Enhanced coronary vasoconstriction to oxidative stress product, 8-epi-prostaglandinF\(_{2\alpha}\), in experimental hypercholesterolemia

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Abstract

Objectives: The \(F_2\)-isoprostanes are a family of novel prostaglandin isomers and a stable product of in vivo oxidative stress. 8-epi-prostaglandinF\(_{2\alpha}\), a member of this isoprostane family, is a vasoconstrictor and its local release may contribute to the abnormal vasomotor tone associated with hypercholesterolemia. We therefore aimed to outline the role of 8-epi-prostaglandinF\(_{2\alpha}\) as a coronary vasoconstrictor in experimental hypercholesterolemia.

Methods and results: Pigs were randomized to two experimental groups (each \(n=9\): normal (N) and high cholesterol (HC) diet). To determine the vasoconstrictive effects of 8-epi-prostaglandinF\(_{2\alpha}\) in vitro, doses from \(10^{-9}\) to \(10^{-5}\) M were used to constrict coronary epicardial rings. Plasma total and LDL cholesterol levels were significantly higher in the HC group compared with the N group (\(P<0.005\)) as were plasma 8-epi-prostaglandinF\(_{2\alpha}\) levels (\(P<0.001\)). 8-epi-prostaglandinF\(_{2\alpha}\) immunoreactivity was present in the vessel wall in both groups. Normal vessels with intact endothelium (\(n=8\) rings) contracted to 8-epi-prostaglandinF\(_{2\alpha}\) (maximal contraction 15.5\(\pm\)8.74%). In the HC group, rings with intact endothelium had a greater contractile response to 8-epi-prostaglandinF\(_{2\alpha}\) compared to normals (72.3\(\pm\)7.9%; \(n=8\); \(P<0.0001\)). This was reversed by preincubation with NOR-3, a NO donor (maximal contraction 6.7\(\pm\)1.56%; \(n=5\); \(P<0.0001\)). Enhanced contraction in normal vessels occurred with endothelial denudation (98.4\(\pm\)3.56%; \(n=6\); \(P<0.0001\)) and with preincubation of the endothelium-intact rings with \(L-\text{N}^\text{methyl-L-arginine}\), an NO synthase inhibitor (85.5\(\pm\)10.3%; \(n=6\); \(P<0.001\)). The enhanced contraction seen with hypercholesterolemia did not occur with other prostanoid vasoconstrictors.

Conclusion: Experimental hypercholesterolemia leads to a significant increase in 8-epi-prostaglandinF\(_{2\alpha}\) levels in addition to enhanced 8-epi-prostaglandinF\(_{2\alpha}\)-induced coronary vasoconstriction, in vitro. These findings support a role for the \(F_2\)-isoprostanes in the regulation of coronary vasomotor tone in pathophysiologic states.

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1. Introduction

The overproduction of reactive oxygen species, or oxidative stress, has been implicated in the pathogenesis of atherosclerosis [1,2]. An increase in oxidative stress has been demonstrated in experimental hypercholesterolemia with oxidized LDL leading to impaired endothelium-dependent vasorelaxation [3]. The \(F_2\)-isoprostanes, a family of novel prostaglandin isomers and a stable product of in vivo oxidative stress, have recently been described [4,5]. These isoprostanes are chemically stable products of oxidative modification of arachidonic acid, formed through a free radical-catalyzed mechanism, independent of the cyclooxygenase enzyme. 8-epi-prostaglandinF\(_{2\alpha}\), a
member of this isoprostane family, causes vasoconstriction in the aortic, renal and pulmonary vascular beds in vitro [6,7]. However, the exact role this family of compounds plays in vivo is still unclear.

Previous studies have demonstrated increased circulating levels of the F2-isoprostanes in hypercholesterolemia. In addition, the presence of F2-isoprostanes has been noted in atherosclerotic plaque [8]. Since lipid peroxidation is thought to occur in the microenvironment of atherosclerotic plaques, local release of 8-epi-prostaglandinF2α, in addition to increased circulating levels, may contribute to the abnormal vasomotor tone associated with hypercholesterolemia. Experimental hypercholesterolemia is also associated with a decrease in nitric oxide (NO) bioavailability [9,10] which plays a major role in the altered coronary vascular reactivity characteristic of this disease state. Therefore, we postulated that F2-isoprostane-induced coronary vasoconstriction may be enhanced in experimental porcine hypercholesterolemia and modulated via the NO pathway.

2. Methods

2.1. Animals

The study procedures and handling of animals were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee and the investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experiments were conducted on female juvenile domestic crossbred pigs weighing 30–40 kg each. Nine animals were placed on a diet of 2% cholesterol and 15% lard by weight (TD 93296, Harlan Teklad, Madison, WI, USA) for a total of 12 weeks, as previously described [11,12]. A group of nine pigs fed a normal diet were used as controls (N). Both the normal and the high cholesterol diet contained the same amounts of zinc oxide/selenium and copper sulphate, all potential cofactors for antioxidant enzymes, in addition to the same amounts of the antioxidant, vitamin E. After 12 weeks the animals were euthanized with an intravenous overdose of pentobarbital sodium (intravenous 30 mg/kg) and the coronary arteries were immediately harvested.

2.2. Lipid parameters

Plasma total, LDL and HDL cholesterol were measured after completion of 12 weeks of feeding. Plasma total cholesterol and low-density lipoprotein levels were determined by applying the technique of Alain et al. [13], using a commercial reagent (Roche, Nutley, NJ, USA).

2.3. Plasma 8-epi-prostaglandin F2α

Blood samples were taken from five pigs in each group. Samples were collected in EDTA tubes and the plasma was stored at ~80°C until the time of the assay. The total levels of 8-epi-prostaglandinF2α were measured with an enzyme immunoassay kit (EIA, Cayman). Prior to the enzyme immunoassay an alkaline hydrolysis was utilized [14]. Plasma samples were purified by Sep-Pak C-18 columns (Milford, MA, USA) prior to analysis. The samples, tracer, and antiserum were added to wells pre-coated with mouse monoclonal antibody. The plates were washed to remove all unbound reagents. Ellman’s Reagent (containing the substrate to acetylcholinesterase) was added to the wells. The intensity of the distinct yellow color produced by this enzymatic reaction was determined using a spectrophotometer at 405 nm.

2.4. Immunohistochemistry for 8-epi-prostaglandin F2α

Sections were taken from the circumflex artery in normal and high cholesterol groups. The sections were then deparaffinized in xylene and dehydrated using 95% ethanol. Endogenous peroxidase activity was blocked by incubating slides in 1.5% hydrogen peroxide/50% absolute methanol for 10 min. The slides were then steamed for 30 min in 1 mM acetic acid buffer. Nonspecific protein binding sites were blocked with 5% goat serum diluted in 1/2 times the sample dilution. After further washing, color development was performed using an organ chamber filled with 25 ml of 1% goat serum and PBS/0.05% Tween 20 and applied to the slides for 1 h at room temperature and rinsed. A biotinylated horse anti-rabbit IgG at a 1/400 dilution was then incubated on the slides for 30 min and rinsed. The slides were subsequently incubated for 30 min with streptavidin-horseradish peroxidase (Dako) (1/500 dilution). After further washing, color development was performed using 3-amino-9-ethylcarbazole substrate solution for 15 min at room temperature. A counterstain was then performed using hematoxylin for 30 s. The slides were then rinsed for 5 min in running tap water, and mounted in aqueous glycerol gelatin media.

2.5. In vitro determination of vascular reactivity

Vascular reactivity studies were performed as previously described [15]. In brief, epicardial arteries were harvested from hypercholesterolemic and normal diet pigs. No more than two rings were taken from each pig for each protocol. The hearts were placed into cold modified Krebs–Ringer bicarbonate solution of the following composition (control solution in mmol/L): 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 0.026 calcium ethylenediamine-tetraacetic acid, and 11.1 glucose. Endothelium was manually removed by rolling the vessel with wire. Segments of the left circumflex artery 2–3 mm long were dissected. Each vessel was connected to an isometric force transducer (Grass Instruments, West Warwick, RI, USA), suspended in an organ chamber filled with 25 ml of
control solution (37°C; pH 7.4) and gassed with 94% O₂ and 6% CO₂. The isometric tension was recorded continuously. The arteries were allowed to stabilize at a resting tension of 2 g for 1 h. The viability of the rings was confirmed by exhibition of a contractile response to 20 mM KCl. After an equilibration period of 30 min, freshly prepared solutions of the agents detailed in the specific protocols below were added. Drugs were dissolved in distilled water such that volumes of <0.2 ml were added to the organ chambers. All concentrations are expressed as the concentration within the bath solution.

To compare the vasoconstrictive effects of U46619 (a thromboxane A₂ agonist), 8-epi-prostaglandinF₂₀ and 8-epi-prostaglandinE₂ (each from Cayman Chemicals, Ann Arbor, MI, USA), cumulative doses from 10⁻⁹ to 10⁻⁵ M of each were used. Contraction with 60 mM KCl was used to determine 100% contraction and subsequent results were expressed as a percentage of this. The experiments were carried out in vessels with and without the presence of the endothelium. Substance P (10⁻⁷ M, Sigma, St. Louis, MO, USA) was used to confirm the viability of the endothelium. The thromboxane A₂ blocker SQ29548 (10⁻⁷ M, Cayman Chemicals) was used 20 min prior to contraction with 8-epi-prostaglandinF₂₀, to test for antagonist activity. In addition, in vessels with intact endothelium L-NNMMA (10⁻⁴ M, Sigma) was used prior to contraction with 8-epi-prostaglandinF₂₀, to assess the contribution of nitric oxide release in attenuation of the contraction. NOR-3 (FK 409, 10⁻⁵ M, Cayman Chemicals), a slow acting NO donor, was also used prior to contraction with 8-epi-prostaglandinF₂₀. ODQ (H-(1,2,4)oxadiozolo(4,3-a)quinoxallin-1-one) (10⁻⁵ M; Biomol, Plymouth, PA, USA), a selective soluble guanylate cyclase inhibitor, was used prior to preincubation with NOR-3 prior to contraction with 8-epi-prostaglandinF₂₀. l-arginine (10⁻⁵ M; Sigma) and d-arginine (10⁻⁵ M; Sigma), were also used prior to contraction with 8-epi-prostaglandinF₂₀. At the end of these experiments, 10⁻⁵ M papaverine (Sigma) was added to verify that the rings had maintained their vasodilating capacity.

2.6. Data analysis

Results are expressed as mean±SEM. For statistical analysis, curves were compared using a 2-way ANOVA, followed by a Bonferroni’s or Student–Neuman–Keul’s t-test. Statistical significance was assumed if P<0.05.

3. Results

3.1. Lipid profile

There was a significant increase in the total, LDL and HDL cholesterol in the cholesterol fed pigs (HC) compared with the pigs fed normal diet (N) (P<0.005 in all cases; see Table 1) but no difference in the triglyceride levels.

3.2. Plasma 8-epi-prostaglandinF₂₀

There was a significant increase in the plasma total 8-epi-prostaglandinF₂₀ levels in the HC group (233±24.7 pg/mL) compared with the N group (84±6.8 pg/mL; P<0.001; see Table 1).

3.3. Immunohistochemistry for 8-epi-prostaglandinF₂₀

8-epi-prostaglandinF₂₀ was present in the vessel wall in both the normal and hypercholesteremic groups. The majority of the staining was localized to the intima of the artery (see Fig. 1).

3.4. Normal diet group (N)

Rings from normal diet pigs contracted in response to 8-epi-prostaglandinF₂₀ (maximal contraction 15.5±8.74%; n=8). After removal of the endothelium, contraction was significantly enhanced (maximal contraction 98.4±5.56%; n=6; P<0.0001) (Fig. 2(a)), suggesting that the endothelium modulates the contractile response. When the endothelium-intact vessels were preincubated with l-NNMMA (N-monomethyl-l-arginine) (n=6), a NO synthase inhibitor, the attenuation of contraction induced by the endothelium was completely reversed (maximal contraction 85.5±10.3%), suggesting that the endothelium-dependent vasorelaxant was NO (Fig. 2(a)). In addition, following preincubation of endothelium-denuded vessels with the slow acting NO donor, NOR-3, contraction of the vessels was attenuated to the level of the endothelium-intact vessels (maximal contraction 15.4±6.6%) (Fig. 2(b)). The selective soluble guanylate cyclase inhibitor, ODQ (n=6) significantly blocked the attenuation of contraction in endothelium-denuded vessels caused by NOR-3 (maximal contraction 82±7.0%; P<0.0001) (Fig. 2(b)). Preincubation of endothelium-intact vessels with l-arginine did not attenuate contraction to 8-epi-prostaglandinF₂₀. In contrast to 8-epi-prostaglandinF₂₀, there was no difference in the contractile response to U46619, another prostanoid vasconstrictor, between endothelium intact and denuded vessels (Fig. 3).

<table>
<thead>
<tr>
<th>Lipid parameters in normal and cholesterol fed pigs</th>
<th>Normal (N)</th>
<th>High cholesterol (HC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>83±4.8</td>
<td>395±58.7*</td>
</tr>
<tr>
<td>Low density lipoprotein (mg/dL)</td>
<td>32±4.6</td>
<td>310±58.1*</td>
</tr>
<tr>
<td>High density lipoprotein (mg/dL)</td>
<td>39±1.3</td>
<td>89±8.1*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>64±9.4</td>
<td>43±4.3</td>
</tr>
<tr>
<td>Plasma F₂₀-isoprostanes (pg/mL)</td>
<td>84±6.8</td>
<td>233±24.7*</td>
</tr>
</tbody>
</table>

*P<0.05 vs. normal
3.5. Cholesterol fed group (HC)

In epicardial coronary vessels from the HC group with intact endothelium, the contraction in response to 8-epi-prostaglandinF$_{2\alpha}$ was significantly greater compared with vessels from the normal group (72.3±7.9%; n=8; P<0.0001) (Fig. 4). In addition, preincubation of the endothelium-intact vessels with the NO donor, NOR-3, led to complete attenuation in the contraction to 8-epi-prostaglandinF$_{2\alpha}$ (maximum contraction 6.7±1.56%; n=5; P<0.0001) (Fig. 4). Preincubation of the endothelium-intact vessels with L-arginine led to an attenuation in the contraction to 8-epi-prostaglandinF$_{2\alpha}$ (maximum contraction 38.2±7.9%; n=12; P<0.01) (Fig. 5). D-arginine had no effect on contraction to 8-epi-prostaglandinF$_{2\alpha}$ (maximum contraction 81.3±7.5; n=6; P=NS). In the high cholesterol group there was no enhanced vasoconstrictor response to endothelin-1 (maximum contraction 154±8.7% (N) vs. 133±8.4 (HC)), U46619 (Fig. 3) or 8-epi-prostaglandinE$_2$ (maximum contraction 61.0±10.7% (N) vs. 32.0±7.1% (HC).

4. Discussion

This study demonstrates that 8-epi-prostaglandinF$_{2\alpha}$, a novel prostanoid and marker of oxidative stress, causes coronary vasoconstriction, in vitro. Moreover, we have demonstrated for the first time, enhanced vasoconstriction to the F$_2$-isoprostanes in experimental hypercholesterolemia, a state characterized by increased levels of circulating isoprostanes. This vasoconstriction was modulated by the NO pathway. These findings support a role for oxidative stress products in the regulation of coronary vasomotor tone in pathophysiologic states.

Oxidative stress and the oxidative modification of LDL have been postulated to play a central role in the pathogenesis of atherosclerosis [1]. Hypercholesterolemia is associated with an alteration in the NO pathway in both animal models and humans [16,17]. In addition, oxidized LDL impairs endothelium-dependent vasorelaxation, which may play a role in the atherosclerotic process [3]. A novel family of prostaglandin isomers, the F$_2$-isoprostanes, have recently been described as a reliable measure of in vivo oxidative stress [4,5]. However, the role they play in cellular activation and the regulation of coronary vasomotor tone in hypercholesterolemia is still being investigated. We demonstrated the presence of 8-epi-prostaglandinF$_{2\alpha}$ immunoreactivity in both normal and hypercholesterolemic vessels, suggesting that this compound may play a role in the regulation of vascular tone in both physiologic and pathophysiologic states. In this early stage of atheromatous disease, characterized by minor intimal proliferation and abnormal endothelial function [9], we found immunoreactivity only in the intima of vessels, unlike previous studies of atherosclerotic plaque [8], where the presence of 8-epi-prostaglandinF$_{2\alpha}$ was more widespread, consistent with the more advanced stage of disease.

Previous studies have shown that 8-epi-prostaglandinF$_{2\alpha}$ is a renal, aortic and coronary vasoconstrictor under physiologic condition. We confirmed these findings in porcine coronary epicardial vessels, with a greater contractile response occurring in the endothelium-denuded coronary rings. This suggests that under normal conditions, the contraction of intact vessels to 8-epi-prostaglandinF$_{2\alpha}$ is attenuated by an endothelium-derived vasodilator. More-
over, L-NMMA, a competitive inhibitor of NO, enhanced the vasoconstrictive response to 8-epi-prostaglandinF₂α, while NOR-3, a slow acting NO donor [18,19], attenuated this response. This implies that NO may serve as a modulator of the vasoconstrictor effects of 8-epi-prostaglandinF₂α, in addition to a previous study suggesting that prostacyclin was the relevant endothelium-dependent vasorelaxant [6]. However, in that study, the effect of L-NMMA was not examined and aortic and pulmonary vessels were utilized rather than the coronary vasculature. We observed no difference in the contractile effect of U46619, another prostanoid, between endothelium intact and denuded vessels. This suggests that the counteracting effect of NO was specific to 8-epi-prostaglandinF₂α and may involve a direct interaction between NO and 8-epi-prostaglandinF₂α or an interaction at the level of a specific isoprostane receptor [20]. However, ODQ, a specific soluble guanylate cyclase inhibitor, blocked the attenuation of isoprostane contraction by NOR-3, the NO donor, suggesting that the modulating effects of NO on 8-epi-prostaglandinF₂α contraction occur via cGMP-dependent mechanisms, making a direct interaction between NO and 8-epi-prostaglandinF₂α unlikely to play a major role.

The effects of 8-epi-prostaglandinF₂α on vascular function in a hypercholesterolemic milieu have not been previously studied. This issue is particularly relevant in view of the elevated levels of F₂-isoprostanes found in hypercholesterolemia in vivo [21] and confirmed in our study. We found that vasoconstriction in endothelium-intact vessels in response to 8-epi-prostaglandinF₂α was markedly increased in vessels from hypercholesterolemic animals with endothelium preincubated with NOR-3 (10⁻⁵ M) (n=5). *P<0.05 for (2) compared with (1) and (3).

Fig. 2. (a) Contraction to the F₂-isoprostane, 8-epi-prostaglandinF₂α (10⁻⁷ to 10⁻⁵ M) in normal vessels: (1) with endothelium (n=8 rings); (2) without endothelium (n=6); and (3) with endothelium, preincubated with the NO inhibitor, L-NMMA (10⁻⁵ M) (n=6). *P<0.05 for (1) compared with (2) and (3). (b) Contraction to the F₂-isoprostane, 8-epi-prostaglandinF₂α (10⁻⁷ to 10⁻⁵ M) in normal vessels: (1) with endothelium (n=8); (2) without endothelium (n=6); (3) without endothelium, preincubated with NOR-3 (10⁻⁵ M), a NO donor (n=4); and (4) without endothelium, preincubated with ODQ, a selective soluble guanylate cyclase inhibitor (10⁻⁷ M) and NOR-3 (10⁻⁷ M), a NO donor (n=6). *P<0.05 for (2) and (4) compared with (1) and (3).

Fig. 3. Contraction to the thromboxane A₂ analogue, U46619 (10⁻⁹–10⁻⁵ M), in normal vessels with (n=6) and without (n=4) endothelium and from endothelium intact vessels from hypercholesterolemic animals (n=4).
pigs compared with normal controls. This effect was specific to 8-epi-prostaglandinF₂α, with no enhancement in the response to other vasoconstrictors, including endothelin-1, U46619 or 8-epi-prostaglandinE₂, in hypercholesterolemia, as previously reported [15,22]. In contrast, in the endothelium-denuded vessels, there was no significant difference between the hypercholesterolemic and normal groups. This again implicates NO in a modulating vasodilatory role, specifically attenuating the isoprostane-induced vasoconstriction. However, this modulation appears to be less effective in a hypercholesterolemic milieu, consistent with previous findings of a decrease in functional NO activity in experimental hypercholesterolemia [9]. Preincubation of hypercholesterolemic endothelium-intact vessels with L-arginine, the physiologic substrate of NO, and NOR-3, a slow acting NO donor, attenuated the enhanced vasoconstriction, supporting a role for NO in altering the vasoconstrictor response to 8-epi-prostaglandinF₂α in hypercholesterolemia. D-arginine, the inactive isomer of L-arginine, had no effect. The more marked response of hypercholesterolemic vessels to 8-epi-prostaglandinF₂α compared with normal vessels may contribute to the abnormal vasomotor tone found in hypercholesterolemia.

The plasma levels of 8-epi-prostaglandinF₂α found in hypercholesterolemia in our model were at least 100 fold lower than levels associated with a vasoconstrictive response in vitro. However, the levels in the vessel wall may be more relevant to vasomotor tone and would be expected to be higher than those in the plasma. In addition, previous data has shown high levels of 8-epi-prostaglandinF₂α in plaque compared with normal vessel [8], suggesting that isoprostanes may play an even greater role in control of vasomotion in the presence of established atherosclerosis.

In summary, this study demonstrates that 8-epi-prostaglandinF₂α, a novel prostanoid and marker of oxidative stress, causes coronary vasoconstriction that is modulated by the endothelial NO pathway. Experimental hypercholesterolemia leads to a significant increase in this 8-epi-prostaglandinF₂α-induced coronary vasoconstriction, supporting a role for oxidative stress products in the regulation of coronary vasomotor tone.

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