Prostacyclin, Thromboxane A2, and Prostaglandin E2 Formation in Atherosclerotic Human Carotid Artery

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Prostaglandin (PG) formation in 16 atherosclerotic human carotid endarterectomy specimens was compared systematically with that of normal carotid artery from seven white pigs and six rhesus monkeys. Prostacyclin (PGI2) formation (picomoles of 6-keto-PGF1α/2 min/100 μg homogenate protein plus 2 mM glutathione [GSH]) of nonatheromatous intima adjacent proximal (276 ± 32, mean ± SEM) or distal (271 ± 14) to carotid plaque was comparable to that of normal carotid artery from white pig (272 ± 25, NS) and rhesus monkey (219 ± 41, NS), and was greater than stenotic intima (156 ± 17, p < 0.01), subintimal plaque (188 ± 14, p < 0.01), and ulceration (65 ± 16, p < 0.01). GSH-modulated PGI2 synthesis in all carotid specimens except areas of ulceration (p < 0.05), but did not restore PGI2 formation in atheromatous fractions to basal level. No detectable arterial thromboxane A2 (TXA2) formation or GSH-dependent PGE2 isomerase activity was observed. The decrement in atherosclerotic carotid artery PGI2 formation was focal (confined to the plaque) and may have been related to loss of effective GSH modulation. These conditions could contribute to a localized imbalance between arterial PGI2 and platelet TXA2 with adverse vascular thromboregulatory consequences. (Arteriosclerosis 8:73–78, January/February 1988)

Arterial prostaglandin (PG) synthesis is thought to be important in the maintenance of circulatory homeostasis by contributing to regulation of vascular tone and endothelial platelet adherence. Prostacyclin (PGI2), the major product of arachidonic acid metabolism in normal vascular endothelium, is a potent vasodilator and inhibitor of platelet aggregation and adhesion to the vessel wall.1 It has been hypothesized that PGI2 counterbalances the proaggregatory and vasoconstrictive effects of predominantly platelet-synthesized thromboxane A2 (TXA2).1 Recent reports2,3 suggest that atherosclerotic arteries differ from normal arterial tissues by producing substantially less PGI2, more PGE2,4 and detectable levels of TXA2.5 Such alterations in PG synthesis could create a localized imbalance between the intravascular actions of PGI2 and TXA2, resulting in increased platelet deposition and arterial thrombogenicity at atherosclerotic sites.

The underlying biochemical basis for reported alterations in arterial PG synthesis in atherosclerosis is unclear. Furthermore, it has not been demonstrated whether such changes in atherosclerotic patients are focal (localized only to plaque) or systemic (also found in nonatheromatous arterial tissues). The purpose of this study was to identify enzymatic changes in carotid endarterectomy specimens that might account for these previously observed disturbances of eicosanoid metabolism and investigate the potential of glutathione (GSH), an endogenous antioxidant, to modulate PG synthesis.

Methods

Normal Carotid Artery Specimens

A single specimen of nonatherosclerotic human carotid artery (n = 1) was harvested with appropriate authorization from a brain-dead cadaver kidney donor. Fresh carotid arteries from young adult, white (cross-bred, market grade) pigs (n = 7) and rhesus monkeys (n = 6) were used as control specimens. The animal experiments conformed to principles of animal care established by the Tulane Medical Center Advisory Committee for Animal Resources, “Principles of Laboratory Animal Care,” and the “Guide for the Care and Use of Laboratory Animals” (NIH publication No. 80-23, revised 1978).

Atherosclerotic Carotid Artery Specimens

Oclusive atheromatous internal carotid artery plaques were removed from patients (n = 16) by standard carotid endarterectomy with appropriate informed consent. Human specimens were obtained in accordance with guidelines established by the Code of Federal Regulations (46.101,b,5) and with approval of the Tulane Medical Center Committee on Use of Human Subjects.

Carotid plaques were dissected into the following fractions for systematic analysis of eicosanoid formation (Figure 1): 1) atherosclerotic, but nonatheromatous, intima adjacent proximal (n = 10) and distal (n = 7) to the occlusive internal carotid plaque; 2) atherosclerotic stenotic intima overlying plaque in the point of arterial narrowing (n = 9);
ed by addition of 100 µl of 0.1 M potassium phosphate buffer. Homogenate fractions, blotted dry, weighed while maintaining a temperature of 0°C to 4°C, minced, and then homogenized in potassium phosphate buffer (pH 7.4) and either assayed immediately or stored at -45°C. No statistical difference (NS) in enzyme activity was observed between specimens assayed immediately and those stored for assay at a later date. At the time of analysis, specimens were divided into fractions, blotted dry, weighed while maintaining a temperature of 0°C to 4°C, minced, and then homogenized in ten volumes of cold phosphate buffer. Homogenate protein concentrations were determined using the Bio-Rad protein concentration assay. Radiolabelled PG endoperoxide H2 (14C-PGH2) was prepared from radiolabelled arachidonic acid in our laboratory using a previously published protocol. 

All vascular specimens were immersed in ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and either assayed immediately or stored at -45°C. No statistical difference (NS) in enzyme activity was observed between specimens assayed immediately and those stored for assay at a later date. At the time of analysis, specimens were divided into fractions, blotted dry, weighed while maintaining a temperature of 0°C to 4°C, minced, and then homogenized in ten volumes of cold phosphate buffer. Homogenate protein concentrations were determined using the Bio-Rad protein concentration assay. Radiolabelled PG endoperoxide H2 (14C-PGH2) was prepared from radiolabelled arachidonic acid in our laboratory using a previously published protocol. 

Quantitative radiochromatography was used to study 14C-PGH2 metabolism by the following methodology. The reaction substrate, 10 µM 14C-PGH2 (15,000 cpm), was evaporated to dryness under a stream of nitrogen in a cold (0°C) Brinkman centrifuge tube. The assay was then started by addition of 100 µl of 0.1 M potassium phosphate buffer (pH 7.4) containing varying concentrations of tissue homogenate protein with or without 2 mM GSH and vortexed immediately. After a 2-minute incubation at 37°C, the reaction was stopped and PG products were extracted by addition of 400 µl of an ice-cold solution of ethyl acetate/methanol/0.2 M citric acid (pH 2.0) (15:2:1). The reaction tubes were again vortexed, centrifuged at 12,000 g for 30 seconds, and frozen quickly on dry ice. The upper organic layer (containing 85% to 95% of the 14C-radioactivity) was spotted for thin-layer chromatography (TLC) on Analtech silica gel GHL plates and then developed with the solvent system, ethyl acetate/acetic acid/hexane/water (54:12:25:60, organic phase). PG products were identified by exposure to iodine vapor and comparison with the migration of authentic PG standards on the TLC plate. Radiolabelled PG products were quantitated using a radiochromatogram scanner (Packard 7201B or Technical Associates Hydra HY-3) mated to a computer. Duplicate determinations were performed at each homogenate protein concentration for the various tissue samples. The lowest detectable level of PGH2 product formation by radiochromatographic TLC was 1% of substrate conversion (10 picomoles).

**Assay of Prostaglandin Formation**

A general discussion of eicosanoid metabolism, PG synthetic pathways, and the pivotal role of PGH2 (the product of arachidonic acid metabolism by cyclooxygenase, and substrate for the terminal enzymes PGI2 synthase in vascular tissues and TXA2 synthase in platelets) is available from other sources. All of the patients in this study were placed on antiplatelet therapy preoperatively and, therefore, determination of cyclooxygenase activity could not be undertaken.

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**Data Analysis**

PG formation for each specimen is expressed as mean ± standard error of the mean (SEM) in picomoles of stable PG breakdown product formed per 2-minute incubation of PGH2 at various homogenate protein concentrations, with or without 2 mM GSH. Data were analyzed using both parametric and nonparametric methods with comparable results; p<0.05 was considered statistically significant. One-way analysis of variance with Newman-Keuls post hoc test and the Kruskal-Wallis test were used for group comparisons. Student’s paired t test and Wilcoxon signed rank test were used to determine the effects of GSH.

**Results**

**Prostacyclin Synthase Activity**

PGI2 synthesis was determined by identification of 6-keto-PGF1α, the only product of spontaneous PGI2 hydrolysis under these incubation conditions. Basal PGI2 synthase activity (picomoles 6-keto-PGF1α formed during 2-minute incubation of PGH2 at each homogenate protein concentration with 2 mM GSH) was determined over a wide range of protein levels and demonstrated increasing PGI2 formation in proportion to the homogenate protein concentration (Figure 2). These data indicate the presence of an active PGI2 synthase in carotid artery specimens. An increase in PGI2 synthase activity was found with the addition of 2 mM GSH at each homogenate protein level (p<0.01, data not shown).

No difference in PGI2 synthase activity was identified at any protein concentration between normal carotid artery from white pigs or rhesus monkeys and nonatheromatous intima adjacent (proximal or distal) to carotid plaque from atherosclerotic patients (Figure 2) (NS). Basal PGI2 formation (picomoles 6-keto-PGF1α/2 min/100 µg homogenate protein with 2 mM GSH) of arteriosclerotic, nonatheromatous, intima adjacent proximal (276 ± 32) and distal intima proximal and distal to internal carotid artery plaque, subintimal plaque, intima in the region of arterial stenosis overlying plaque, and an area of intimal ulceration.
(271 ± 14) to the internal carotid plaque was comparable to that of normal carotid from young adult white pigs (272 ± 25) (NS) and rhesus monkeys (219 ± 41) (NS). Data from the single specimen of normal human carotid artery (not entered into the statistical analysis) indicate values of PG12 formation also within the range of these specimens.

Figure 3 shows the 6-keto-PGF1α formation by fractions of human carotid endarterectomy specimens at various homogenate protein concentrations. PG12 formation by nonatheromatous intima proximal and distal to carotid plaque was similar at all protein concentrations (NS, data not shown). A decrement in PG12 formation was demonstrated between nonatheromatous intima adjacent proximal or distal to the carotid plaque and stenotic intima overlying the plaque, subintimal plaque, and areas of intimal ulceration. It should be noted that PG12 formation in subintimal plaque was comparable to stenotic intima overlying plaque at the point of arterial narrowing; the least activity was found in intimal ulcerations. Other than areas of ulceration, all specimens increased PG12 formation with the addition of 2 mM GSH (p<0.05) (Table 1).

Thromboxane A2 Synthase Activity

TXA2 formation determined by identification of TXB2, the stable product of TXA2 hydrolysis, was not detected at any protein concentration in homogenates of white pigs, rhesus monkeys, or human carotid artery specimens, and if present was, thus, less than 10 picomoles.

Glutathione-Dependent Prostaglandin E2 Isomerase Activity

Data in Tables 1 and 2 demonstrate an inverse relationship between PG12 and PGE2 formation for all carotid artery specimens in both degree of plaque pathology and homogenate protein concentration. In all cases in which PG12 formation was low, PGE2 formation was high; a reciprocal relationship was observed when PG12 formation was high. PGE2 formation decreased with increasing homogenate protein concentration, although no difference in PGE2 formation by carotid artery from white pigs, rhesus monkeys, or human specimens was found at any given protein concentration (NS). Additionally, no increase in PGE2 formation was detected after addition of 2 mM GSH (NS).

Discussion

This study demonstrated the presence of an active PG12 synthase in carotid artery from white pigs, rhesus monkeys, and in human normal and atherosclerotic tissue. Nonatheromatous arterial intima adjacent to atherosclerotic carotid plaque formed PG12 in quantities indistinguishable from that of normal carotid specimens from young adult white pigs and rhesus monkeys. In contrast, atheromatous fractions of human carotid plaque formed significantly less PG12 than adjacent arteriosclerotic, nonatheromatous, arterial intima and was least in areas of plaque ulceration. Although other authors3,4 have reported decreased PG12 formation relative to the severity of arteriosclerosis, this study was the first to systematically evaluate
The significance of in vitro studies of PGI2 formation by atherosclerotic arterial specimens has recently been questioned. Quantitative correlation between the in vitro capacity of vascular tissues to synthesize eicosanoids and actual endogenous biosynthetic rates may be tenuous, and discrepancies between the two have been suggested.15 Direct in vivo assay of PGI2 formation by atherosclerotic arteries is not currently possible and, thus, other authors have investigated endogenous PGI2 synthesis by indirect methods.15,16 Basal production of PGI2 under physiologic conditions is reported to be low. Patients with severe atherosclerosis and evidence of platelet activation were observed to excrete higher urinary levels of 2,3-dinor-6-keto-PGF1α (one of a number of in vivo PGI2 metabolites) than normal subjects;15 these authors concluded that reduced PGI2 formation by atherosclerotic specimens observed in vitro was, therefore, physiologically irrelevant. It was proposed that endogenous production of PGI2 was lower than normal vascular tissues are capable of producing because of the lack of a stimulus and, conversely, higher in atherosclerotic patients. Possible mechanisms for stimulation of PGI2 formation (increased platelet/vascular interactions) are speculative.15

Increased urinary excretion of a PGI2 metabolite from unknown tissue origin in atherosclerotic patients and studies indicating a localized reduction in PGI2 synthesis by atheromatous plaques are not necessarily contradictory. Whether reported in vivo increases in PGI2 formation in patients with advanced atherosclerosis occur in atheromatous or nonatheromatous arterial regions is currently unknown and cannot be determined conclusively by indirect assay methods. If, as suggested by urinary PGI2 metabolite excretion studies, increased PGI2 is produced in atherosclerotic patients, the site of PGI2 formation may not be from plaques themselves, but from neighboring nonatheromatous arterial intima. Definitive conclusions regarding endogenous vascular PGI2 biosynthesis in atherosclerotic patients, based on a quantitative correlation with urinary eicosanoid metabolite levels, may be premature.14

Deficiencies in PGI2 synthase activity may set limits on endogenous PGI2 biosynthesis by atheromatous plaques.
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regardless of the level of stimulation. Although the theory is speculative and requires further experimentation, the non-atheromatous portions of the arterial system may be subjected to increased traumatic or chemically mediated platelet/vascular interactions by proximity to neighboring plaques and, thus, stimulated to produce PGI₂. 13, 14, 17 Additionally, hemodynamic factors such as increased flow velocity or wall shear stress in nonatheromatous regions adjacent to atherosclerotic arterial stenoses could stimulate PGI₂ formation. 18, 19 Whether PGI₂ synthesis by non-atheromatous intima can regulate platelet adherence to nearby plaques is currently unknown. Flow separation and other complex fluid disturbances at the carotid bifurcation and in the proximity of occlusive plaques may interfere with the local dispersion of PGI₂ in the blood stream, precluding inhibition of platelet aggregation and adherence. 20, 21 It has been shown in vitro that small concentrations of PGI₂ prevent thrombus formation, but larger concentrations are needed to reduce platelet adhesion. 9 Imbalances between PGI₂ and TXA₂ could have localized adverse thromboregulatory consequences in the immediate environment of atherosclerotic plaques, not necessarily reflected in circulating or urinary PGI₂ metabolites.

It has been proposed 7 that lipid peroxides or other reactive oxygen species found in atherosclerotic vessels inhibit PG I₂ formation. PGI₂ synthase is uniquely sensitive to oxidant (O₂⁻) released after peroxidation of 15-hydroperoxy-eicosatetraenoic acid (15-HPETE), a lipoygenase product of arachidonic acid found in atherosclerotic plaques. 22 A recent study from our laboratory found that PG I₂ formation in coronary artery microsomes could be modulated in a dose-dependent manner by GSH. 8 We suggested that PG I₂ synthase activity determinations with the addition of GSH is present intracellularly in millimolar concentrations. 8 Both normal and atherosclerotic carotid artery formed more PG I₂ in the presence of GSH; however, GSH was unable to restore PG I₂ formation in carotid plaque to a level comparable to that of the surrounding nonatheromatous intima. These data suggest that a reduction in, or loss of, the effectiveness of intracellular GSH to maintain basal PG I₂ synthase activity as a consequence of oxidative or other mechanisms could contribute to diminished PG I₂ formation.

TXA₂ synthesis by arterial tissues is controversial, but TXA₂ formation by whole atherosclerotic human carotid plaque specimens has been recently reported. 3 We found no detectable TXA₂ formation in any normal or atherosclerotic carotid artery homogenates. Because of the restricted quantity of material in each carotid plaque specimen, it is possible that homogenate protein concentrations used in this study were too low to support detectable levels (10 picomoles) of TXA₂ formation by radiometric TLC. Nevertheless, current data suggest that neither normal nor atherosclerotic carotid artery have substantial capability to form TXA₂. Arterial TXA₂ formation is, thus, unlikely to be a major contributing factor in platelet adherence to carotid plaque.

The reciprocal relationship between PG I₂ and PGE₂ formation has been observed previously in atherosclerotic human aorta. 4 It was proposed that atherosclerotic aorta

shifts eicosanoid metabolism from PG I₂ synthesis to produce PGE₂ predominantly. 4 PGE₂ formation can occur by spontaneous hydrolysis of PGH₂ or enzymatically by GSH-dependent PGE₂ isomerase of PGH₂. 8 Our data demonstrate substantially less PGE₂ formation when PGH₂ is converted rapidly to PG I₂ by PG I₂ synthase, and vice versa; additionally, no GSH-dependent PGE₂ formation was observed. We conclude that in carotid artery homogenates most, if not all, PGE₂ was formed nonenzymatically secondary to spontaneous hydrolysis of PGH₂. The significance of this apparent lack of GSH-dependent PGE₂ isomerase in carotid artery is currently unknown, but could preclude substantial endogenous PGE₂ formation. PGE₂ reportedly promotes the second phase of platelet aggregation. 23, 24 While it is doubtful that unregulated PGE₂ formation by spontaneous hydrolysis of PGH₂ contributes to carotid arterial thromboregulatory mechanisms, PGE₂ formed nonenzymatically could oppose platelet inhibitory effects of PG I₂. Previous work in our laboratory, which unmasked the presence of GSH-dependent PGE₂ isomerase activity in bovine coronary artery microsomes, demonstrated the need for appropriately controlled experiments before the presence of GSH-dependent PGE₂ isomerase in vascular tissues could be definitely excluded. 3, 14, 15 A study reports significant focal alterations in eicosanoid metabolism, particularly diminished PG I₂ formation, with ad to atheromatous fractions of human carotid enda. 14, 15 Studies suggest that the reciprocal relationship between PG I₂ and PGE₂ formation with spontaneous hydrolysis of PGH₂ contributes to carotid arterial thromboregulatory mechanisms, PGE₂ formed nonenzymatically could oppose platelet inhibitory effects of PG I₂. Previous work in our laboratory, which unmasked the presence of GSH-dependent PGE₂ isomerase activity in bovine coronary artery microsomes, demonstrated the need for appropriately controlled experiments before the presence of GSH-dependent PGE₂ isomerase in vascular tissues could be definitely excluded. 3, 14, 15

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