aortic segment.

PG purification and analysis. Labeled internal standards (600–900 ng, 150,000 dpm) were added to each sample. Samples were acidified to pH 3 with formic acid, and PG extracted with ethyl acetate (1 vol, three times). Combined ethyl acetate extracts were evaporated to dryness under reduced pressure at 35°–40°C. Samples analyzed for PGI_2 only (assayed as its stable hydrolysis end product, 6-keto-$PGF_{1\alpha}$) were redissolved in 1 ml chloroform, placed on an open bed silicic acid column (0.5 g), washed with 2 ml chloroform and 5 ml 50% chloroform/50% ethyl acetate, and eluted with 10 ml 90% ethyl acetate/10% methanol. The eluates were evaporated to dryness under N_2 and 6-keto-$PGF_{1\alpha}$ derivatized by reaction with 0.2 ml ethereal diazomethane for 30 min at room temperature followed by reaction with 0.2 ml of a saturated solution of methoxyamine HCl in pyridine overnight at room temperature. The methyl ester-methoxime 6-keto-$PGF_{1\alpha}$ derivatives, dissolved in 0.01 ml pyridine, were then reacted with 0.01 ml BSTFA (*N,O-bis*-[trimethylsilyl]-trifluoroacetamide, Pierce Chemical Co., Rockford, IL) for

2 h at room temperature before mass spectrometric analysis. Samples analyzed for PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ were dissolved in 23% acetonitrile/76.7% H_2O/0.2% toluene/0.1% glacial acetic acid and applied to a fatty acid analysis column (Waters Associates, Millford, MA). Chromatographic separation of PG was performed isocratically using a solvent delivery pump (Constametric II, Laboratory Data Control, Riviera Beach, FL). Fractions containing [^{3}H]PG were collected, extracted with ethyl acetate (1 vol, two times), evaporated to dryness under N_2, and derivatized. Purified PGE_2 and 6-keto-$PGF_{1\alpha}$ were methylated, methoxylated, and silylated, while for $PGF_{2\alpha}$ the methoxylation step was omitted. Samples containing purified, derivatized PG were analyzed by selected ion monitoring (27) using a gas chromatograph-mass spectrometer (model 5992B, Hewlett-Packard Co., Palo Alto, CA) operated with GC oven at 250°C using a 6-ft glass column packed with 3% OV-1 on 80–100 Supelcoport (Supelco, Inc., Bellefonte, PA) and a helium flow of 20–30 ml/min. The ion source and jet separator were maintained at 250°C. Monitored ions (endogenous/deuterated) were (mass/charge, m/z) 598/602 and 508/512 for the methyl ester-methoxime-trimethylsilyl ethers of 6-keto-$PGF_{1\alpha}$ and PGE_2, respectively, and 494/498 for the methyl ester-trimethylsilyl ether of $PGF_{2\alpha}$.

RESULTS

We initially investigated the rate of PGI_2 synthesis by rabbit aortas in response to a continuous infusion of arachidonic acid. The time course of PGI_2 production from infused arachidonate is shown in Fig. 1. The peak rate of synthesis occurred at 4 min and was followed by a progressive decline in the rate of arachidonate conversion to PGI_2. By 90 min the rate of PGI_2 production had decreased to 3% of the peak rate. The stability of this model was tested by preperfusing aortas with HBSS for 2 h before arachidonate infusion. We observed no difference in response between these preperfused aortas and those reported in Fig. 1 (data not shown). Thus, autoinactivation of PG cascade enzymes occurs during arachidonic acid metabolism and leads to a marked reduction in the further metabolism of arachidonate to PGI_2.

Under the conditions of continuous arachidonate infusion, the time-dependent ability of these aortas to produce PGI_2 must be a complex function of substrate delivery, enzymatic activity (perhaps determined by ambient levels of hydroperoxides and the stimulatory effects of enzymatically produced PGG_2), and enzymatic self-destruction. To assess the effect of substrate delivery, we repeated the above studies using a perfusion rate of 20 ml/min (Fig. 1 *inset*). Despite the increased flow, there was no distension of the aortic segments that might tend to increase PGI_2 production. At the higher infusion rate peak production of PGI_2 was 28.9 ng/cm^2 per min. This peak occurred at 2 min. These values represent the maximal production rate and minimal time to peak rate achievable at 25°C in this preparation. The velocity profile of enzymatic

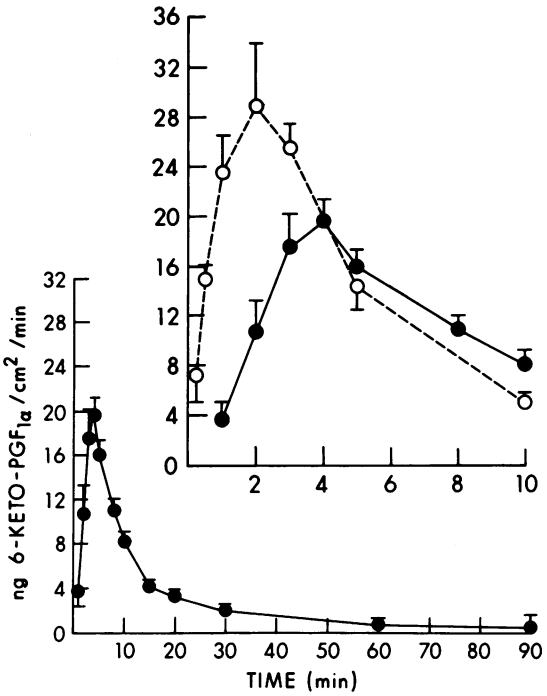

FIGURE 1 Aortic PGI_2 production rate in response to a continuous infusion of arachidonic acid, 10 μg/ml. PGI_2 was measured as its stable hydrolysis product, 6-keto-$PGF_{1\alpha}$. Each point represents a 1-min aliquot of perfusate for aortas perfused at 1 ml/min, 25°C. Data shown as mean±SEM, n = 7. *Inset:* aortic PGI_2 production rate in response to continuous infusions of arachidonic acid, 10 μg/ml, at two different rates. The closed circles represent the first seven time points from Fig. 1 (perfusion rate 1 ml/min). The open circles are data in five additional rabbit aortas perfused at 20 ml/min, 25°C. These points represent 0.25–1-min aliquots of perfusate. Data shown as mean±SEM.

activity in these aortas is very similar to that found in cyclooxygenase holoenzyme assayed in vitro (20) and suggests that acceleration of arachidonate metabolism results from the stimulatory effect of newly generated PGG_2 on cyclooxygenase, while subsequent deceleration of the reaction is caused by enzymatic autoinactivation. The peak reaction velocity and time to peak velocity are determined by the balance of these processes, which in turn is affected by the rate of substrate delivery.

Fig. 2 is a dose-response curve of maximal PGI_2 production rate plotted against concentration of infused arachidonic acid. The arachidonate EC_{50} in this model is ~0.4 μg/ml. No decrease in maximal response was seen with increasing concentrations of infused arachidonate up to 10 μg/ml (cf Fig. 1 *inset*).

To investigate the relative inhibitory effects of arachidonate metabolism on cyclooxygenase and prostacyclin synthetase, we measured 6-keto-$PGF_{1\alpha}$ and

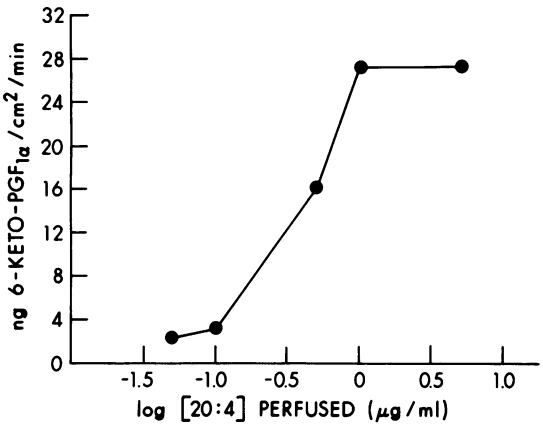

FIGURE 2 Dose-response curve of maximal aortic PGI_2 production rate vs. concentration of infused arachidonic acid. Each point represents the maximal aortic PGI_2 (6-keto-$PGF_{1\alpha}$) production rate achieved during an infusion of arachidonic acid, 20 ml/min, 25°C, in a single rabbit.

PGE_2 production rates both initially (maximal rates, determined as in Fig. 1 *inset*) and after 1-h infusion of arachidonic acid (Fig. 3). No $PGF_{2\alpha}$ was detected. As shown in Fig. 3, total cyclooxygenase products (6-keto-$PGF_{1\alpha}$ plus PGE_2) decreased by 93% from 39.1 ng/cm^2 per min initially to 2.8 ng/cm^2 per min at the end of the arachidonic acid infusion. The rate of 6-keto-$PGF_{1\alpha}$ production decreased by 97% while the PGE_2 production rate decreased by 80%, resulting in a reversal of the 6-keto-$PGF_{1\alpha}$/PGE_2 ratio. These data are consistent with a 93% decrease in cyclooxygenase activity and a 57% decrease in prostacyclin synthetase activity.[2] The greater inhibition of cyclooxygenase was statistically significant ($P < 0.01$ by Student's t test).

To confirm the preferential inhibition of cyclooxygenase compared with prostacyclin synthetase during the metabolism of arachidonic acid we studied the activity of prostacyclin synthetase directly (Fig. 4). Cyclic endoperoxides, a mixture of PGG_2 and PGH_2, 2 μg/ml, were infused before and after a 1-h infusion of arachidonic acid 10 μg/ml. The production rate of 6-keto-$PGF_{1\alpha}$ at the end of the arachidonic acid infusion period, 1.5 ng/cm^2 per min (Fig. 4), represented a 95% inhibition of the maximal 6-keto-$PGF_{1\alpha}$ production rate in this model (cf Figs. 1 *inset* and 3). Despite this 95% decrease in the metabolism of arachidonic acid to PGI_2, however, prostacyclin synthetase activity, assessed by the metabolism of cyclic endoperoxides to PGI_2, decreased by only 59% (7.87 ng/cm^2 per min to 3.24 ng/cm^2 per min, Fig. 4). These results confirm a relative sparing of prostacyclin synthetase compared with cyclooxygenase and are consistent with the data presented in Fig. 3, where a 57% decrease in prostacyclin synthetase was inferred. The findings also indicate that in this model, changes in the activities of cyclooxygenase and prostacyclin synthetase can be predicted by changes in PGI_2 and PGE_2 production rates.

The next set of experiments was performed to establish a "dose-response" relationship between total arachidonate metabolism and the extent of arachidonic acid cascade enzyme inactivation. Rabbit aortas were preperfused for 1 h with concentrations of arachidonic acid ranging from 0 (control) to 1.0 μg/ml. At the end of 1 h residual PGI_2 production was determined by an infusion of arachidonic acid 10 μg/ml, 20 ml/min. Fig. 5 clearly shows the progressive decline in remaining enzymatic activity with increasing arachidonate preperfusion concentration. Also of note are the higher peak rates of 6-keto-$PGF_{1\alpha}$ production achieved, and the more rapid acceleration/deceleration of these reactions at 37°C compared with 25°C (cf Fig. 1 *inset*).

To assess the arachidonate "dose-related" inhibition of cyclooxygenase and prostacyclin synthetase specifically, the maximal production rates of PGI_2 (2-min time points, Fig. 5) and simultaneously measured PGE_2 production rates for each arachidonate preperfusion concentration were analyzed similarly to the data in Fig. 3. The data in Fig. 6 indicate a progressive decrease in the 6-keto-$PGF_{1\alpha}$/PGE_2 ratio with increasing preperfusion concentrations of arachidonic acid. Interestingly, although there is a progressive decline in PGI_2 production, PGE_2 production actually increases at low arachidonate preperfusion concentrations with a subsequent slower decline as arachidonate preperfusion concentration increases. We interpret these data to indicate diversion of cyclic endoperoxides from PGI_2 to PGE_2 synthesis secondary to prostacyclin synthetase inactivation. With progressive inhibition of cyclooxygenase, PGE_2 synthesis eventually declines as well.

[2] We have determined that 6-keto-$PGF_{1\alpha}$ and PGE_2 are the only cyclooxygenase products produced, and that cyclic endoperoxides not metabolized to 6-keto-$PGF_{1\alpha}$ (PGI_2) by prostacyclin synthetase are converted to PGE_2. Were the decrease in PGI_2 and PGE_2 production rates due solely to cyclooxygenase inhibition, we would expect a 93% decrease in both, with preservation of the initial 6-keto-$PGF_{1\alpha}$/PGE_2 ratio. The expected production rates for PGI_2 and PGE_2 would therefore be 2.1 ng/cm^2 per min and 0.7 ng/cm^2 per min (7% of 29.7 ng/cm^2 per min and 9.4 ng/cm^2 per min, respectively; Fig. 3). The actual postarachidonate production rate for PGI_2, 0.9 ng/cm^2 per min, is thus consistent with an additional 57% decrease in prostacyclin synthetase activity (43% of 2.1 ng/cm^2 per min). The actual postarachidonate production rate for PGE_2, 1.9 ng/cm^2 per min, represents its expected rate of 0.7 ng/cm^2 per min plus the 1.2 ng/cm^2 per min of endoperoxide diverted from prostacyclin synthetase (2.1 ng/cm^2 per min PGI_2 expected—0.9 ng/cm^2 per min PGI_2 found) to PGE_2 formation.

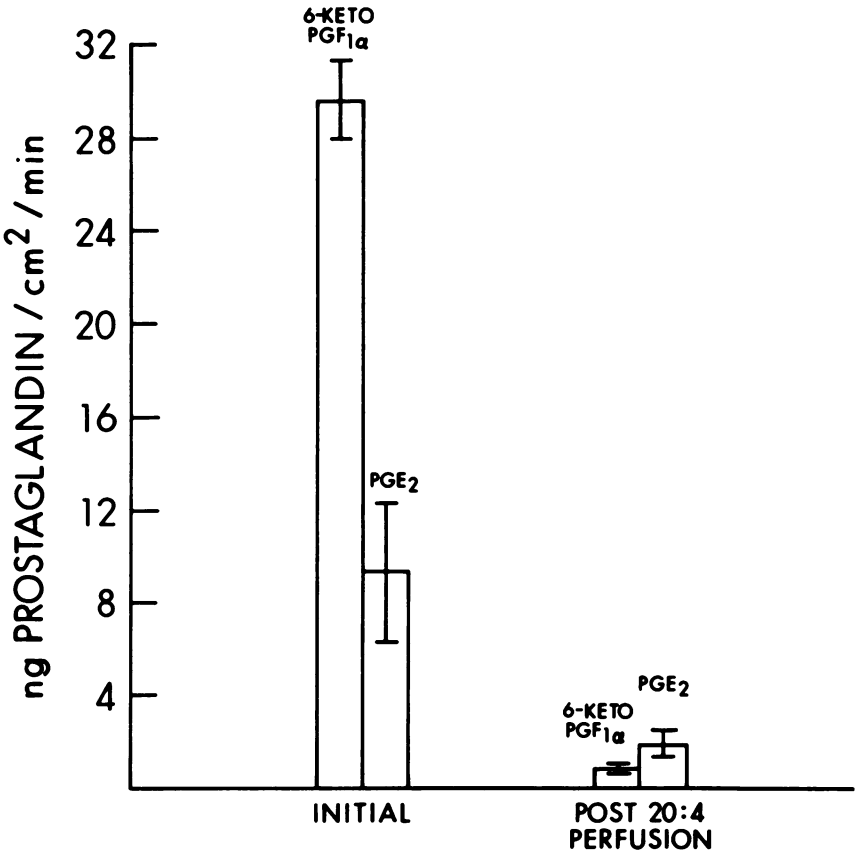

FIGURE 3 Effect of aortic arachidonic acid metabolism on PGI_2 and PGE_2 production rates. Initial: arachidonic acid, 10 μg/ml, was infused at 20 ml/min, 25°C, for determination of the maximal rate of PG production (as in Fig. 1 *inset*). Postarachidonic acid (20:4) infusion: the infusion was continued for 62 min at 2 ml/min. From 58 to 62 min the perfusate was collected for PG analysis. Data shown as mean±SEM, $n = 5$.

The data in Fig. 6 were analyzed to determine the inhibition of both cyclooxygenase and prostacyclin synthetase. These results, which are shown in Table I, indicate the "dose-related" nature of enzymatic inactivation by arachidonate metabolites. There is a close correlation between total arachidonic acid metabolism and the inhibition of both cyclooxygenase and prostacyclin synthetase activities. Furthermore, it appears that although both enzymes are inactivated, the metabolism of exogenous arachidonic acid leads to the preferential inactivation of cyclooxygenase. This trend reaches statistical significance for the preperfusion concentrations of 0.10 μg/ml (Table I) and 10 μg/ml (analysis of data in Fig. 3). These results are not surprising given in vitro findings demonstrating the generation of reactive oxidizing intermediates in the reduction of PGG_2 to PGH_2 by the peroxidase moiety of cyclooxygenase (13, 14, 19, 20), the possible situation of prostacyclin synthetase at some distance from the free radical-generating site, and the fact that prostacyclin synthetase does not generate inactivating free radicals (28).

To compare the metabolic effects of endogenously released arachidonic acid with those of exogenously added arachidonate we infused ionophore A23187, 4 μM, into five aortas (Fig. 7). The peak 6-keto-$PGF_{1\alpha}$ production rate, 6.9 ng/cm^2 per min, represented ~25% of the maximum obtained with exogenous arachidonic acid (Fig. 1 *inset*). The rate of PGI_2 production progressively declined, reaching a minimum at 30 min. At the end of a 90-min infusion and 15-min washout period, arachidonic acid was infused. Interestingly, these aortas demonstrated considerable residual capacity to metabolize arachidonate to PGI_2. The resultant 6-keto-$PGF_{1\alpha}$ production rate, represented 35% of maximal production capacity (Fig. 1 *inset*). Thus, it appears that in these experiments the metabolism of endogenously released arachidonic acid did

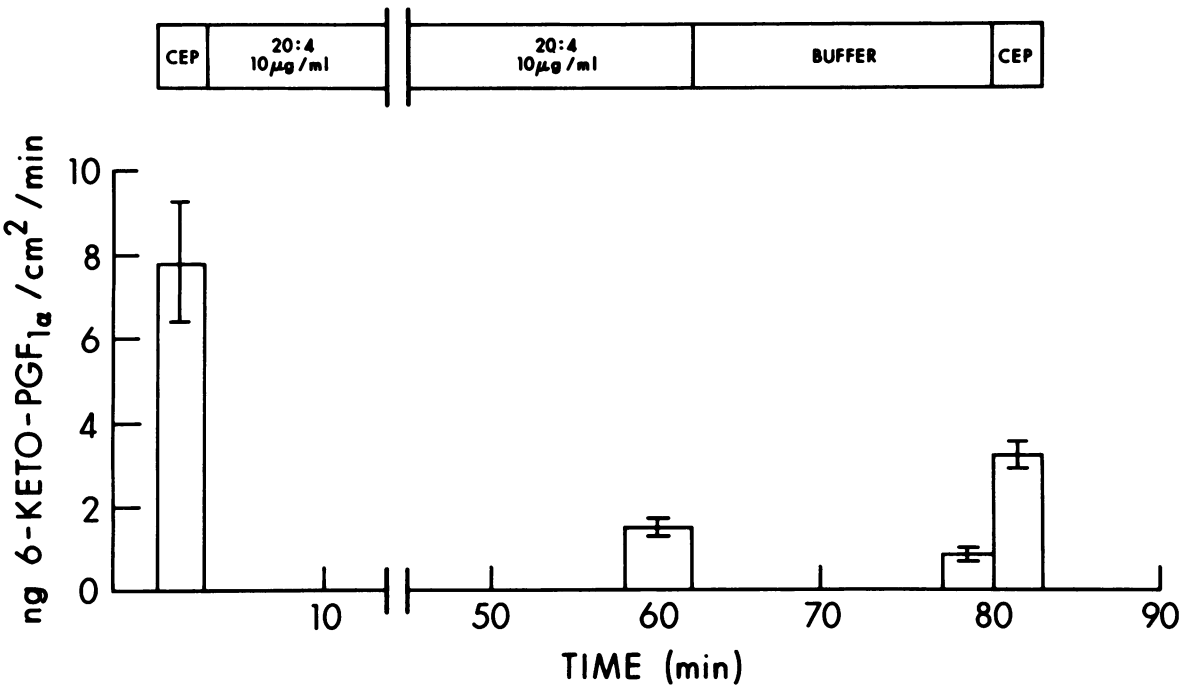

FIGURE 4 Effect of aortic arachidonic acid metabolism on the conversion of prostaglandin cyclic endoperoxides (CEP) to PGI_2. CEP, 2 μg/ml, were infused at 20 ml/min, 25°C, for 3 min, and the perfusate collected for analysis. Arachidonic acid (20:4), 10 μg/ml, was then infused for 1 h at 2 ml/min. Following a 15-min washout period, CEP, 2 μg/ml, were again infused at 20 ml/min. Data shown as mean±SEM, n = 7.

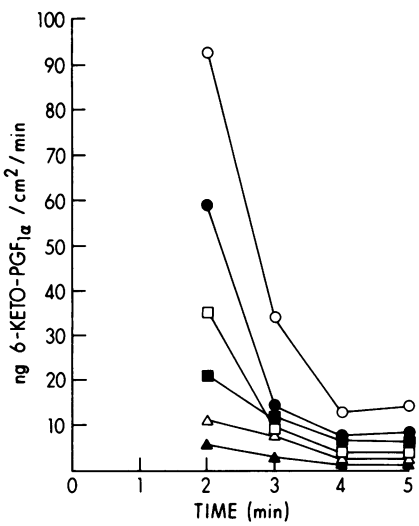

FIGURE 5 Relationship of total arachidonate metabolism to residual PGI_2 production. Aortas were preperfused for 60 min with various concentrations of arachidonic acid (μg/ml) (0.00, ○; 0.01, ●; 0.05, □; 0.10, ■; 0.5, △; 1.0, ▲). At 60 min (time 0) all aortas were perfused with arachidonic acid, 10 μg/ml to determine residual enzymatic activity. All perfusions were done at 37°C. n = 3–5 for each group. Error bars omitted for clarity.

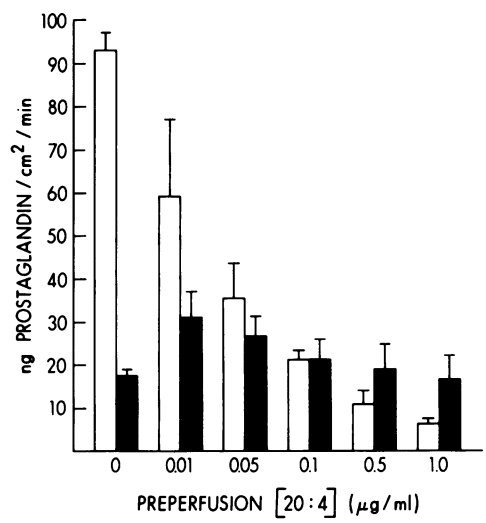

FIGURE 6 Relationship of total arachidonate metabolism to residual PGI_2 and PGE_2 production rates. Peak PGI_2 (open bars; data taken from the 2-min time points of Fig. 5) and PGE_2 (solid bars) production rates in response to an infusion of arachidonic acid, 10 μg/ml, 20 ml/min, 37°C, were determined after 60-min preperfusions with the indicated arachidonate (20:4) concentrations. Data shown as mean±SEM; n = 3–5 for each group.

TABLE I
Enzymatic Inhibition by Metabolites of Arachidonic Acid

20:4 1-h preperfusion	n*	Percentage of control	
		Cyclooxygenase activity	Prostacyclin synthetase activity
μg/ml			
0.00	(3)	100	100
0.01	(4)	81.5±14.6‡	81.9±9.1
0.05	(4)	56.2± 9.4	68.2±3.4
0.10	(5)	39.0± 4.8	61.7±5.2§
0.50	(3)	27.2± 7.2	43.3±5.9
1.00	(3)	20.9± 5.1	34.7±6.6

20:4, arachidonic acid.
* Number of experiments.
‡ Data represent mean±SEM.
§ $P < 0.01$ by Student's t test.

indeed lead to arachidonic cascade enzymatic inactivation, but, in addition, progressive exhaustion of accessible arachidonic acid stores occurred, limiting the total amount of substrate metabolized and the extent of enzyme inhibition.

The metabolism of arachidonic acid by cyclooxygenase provides an endogenous source of peroxidized fatty acids and free radicals. To compare the effects of endogenously produced and exogenously added peroxidized fatty acids we infused 15-hydroperoxy eicosatetraenoic acid (15-HPETE), 2 μg/ml, into six rabbit aortas. After a washout period, arachidonic acid was infused and peak 6-keto-$PGF_{1\alpha}$ and PGE_2 production rates determined. The results are shown in Table II. Of note is the fact that 15-HPETE actually mildly stimulated total cyclooxygenase activity (6-keto-$PGF_{1\alpha}$ plus PGE_2), but markedly inhibited PGI_2 synthesis. The reason for the increase in cyclooxygenase activity is not clear but could conceivably involve alterations in "peroxide tone." When the preperfusion concentration of 15-HPETE was increased to 10 μg/ml, the post-15-HPETE production rates in response to arachidonate were: 6-keto-$PGF_{1\alpha}$, 1.3±0.5 ng/cm² per min and PGE_2, 53.5±14.1 ng/cm² per min (mean±SEM, n = 3). Thus, the inhibitory effects of

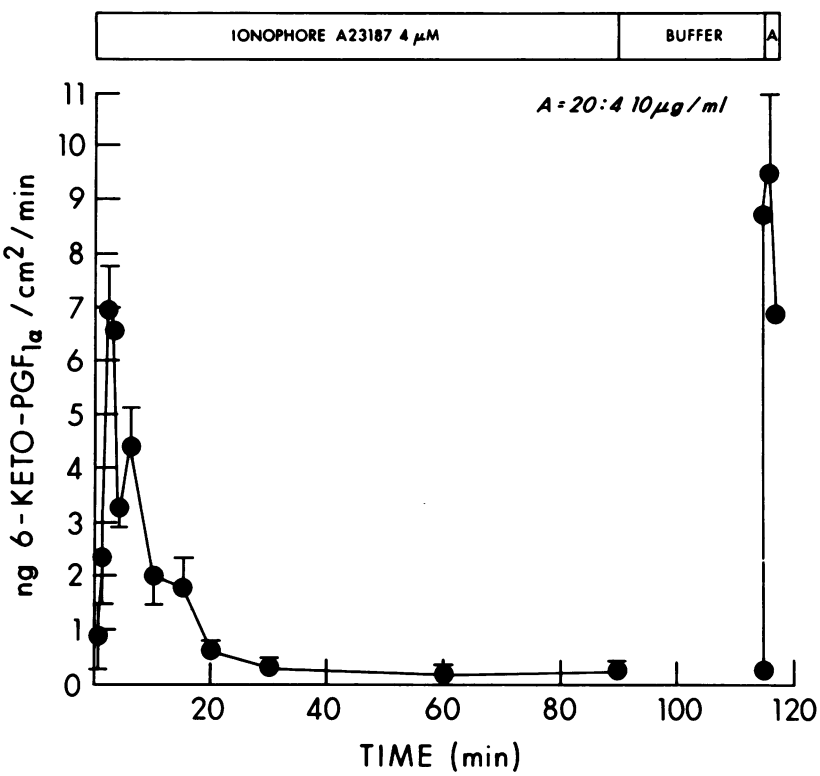

FIGURE 7 Aortic PGI_2 production rate in response to a continuous infusion of ionophore A23187, 4 μM. The initial infusion rate, 20 ml/min, was maintained for 5 min and then lowered in a stepwise manner over the next 5 min to 2 ml/min. After a 90-min infusion and a 15-min washout, arachidonic acid (20:4), 10 μg/ml, was infused at 20 ml/min. Each point represents a 0.5–2-min aliquot of perfusate for five aortas perfused at 25°C. Data shown as mean±SEM.

TABLE II

Effect of Preperfused 15-HPETE on Subsequent Rates of PG Production from Exogenous Arachidonic Acid

	6-keto-$PGF_{1\alpha}$	PGE_2	Total products	n
Control	92.9±4.1	17.5± 1.2	110.4± 3.2	3
15-HPETE	19.7±6.4	123.9±11.9	143.5±10.2*	6

15-HPETE, 2 μg/ml, was infused at 37°C for 15 min. After a 15-min washout, arachidonic acid, 10 μg/ml, was infused at 20 ml/min and peak PGI_2 (6-keto-$PGF_{1\alpha}$) and PGE_2 production rates (ng/cm^2 per min) determined. Data shown as mean±SEM. *n*, number of experiments. Control data taken from control (arachidonic acid = 0) points of Fig. 6.

* $P = 0.06$ compared with control (two-sample, unpaired *t* test).

exogenously supplied oxidizing intermediates (greater inhibition of prostacyclin synthetase than cyclooxygenase) are opposite that found with the generation of endogenous oxidizing intermediates from arachidonic acid metabolism.

DISCUSSION

These experiments provide evidence that the metabolism of exogenous or endogenous arachidonic acid in intact vascular tissue leads to the progressive inactivation of both cyclooxygenase and prostacyclin synthetase. The extent of inhibition of these enzymes is related to both arachidonate concentration and time of exposure in a dose-dependent manner. Our results indicate that cyclooxygenase is especially vulnerable to irreversible inactivation from endogenously produced oxidative metabolites of arachidonic acid, but is actually stimulated by low doses of exogenous hydroperoxy fatty acids. Prostacyclin synthetase, on the other hand, is inactivated to a lesser extent by endogenously produced oxidative intermediates, but, in agreement with previous studies (14, 29), is readily inactivated by exogenous peroxidized fatty acids. These findings are thus consistent with the hypothesis that in blood vessels, intermediates of arachidonic acid metabolism are potentially important regulators of the arachidonic acid cascade, and therefore of the vascular response to a variety of stimuli.

The cyclooxygenase enzyme complex actually catalyzes two specific reactions: a double dioxygenase reaction during which PGG_2 is formed from arachidonic acid (30, 31), and a peroxidase reaction which reduces the 15-hydroperoxy on PGG_2 to a hydroxyl on PGH_2 (13, 32, 33). The inactivation of cyclooxygenase, which occurs as a concomitant of arachidonic acid metabolism in cell-free systems, is due to the production of a highly reactive free radical during the reduction of PGG_2 to PGH_2 (13, 16, 20, 33). Although the exact nature of this reactive oxidant remains undefined, it is responsible for the inactivation of both oxygenase and peroxidase activities of cyclooxygenase, as well as prostacyclin synthetase (13, 14, 33). Recent evidence in cultured cells suggests that prostacyclin synthetase is an "innocent bystander" in this process; being inactivated by cyclooxygenase-derived free radicals, but not itself producing enzyme-inactivating intermediates in the metabolism of PGH_2 to PGI_2 (28). Antioxidants, such as ascorbic acid and propylgallate, have been shown to "protect" prostacyclin synthetase from inactivation in microsomal preparations during arachidonate metabolism, presumably by quenching oxidative radicals (18).

Despite the self-destructive nature of its catalytic activity, cyclooxygenase actually requires a certain ambient level of peroxides to function (19, 20) and is, in fact, inhibited by conditions in which peroxides are not allowed to form. From these observations, Hemler et al. (19, 20) have postulated that the balance between formation and removal of cellular lipid peroxides sets a "peroxide tone" that can regulate the rate of PG formation in cells.

Our data confirm that these concepts, developed from work with cell-free, microsomal preparations, extend to the intact blood vessel. We observed, even with very rapid arachidonate infusion rates, a lag period of ~2 min before the peak velocity of PGI_2 production occurred. Although diffusion phenomena may have contributed to some extent to this delay, the acceleration of arachidonate metabolism during this period is also consistent with low initial levels of hydroperoxide, which gradually increase as product hydroperoxide accumulates (20). The duration of cyclooxygenase activity before total self-destruction is markedly longer in intact vascular tissue (where we detected some activity even after 90 min) than in cell-

free in vitro systems (where total inactivation generally occurs between 1 and 5 min (15, 20, 34)). This difference may be due to antioxidants in intact tissue that are lost in microsomal preparations.

Our results suggest a close dose-response relationship between the amount of arachidonate metabolized and the extent of enzymatic inactivation. The data in Fig. 5 indicate significant enzyme inhibition by infusions of arachidonic acid at concentrations as low as 10 ng/ml. These data are consistent with a dose-response relationship that may extend to arachidonate levels far <10 ng/ml. These levels may be found in the vicinity of the cyclooxygenase following phospholipase activation and subsequent arachidonic acid release from membrane lipids. Our data do not, however, indicate whether a "critical level" of arachidonate metabolism exists below which antioxidant mechanisms are sufficient to prevent oxidative enzyme damage, or whether this level can be modified by antioxidant treatment. Previous studies of the effects of antioxidants on arachidonic acid metabolism have yielded variable results, depending on tissue, species, and specific antioxidant (18, 21, 33, 35–40). This finding is not surprising given the potential of oxidative intermediates to both stimulate and inhibit the enzymes of the arachidonic acid cascade. It remains unclear, therefore, whether the progressive inhibition of vascular PGI_2 production seen with age (23) and in atherosclerosis (22) is preventable by antioxidant treatment.

The differential sensitivity of cyclooxygenase and prostacyclin synthetase to both exogenous and endogenous oxidative intermediates results in a reversal of the normal 6-keto-$PGF_{1\alpha}$/PGE_2 ratio. Indeed, in experiments in which we infused 15-HPETE at 2 μg/ml (6.5 μM), cyclooxygenase was actually stimulated while prostacyclin synthetase was markedly inhibited, leading to very high PGE_2 levels. These results are similar to those of Hemler et al. (19), who found potentiation of cyclooxygenase activity in a purified enzyme preparation by various peroxides in the range of 0 to 2 μM. In general, PGE_2 does not have the potent vasodilatory or antiplatelet effects of PGI_2 (41–43), and in the example of cholesterol ester hydrolytic activity, is inactive (4). Thus, despite increased cyclooxygenase activity due to enhanced "peroxide tone," arachidonic acid metabolism is diverted to biologically less active or inactive products as cyclic endoperoxides are diverted away from inactivated prostacyclin synthetase. An interesting study bearing on these phenomena is that of Chang et al. (44) who examined PG production in cultured rat aortic smooth muscle cells as a function of animal age. They demonstrated that decreased PGI_2 synthesis was due to lower prostacyclin synthetase activity. No change in total cyclooxygenase activity was observed. When compared to our data, these findings are consistent with the effects of an exogenous, non-cyclooxygenase source of oxidative intermediates, but whether such enzyme alterations can be caused by age-related increases in cellular lipid peroxides is speculative.

The ability of vascular tissue to metabolize arachidonic acid clearly depends on substrate availability. With potent continuing stimulation of phospholipase, the available pools may become depleted. Our data suggest that normal rabbit aorta contains sufficient arachidonate to significantly inactivate cyclooxygenase during maximal phospholipase stimulation with ionophore A23187. We did not, however, examine the inactivation of individual enzymes, and also cannot exclude a direct effect of the ionophore.

We have shown that the proposed mechanisms regulating PG synthesis in cell-free preparations are also applicable to intact vascular tissue. Because of the involvement of arachidonic acid metabolites in the response of the vasculature to a number of physiological, pharmacological, and pathological stimuli, the mechanisms regulating the production of arachidonate metabolites may be important components in the functioning of blood vessels in a variety of disease states.

ACKNOWLEDGMENTS

This work was supported by grant-in-aid 79-923 from the American Heart Association, with funds contributed in part by the North Carolina Affiliate, by a National Institutes of Health grant AG 02868, and by a grant-in-aid from the Burroughs Wellcome Fund. Dr. R. S. Kent was supported by a Clinician-Scientist Award from the American Heart Association and Dr. A. R. Whorton by a Research Career Development Award, HL00857 from the National Institutes of Health.

REFERENCES

1. Czervionke, R. L., J. B. Smith, G. L. Fry, J. C. Hoak, and D. L. Haycraft. 1979. Inhibition of prostacyclin by treatment of endothelium with aspirin: correlation with platelet adherence. *J. Clin. Invest.* 63:1089–1092.
2. Eldor, A., D. J. Falcone, D. P. Hajjar, C. R. Minick, and B. B. Weksler. 1981. Recovery of prostacyclin production by deendothelialized rabbit aorta: critical role of neointimal smooth muscle cells. *J. Clin. Invest.* 67:735–741.
3. Huttner, J. J., E. T. Gwebu, R. V. Panganamala, G. E. Milo, D. G. Cornwell, H. M. Sharma, and J. C. Geer. 1977. Fatty acids and their prostaglandin derivatives: inhibitors of proliferation in aortic smooth muscle cells. *Science (Wash. DC).* 197:289–291.
4. Hajjar, D. P., B. B. Weksler, D. J. Falcone, J. M. Hefton, K. Tack-Goldman, and C. R. Minick. 1982. Prostacyclin modulates cholesterol ester hydrolytic activity by its effect on cyclic adenosine monophosphate in rabbit aortic smooth muscle cells. *J. Clin. Invest.* 70:479–488.

5. Levin, R. I., E. A. Jaffe, B. B. Weksler, and K. Tack-Goldman. 1981. Nitroglycerin stimulates synthesis of prostacyclin by cultured human endothelial cells. *J. Clin. Invest.* 67:762–769.
6. Morcillo, E., P. R. Reid, N. Dubin, R. Ghodgaonkar, and B. Pitt. 1980. Myocardial prostaglandin E release by nitroglycerin and modification by indomethacin. *Am. J. Cardiol.* 45:53–57.
7. Rubin, L. J., and J. D. Lazar. 1981. Influence of prostaglandin synthesis inhibitors on pulmonary vasodilatory effects of hydralazine in dogs with hypoxic pulmonary vasoconstriction. *J. Clin. Invest.* 67:193–200.
8. Swartz, S. L., G. H. Williams, N. K. Hollenberg, L. Levine, R. G. Dluhy, and T. J. Moore. 1980. Captopril-induced changes in prostaglandin production: relationship to vascular responses in normal man. *J. Clin. Invest.* 65:1257–1264.
9. Whorton, A. R., S. L. Young, J. L. Data, A. Barchowsky, and R. S. Kent. 1982. Mechanism of bradykinin-stimulated prostacyclin synthesis in porcine aortic endothelial cells. *Biochim. Biophys. Acta.* 712:79–87.
10. Needleman, P., G. R. Marshall, and B. E. Sobel. 1975. Hormone interactions in the isolated rabbit heart; synthesis and coronary vasomotor effects of prostaglandins, angiotensin, and bradykinin. *Circ. Res.* 37:802–808.
11. Blumberg, A. L., S. E. Denny, G. R. Marshall, and P. Needleman. 1977. Blood vessel-hormone interactions: angiotensin, bradykinin, and prostaglandins. *Am. J. Physiol.* 232:H305–H310.
12. Fitzgerald, G. A., A. R. Brash, P. Falardeau, and J. A. Oates. 1981. Estimated role of prostacyclin secretion into the circulation of normal man. *J. Clin. Invest.* 68:1272–1276.
13. Egan, R. W., J. Paxton, and F. A. Kuehl, Jr. 1976. Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J. Biol. Chem.* 251:7329–7335.
14. Ham, E. A., R. W. Egan, E. E. Soderman, P. H. Gale, and F. A. Kuehl, Jr. 1979. Peroxidase-dependent deactivation of prostacyclin synthetase. *J. Biol. Chem.* 254:2191–2194.
15. Lapetina, E. G., and P. Cuatrecasas. 1979. Rapid inactivation of cyclooxygenase after stimulation of intact platelets. *Proc. Natl. Acad. Sci. USA.* 76:121–125.
16. Kalyanaraman, B., R. P. Mason, B. Tainer, and T. E. Eling. 1982. The free radical formed during the hydroperoxide-mediated deactivation of ram seminal vesicles is hemoprotein derived. *J. Biol. Chem.* 257:4764–4768.
17. Cottee, F., R. J. Flower, S. Moncada, J. A. Salmon, and J. R. Vane. 1977. Synthesis of 6-keto-$PGF_{1\alpha}$ by ram seminal vesicle microsomes. *Prostaglandins.* 14:413–423.
18. Beetens, J. R., M. Claeys, and A. G. Herman. 1981. Antioxidants increase the formation of 6-oxo-$PGF_{1\alpha}$ by ram seminal vesicle microsomes. *Biochem. Pharmacol.* 30:2811–2815.
19. Hemler, M. E., H. W. Cook, and W. E. M. Lands. 1979. Prostaglandin biosynthesis can be triggered by lipid peroxides. *Arch. Biochem. Biophys.* 193:340–345.
20. Hemler, M. E., and W. E. M. Lands. 1980. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* 255:6253–6261.
21. Chan, A. C., and M. K. Leith. 1981. Decreased prostacyclin synthesis in vitamin E-deficient rabbit aorta. *Am. J. Clin. Nutr.* 34:2341–2347.
22. Gryglewski, R. J., A. Dembinska-Kiec, A. Zmuda, and T. Gryglewska. 1978. Prostacyclin and thromboxane A_2 biosynthesis capacities of heart, arteries, and platelets at various stages of experimental atherosclerosis in rabbits. *Atherosclerosis.* 31:385–394.
23. Kent, R. S., B. B. Kitchell, D. G. Shand, and A. R. Whorton. 1981. The ability of vascular tissue to produce prostacyclin decreases with age. *Prostaglandins.* 21:483–490.
24. Green, K., M. Hamberg, B. Samuelsson, M. Smigel, and J. C. Frolich. 1978. Measurement of prostaglandins, thromboxanes, prostacyclin, and their metabolites by gas liquid chromatography-mass spectrometry. *Adv. Prostaglandin Thromboxane Res.* 5:45–47.
25. Crawford, C. G., G. W. H. M. van Alphen, H. W. Cook, and W. E. M. Lands. 1978. The effect of precursors, products, and product analogs of prostaglandin cyclooxygenase upon iris sphincter muscle. *Life Sci.* 23:1255–1262.
26. Goldsmith, J. C., C. T. Jafvert, P. Lollar, W. G. Owen, and J. C. Hoak. 1981. Prostacyclin release from cultured and ex vivo bovine vascular endothelium: studies with thrombin, arachidonic acid, and ionophore A23187. *Lab. Invest.* 45:191–197.
27. Whorton, A. R., B. J. Sweetman, and J. A. Oates. 1979. Application of high-performance liquid chromatography and gas chromatography-mass spectrometry to analysis of prostaglandin E_1 in biological media. *Anal. Biochem.* 98:455–463.
28. Brotherton, A. F. A., and J. C. Hoak. 1982. Deactivation of cyclooxygenase in vascular endothelium during PGI_2 biosynthesis. *Circulation.* 66(Suppl. 2):II-178*a*. (Abstr.)
29. Bunting, S., R. Gryglewski, S. Moncada, and J. R. Vane. 1976. Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin X) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation. *Prostaglandins.* 12:897–913.
30. Hamberg, M., and B. Samuelsson. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. USA.* 71:3400–3404.
31. Nugteren, D. H., and E. Hazelhof. 1973. Isolation and properties of intermediates in prostaglandin biosynthesis. *Biochim. Biophys. Acta.* 326:448–461.
32. Marnett, L. J., P. Wlodawer, and B. Samuelsson. 1975. Cooxygenation of organic substrates by the prostaglandin synthetase of sheep vesicular gland. *J. Biol. Chem.* 250:8510–8517.
33. Egan, R. W., P. H. Gale, and F. A. Kuehl, Jr. 1979. Reduction of hydroperoxides in the prostaglandin biosynthetic pathway by a microsomal peroxidase. *J. Biol. Chem.* 254:3295–3302.
34. Egan, R. W., P. H. Gale, E. M. Baptista, K. L. Kennicott, W. J. A. Vanden Heuvel, R. W. Walker, P. E. Fagerness, and F. A. Kuehl, Jr. 1981. Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase. *J. Biol. Chem.* 256:7352–7361.
35. Vanderhoek, J. Y., and W. E. M. Lands. 1973. The inhibition of the fatty acid oxygenase of sheep vesicular gland by antioxidants. *Biochim. Biophys. Acta.* 296:382–385.
36. Whitaker, M. O., A. Wyche, F. Fitzpatrick, H. Sprecher, and P. Needleman. 1979. Triene prostaglandins: prostaglandin D_3 and icosapentaenoic acid as potential antithrombotic substances. *Proc. Natl. Acad. Sci. USA.* 76:5919–5923.

37. Chan, A. C., C. E. Allen, and P. V. J. Hegarty. 1980. Effects of vitamin E depletion and repletion on prostaglandin synthesis in semitendinosis muscle of young rabbits. *J. Nutr.* 110:66-73.

38. Carpenter, M. P. 1981. Antioxidant effects on the prostaglandin endoperoxide synthetase product profile. *Fed. Proc.* 40:189–194.

39. Doni, M. G., G. L. Aventi, L. Bonadiman, and G. Bonaccorso. 1981. Glutathione peroxidase, selenium, and prostaglandin synthesis in platelets. *Am. J. Physiol.* 240:H800–H803.

40. Machlin, L. J., E. Gabriel, L. R. Horn, D. Woo, R. Filipski, M. Brin, and J. Nelson. 1980. Effects of aspirin and related drugs in vitamin E-deficient rats. *J. Nutr.* 110:1958–1964.

41. Dusting, G. J., and J. R. Vane. 1980. Some cardiovascular properties of prostacyclin (PGI_2) which are not shared by PGE_2. *Circ. Res.* 44:223–227.

42. Weeks, J. R., N. C. Sekhar, and D. W. Ducharme. 1969. Relative activity of prostaglandin E_1, A_1, E_2, and A_2 on lipolysis, platelet aggregation, smooth muscle, and the cardiovascular system. *J. Pharm. Pharmacol.* 21:103–108.

43. Whittle, B. J. R., S. Moncada, and J. R. Vane. 1978. Comparison of the effects of prostacyclin (PGI_2), prostaglandin E_1 and D_2 on platelet aggregation in different species. *Prostaglandins.* 16:373–388.

44. Chang, W. C., S. I. Murota, J. Nakao, and H. Orimo. 1980. Age-related decrease in prostacyclin biosynthetic activity in rat aortic smooth muscle cells. *Biochim. Biophys. Acta.* 620:159–166.