Attenuated In Vitro Coronary Arteriolar Vasorelaxation to Insulin-like Growth Factor I in Experimental Hypercholesterolemia

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Abstract—Insulin and insulin-like growth factor (IGF) 1 affect coronary vasoactivity. Experimental hypercholesterolemia is associated with coronary atherogenesis and altered vasomotor regulation. Because the IGF axis is altered during atherogenesis, we postulated that experimental hypercholesterolemia is associated with an altered coronary vasoactive response to IGF-1 in vitro. Coronary arteries and arterioles from pigs fed either a normal or high-cholesterol diet for 10 weeks were contracted with endothelin-1 and relaxed with cumulative concentrations of insulin or IGF-1 (10⁻¹² to 10⁻⁷ mol/L). Control arterioles were also incubated with the nitric oxide synthase inhibitor 10⁻⁴ mol/L N⁶-monomethyl-L-arginine (L-NMMA) or the potassium channel blocker 10⁻² mol/L tetraethylammonium (TEA), contracted with endothelin-1, and relaxed with insulin or IGF-1. Experimental hypercholesterolemia (1) increased serum cholesterol (9.5±1.0 versus 1.9±0.08 mmol/L; P<0.0001), (2) caused coronary arterial and arteriolar endothelial dysfunction in vitro (attenuated vasorelaxation to bradykinin), (3) did not alter the epicardial response to either insulin (P=0.80) or IGF-1 (P=0.12), and (4) significantly attenuated the arteriolar response to IGF-1 (maximal relaxation of 79±6% versus 42±8%; P=0.01) but not insulin (43±6% versus 53±7%; P=0.99). Control arteriolar vasorelaxation to IGF-1 was attenuated by both L-NMMA (P<0.001) and TEA (P=0.01), whereas only L-NMMA attenuated insulin (P<0.001). Staining for IGF-1 and IGF binding protein 2 was increased (P<0.05) in arterioles of cholesterol-fed pigs. IGF-1 and insulin are therefore coronary arteriolar vasorelaxants through different mechanisms. Experimental hypercholesterolemia is associated with resistance to the coronary arteriolar vasorelaxing effects of IGF-1 but not insulin, in conjunction with increased ligand and binding-protein expression. The IGF axis may contribute to the altered coronary vasoactivity in hypercholesterolemia. (Hypertension. 1999;34:89-95.)

Key Words: pigs ■ arteries ■ insulin ■ insulin growth factor ■ hypercholesterolemia

Insulin-like growth factor (IGF) 1 and 2, 7.5-kDa peptides, are the principal ligands of the IGF axis.¹ Both are highly homologous in structure and function to proinsulin. Unlike insulin, which is only produced in the pancreas, IGF-1 is produced by various cell types, including endothelial² and vascular smooth muscle cells.³ IGF-1 is presumed to affect the structural changes associated with atherosclerosis and related conditions.⁴⁻⁵ Both insulin and IGF have diverse vasoactive actions (see Reference 4). Although the vasoactive effects of the 2 peptides may be similar, each peptide may have unique effects depending on the vascular bed and the pathophysiological state.⁵ We have recently demonstrated that both insulin and IGF-1 are coronary epicardial vasorelaxants; these effects were attenuated by inhibitors of the nitric oxide pathway and of potassium channels. Because coronary blood flow is primarily regulated by arterioles and resistance vessels, the in vitro effect of agents on arterioles may be of greater practical significance than epicardial arteries. Hitherto, we are not aware of any study that characterized the effect of insulin and IGF-1 on coronary arterioles.

Experimental hypercholesterolemia in the pig is associated with early coronary atherogenesis⁶ and altered coronary endothelial function.⁷⁻¹¹ The role of the coronary arterioles in the regulation of vascular tone in early coronary atherosclerosis is emerging. Accordingly, we have shown that the altered vasoreactivity associated with hypercholesterolemia may be differentially affected in porcine coronary epicardial arteries and arterioles,² underscoring the need for the evaluation of both vessel types in pathophysiological states.

The coronary vasoactive effects of insulin-related peptides may be impaired in pathophysiological states associated with
altered endothelial function such as diabetes mellitus and hypertension.\textsuperscript{5,12,13} In particular, a resistance to the renal actions of IGF-1 has recently been reported in spontaneously hypertensive rats, primarily at the arteriolar level.\textsuperscript{5,12,13} The present study was therefore designed to examine the hypothesis that experimental hypercholesterolemia is associated with an altered coronary vasoactive response to IGF-1, primarily at the level of coronary arterioles.

**Methods**

**Animals**

The study procedures and handling of animals were reviewed and approved by the Mayo Foundation Institutional Animal Care and Use Committee. Juvenile domestic crossbred pigs were randomly placed on either a normal diet or a diet of 2\% cholesterol and 15\% lard by weight (TD 93296, Harlan Teklad) for 10 weeks.\textsuperscript{7,11} At 10 weeks, we determined plasma total cholesterol and low-density lipoprotein levels by applying the technique of Allain et al.,\textsuperscript{14} using a commercial reagent (Roche). The animals were then killed with an intravenous overdose of pentobarbital sodium (30 mg/kg IV, Sleepaway, Fort Dodge Laboratories). After the animals were killed, the hearts were harvested for in vitro analysis.

**In Vitro Analysis of Epicardial Arteries**

In vitro determination of epicardial reactivity was performed as we previously described.\textsuperscript{3,8} Viability of the vessels was confirmed by a contractile response to 20 mmol/L KCl at baseline and at 2, 4, and 6 g, each time after the potassium had been washed out. At 6 g, all vessels were then exposed to substance P (10-6 mol/L, Sigma), an endothelium-dependent vasodilator, to verify the functional integrity of the vascular endothelium. All chambers were then washed out with the control solution.

After an equilibration period of 30 minutes, epicardial arteries harvested from normal or hypercholesterolemic animals were contracted with endothelin-1 (7 mol/L, Sigma), and after equilibration for 20 minutes, they were relaxed with cumulative concentrations of either 10-12 to 10-7 mol/L regular insulin (Eli Lilly) or 10-12 to 10-7 mol/L IGF-1 (Pharmaucia Upjohn). Stock solutions of each agent were freshly prepared for each experiment. 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. At the end of all experiments, papaverine (10-4 mol/L, Sigma) was added to verify that the vessels maintained vasodilating capacity.

A control group was established to verify that the loss of tension was due to insulin and IGF-1 and not due to the loss of the vasoconstrictor effect of endothelin-1: distilled water was added to control vessels (n=5) at the same time insulin and IGF-1 were added in the experimental group. In the control group, no significant vasorelaxation was evident.

To determine the endothelium-dependent vasorelaxation properties of epicardial arteries harvested from normal and hypercholesterolemic pigs, epicardial arteries (n=7 for each group) were first contracted with 10-7 mol/L endothelin-1 and then relaxed with 10-11 to 10-6 mol/L bradykinin (Sigma).

**In Vitro Analysis of Coronary Arterioles**

In vitro determination of coronary arteriolar reactivity was performed with the use of a method we previously described.\textsuperscript{7} All vessels were first contracted with 60 mmol/L KCl and then relaxed with 10-6 mol/L bradykinin to determine the integrity of the vessel, as described above for epicardial vessels. Responses of the pressurized arteries (ie, not perfused with flow) were measured at a transmural pressure of 50 mm Hg.

To determine the effects of insulin and IGF-1 on coronary arterioles, vessels harvested from normal or hypercholesterolemic animals were first contracted with 10-8 mol/L endothelin-1 and after equilibration relaxed with cumulative concentrations of either 10-12 to 10-7 mol/L insulin or 10-12 to 10-7 mol/L IGF-1. To determine possible mechanisms for the arteriolar vasorelaxation effects of insulin and IGF-1, in particular mechanisms involving the activation of nitric oxide and potassium channels, vessels harvested from additional control animals were exposed to either the nitric oxide synthase inhibitor 10-4 mol/L N\textsuperscript{\textsuperscript{-}}monomethyl-L-arginine (L-NMMA) (Sigma) or the potassium channel inhibitor 10-4 mol/L tetraethylammonium (TEA) (Sigma) 20 to 30 minutes before the addition of endothelin-1. The experiments with TEA were also performed in arterioles harvested from hypercholesterolemic pigs and exposed to cumulative concentrations of IGF-1, as were experiments with L-NMMA in arterioles harvested from hypercholesterolemic pigs and exposed to insulin. At the end of all experiments, papaverine (10-4 mol/L) was added to verify that the rings maintained vasodilating capacity.

To determine the endothelium-dependent vasorelaxation properties of arterioles harvested from normal and hypercholesterolemic pigs, arterioles (n=6 for each group) were first contracted with 10-8 mol/L endothelin-1 and then relaxed with 10-11 to 10-6 mol/L bradykinin.

**Arteriolar IGF-1 and IGF Binding Protein 2 Immunoreactivity**

IGF binding protein (IGFBP) 2 binds IGF and inhibits its activities.\textsuperscript{1,15,16} Prior studies have demonstrated that IGFBP-2 may have a central role in vascular smooth muscle function.\textsuperscript{13,16} To assess whether experimental hypercholesterolemia altered IGF-1 or IGFBP-2 immunoreactivity in porcine coronary arterioles, arterioles from control (n=5) and hypercholesterolemic animals (n=5) were dissected and embedded in paraffin. IGF-1 and IGFBP-2 immunohistochemistry studies were performed with an antibody for IGF-1 and IGFBP-2 (UBI), respectively. In brief, slides were deparaffinized, and nonspecific binding sites were blocked by applying 5% normal goat serum (Dako) to slides for 10 minutes. The primary antibody for IGF-1 or IGFBP-2 (diluted 1:10 and 1:200, respectively) in 1% normal goat serum serum was applied and incubated overnight at 4°C. The following day the primary antibody was rinsed off and floated, and the biotinylated secondary antiserum cocktail goat anti-rabbit (Dako) diluted at 1:400 and incubated on the slides for 30 minutes. After the slides were rinsed, streptavidin-horseradish peroxidase (Dako) diluted at 1:500 was applied. High-powered photomicrographs were obtained from each specimen.

Two investigators, unaware of the pig’s diet, analyzed the intensity and extent of immunoreactivity from 5 random photomicrographs from each animal. The photomicrographs were each graded on a scale from 1 through 4, with 1 being scant, weak staining and 4 extensive, strong staining, as previously described.\textsuperscript{9} For each animal, an average score was calculated. An average score was then calculated for each group.

**Data Analysis**

Results are presented as mean±SEM. For epicardial vessels, the contraction attained with endothelin-1 for each vessel at baseline was considered baseline (0% relaxation). Subsequent measurements of coronary artery relaxation are expressed as percent reduction in contraction (the maximal relaxation attained, with papaverine being 100% relaxation). In all experiments, n refers to the number of vessels. For epicardial vessels, experiments were performed in parallel to preclude a situation whereby all vessels in 1 experiment were harvested from only 1 animal (on average, each experiment was performed with arterioles from at least 3 to 4 animals). For coronary arterioles, the vessel diameter after contraction with endothelin-1 for each vessel at baseline was considered baseline (0% relaxation). Subsequent measurements of coronary artery relaxation are expressed as percent increase in diameter. For each arteriole, the maximal relaxation in the passive state (after papaverine) was considered 100% relaxation.\textsuperscript{7} For epicardial vessels, each experiment was performed with arterioles from at least 3 to 4 animals.\textsuperscript{7} For statistical analysis, ANOVA or repeated-measures ANOVA followed by Bonferroni’s t test was used. For the immunohistochemistry analyses, an
unpaired t test was used. A 2-tailed P value of ≤0.05 was considered significant.

Results

Lipid Profile
Plasma total cholesterol levels were significantly higher in animals fed a high-cholesterol diet than those in pigs that were given a normal diet (9.5 ± 1.0 versus 1.9 ± 0.08 mmol/L; P < 0.0001).

Mean Maximal Luminal Diameter of Arterioles in Each Protocol
Mean maximal luminal diameters of vessels in the passive state (ie, after papaverine) in each protocol are depicted in the Table. Mean maximal diameters ranged from 289 ± 19 to 484 ± 18 μm in the different study protocols, with a similar mean maximal arteriolar diameter for control and hypercholesterolemic arterioles. As previously reported, there was no correlation between arteriolar diameter and the response to vasoactive agents within this range of diameters (data not shown). The contraction attained with endothelin-1, expressed as the absolute reduction in diameter in micrometers, was not statistically different among the different protocols.

Mean Maximal Contraction of Epicardial Arteries to Endothelin-1 in Each Protocol
The mean contraction of epicardial arteries to endothelin-1 in the different protocols before exposure to insulin or IGF-1 is also presented in the Table. There was no statistically significant difference among the 4 groups.

Figure 1. Epicardial vasorelaxation response to bradykinin after contraction with endothelin-1 in control animals and in animals on a high-cholesterol diet. *P < 0.05.

Endothelial Function
Endothelium-dependent epicardial vasorelaxation to bradykinin was significantly attenuated in hypercholesterolemic pigs, with both a greater EC50 and an attenuated maximal response to bradykinin (Figure 1). Arterioles harvested from hypercholesterolemic pigs had an attenuated vasorelaxing response to bradykinin (Figure 2).

Epicardial Response to Insulin and IGF-1 in Normal and Hypercholesterolemic Animals
As shown in the left panel of Figure 3, insulin caused a similar vasorelaxation response in epicardial vessels harvested from normal and hypercholesterolemic pigs (maximal relaxation of 28 ± 4% and 37 ± 9%, respectively; P = 0.80). IGF-1 also caused epicardial vasorelaxation in normal and hypercholesterolemic pigs (maximal relaxation of 25 ± 3% and 37 ± 5%, respectively; P = 0.12; Figure 3, right). There was no difference in the vasorelaxation response attained with both agents.

Arteriolar Response to Insulin and IGF-1 in Normal and Hypercholesterolemic Animals
In control animals, the vasorelaxation response to IGF-1 was significantly greater than insulin (maximal relaxation of...
79±6% and 43±6%, respectively; \( P=0.01 \)). As shown in the left panel of Figure 4, insulin caused a similar vasorelaxation response in arterioles harvested from normal and hypercholesterolemic pigs (maximal relaxation of 43±6% and 53±7%, respectively; \( P=0.99 \)). Moreover, in hypercholesterolemic arterioles (Figure 4, right), the vasorelaxation response to IGF-1 was significantly attenuated compared with control (maximal relaxation of 79±6% and 42±8%, respectively; \( P=0.01 \)).

**Nitric Oxide Pathway and Arteriolar Vasorelaxation to Insulin and IGF-1**

As shown in the top panel of Figure 5, the incubation of control arterioles with \( 10^{-4} \) mol/L L-NMMA attenuated the vasorelaxation response to insulin at concentrations \( 10^{-10.5} \) mol/L (maximal relaxation of 14±6% and 43±6% with and without L-NMMA, respectively; \( P<0.001 \)). A similar response occurred in hypercholesterolemia (Figure 6; maximal relaxation of 11±8% and 49±7% with and without L-NMMA, respectively; \( P<0.001 \)). The response to IGF-1 at concentrations \( 10^{-12} \) mol/L was also attenuated with L-NMMA (maximal relaxation of 15±5% and 79±6% with and without L-NMMA, respectively; \( P<0.001 \); Figure 5, bottom).

**Potassium Channels and Arteriolar Vasorelaxation to Insulin and IGF-1**

As shown in Figure 5, the incubation of control arterioles with \( 10^{-2} \) mol/L TEA did not attenuate the vasorelaxation response to insulin (maximal relaxation of 52±5% and 43±6% with and without TEA, respectively; \( P=0.93 \)) but significantly attenuated the response to IGF-1 at concentrations \( 10^{-12} \) mol/L (maximal relaxation of 58±7% and 79±6% with and without TEA, respectively; \( P=0.01 \)). The vasorelaxation of arterioles harvested from hypercholesterolemic pigs and incubated with TEA before the exposure to IGF-1 was similar to that of the arterioles that were not incubated with TEA (\( P=0.26 \); Figure 7). Thus, whereas TEA attenuated the vasorelaxation to IGF-1 in the arterioles of control animals, it did not in hypercholesterolemic animals.

**IGFBP-2 and IGF-1 Immunoreactivity**

IGFBP-2 immunoreactivity was significantly more pronounced among arterioles from hypercholesterolemic pigs (Figure 8). Immunoreactivity was most prominent in the medial layer. The mean staining scores for control and hypercholesterolemic animals were 1.1±0.3 and 3.1±0.4, respectively (\( P<0.05 \)). IGF-1 was scantly detected by immunohistochemistry in the endothelial layer and media of arterioles from control animals but was detected in the media of arterioles from hypercholesterolemic pigs (Figure 8).
arterioles harvested from hypercholesterolemic pigs and was more prominent than the control group (3.5 ± 0.3 versus 2.0 ± 0.4; *P*, 0.05; Figure 8).

Discussion

Main Findings

The principal findings of the present study are that both insulin and IGF-1 caused a concentration-dependent vasorelaxing effect of porcine coronary arterioles in vitro, although IGF-1 was more effective. Experimental hypercholesterolemia caused porcine coronary arteriolar endothelial dysfunction in vitro, yet it resulted in an attenuated porcine coronary arteriolar vasorelaxing response only to IGF-1 in conjunction with increased IGF-1 and IGFBP-2 arteriolar expression. Thus, experimental hypercholesterolemia and related pathophysiological states may be associated with a selective resistance to the coronary arteriolar vasorelaxing effects of IGF-1.

Vasorelaxing Effects of Insulin and IGF-1

Studies in animal models and in humans have demonstrated the vasoactive effects of both insulin and IGF-1 (see Reference 4). These prior studies have also highlighted the diverse vasoactive properties of the 2 peptides among different species and even among the different vascular beds of the same organ of a single species. Moreover, although the 2 peptides often have the same vasoactive effect in a particular bed, at times these effects may be different and may be differentially affected by pathophysiological states. The results of the present study extend these prior studies, demonstrating that both insulin and IGF-1 are coronary vasorelaxants of porcine coronary arteries and arterioles in vitro. Furthermore, in contrast to the similar effects exerted by the 2 peptides on epicardial vessels, IGF-1 was more effective in causing vasorelaxation of porcine coronary arterioles.

The peptides belonging to the IGF family interact with specific receptors designated as type 1 and 2 IGF receptors, as well as with the insulin receptor. The type 1 IGF receptor binds IGF-1 with high affinity and insulin with low affinity, whereas the insulin receptor binds insulin with high affinity and IGF-1 with lower affinity. The similar concentration-dependent vasorelaxing effect of insulin and IGF-1 on epicardial vessels therefore suggests that each of the 2 peptides

Figure 5. Coronary arteriolar vasorelaxation response to insulin (A) or IGF-1 (B) after incubation with either vehicle, TEA, or L-NMMA and contraction with endothelin-1 in control animals. *P*<0.05.

Figure 6. Coronary arteriolar vasorelaxation response to insulin after incubation with either vehicle or L-NMMA and contraction with endothelin-1 in animals on a high-cholesterol diet. *P*<0.05.

Figure 7. Coronary arteriolar vasorelaxation to IGF-1 in animals on a high-cholesterol diet with or without incubation with TEA.
exerts its actions through its own receptor and that the postreceptor mechanisms are similar. However, IGF-1 may exert its effects through interactions with the IGFBPs rather than with the IGF receptor.17,18

The greater efficacy of IGF-1 in arterioles compared with insulin even at supraphysiological concentrations of insulin indicates that in porcine coronary arterioles the vasodilator effects of IGF-1 may be mediated through IGF receptors or that IGF receptors are more prevalent at that environment. In addition, the IGFBPs may enhance the IGF effect specifically. For example, IGF-1 may cause the release of IGFBPs with direct effects on the vascular bed through a receptor-independent mechanism.17,18 These IGFBPs, in turn, may inhibit or amplify the effects of IGF-1, thus affecting vaso-reactivity. Given that specific inhibitors of the respective receptors are not available, we were unable to determine whether the mechanism is receptor mediated.

Mechanisms for Arteriolar Vasorelaxation

In prior studies, the vasoactive effects of both insulin and IGF-1 were attenuated or abolished by inhibiting the production of endogenous nitric oxide with L-NMMA (see Reference 4), yet these effects have been reported by others to be endothelium independent (see Reference 4). In our previous study, L-NMMA attenuated the epicardial vasorelaxing effects of both peptides in vessels with and without endothelium.4 In our present study, we found that L-NMMA attenuated the vasorelaxing effects of both insulin and IGF-1 on porcine arterioles. This may indicate that the arteriolar response to insulin and IGF-1 is dependent, at least partially, on the nitric oxide pathway. However, L-NMMA potentially has a direct effect on other pathways such as potassium channels.4,19 This is supported by the fact that L-NMMA also attenuated the arteriolar vasorelaxation to insulin in experimental hypercholesterolemia, a state associated with reduced nitric oxide activity.9

The vasoactive actions of insulin and IGF-1 may also be related to the activation of potassium channels.4 In our previous study, TEA, the nonselective inhibitor of potassium channels, attenuated the epicardial vasorelaxing effect of both insulin and IGF-1.4 In the present study, TEA attenuated the arteriolar vasorelaxing effect of IGF-1 but not insulin, further supporting the hypothesis that insulin and IGF-1 exert their coronary arteriolar vasoactive effects through different mechanisms.

The response to IGF-1 was attenuated in arterioles harvested from hypercholesterolemic pigs but not in epicardial vessels. In contrast, both epicardial vessels and arterioles from these pigs had an impaired in vitro response to the endothelium-dependent vasodilator bradykinin, indicating epicardial endothelial dysfunction. These findings demonstrate that the vasoactive effects of the IGF peptides may be exerted even in the face of an impaired endothelial nitric oxide pathway.

The exact mechanism for the coronary arteriolar effects of insulin and IGF-1 was not specifically examined in the...
Resistance to IGF-1 in Hypercholesterolemia

The selective attenuation of the vasorelaxing effects of IGF-1 on coronary arterioles in hypercholesterolemia may involve 1 or more mechanisms. In our study, TEA attenuated the arteriolar vasorelaxation response to IGF-1 in control animals but not in hypercholesterolemic animals, indicating that the high-cholesterol diet had affected the interaction between IGF-1 and potassium channels at the arteriolar level. Reduced activity of potassium channels in experimental hypercholesterolemia could explain the attenuated IGF-1 activity. Of interest, Najibi et al reported intact potassium channel activity in hypercholesterolemic rabbits, resulting in a preserved response to acetylcholine in the presence of reduced nitric oxide activity. It is therefore more likely that the hypercholesterolemic state altered the ability of IGF-1 to activate arteriolar potassium channels through a receptor-related or prereceptor mechanism.

Grant et al have reported that concomitant with the increase in local concentrations of IGF-1 and its receptor in atherosclerotic coronary arteries, there is also an increase in the IGFBPs. Of interest, in the present study we also detected higher levels of both IGF and IGFBP-2 in hypercholesterolemic pigs. The IGFBPs modulate the systemic and local actions of IGF-1 through their interaction with the IGF ligand and its availability to the IGF receptor, sometimes enhancing the effects of IGF-1 and sometimes attenuating it. This pathway is further modulated by proteases of the IGFBPs. Porcine vascular smooth muscle cells secrete a serine protease for IGFBP-2. Experimental hypercholesterolemia in the pig possibly results in a net inhibitory effect on the actions of IGF-1 at the level of the coronary arterioles through local changes in the activity or concentration of the receptors, IGFBPs, or IGFBP proteases. Because we assessed the expression of only 1 IGFBP in the present study (ie, IGFBP-2) and did not evaluate the other members of the axis, a more complex explanation for the arteriolar IGF resistance may emerge.

Conclusion

This study shows for the first time that a pathophysiological state can be associated with a selective resistance to the coronary vasoactive effects of IGF-1, which is independent of resistance to the vasoactive effects of insulin. Prior studies have implied that changes in the IGF axis may lead to the structural changes associated with atherosclerosis and related diseases. The results of this study complement these studies, demonstrating that the IGF axis may also be involved in the functional changes associated with these conditions.

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References

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