Role of 12-lipoxygenase and oxidant stress in hyperglycaemia-induced acceleration of atherosclerosis in a diabetic pig model

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Abstract

Aims/hypothesis. We previously showed that vascular smooth muscle cells and endothelial cells cultured under high glucose conditions produced more 12(S)-hydroxyeicosatetraenoic acid (12-HETE), the 12-lipoxygenase (12-LO) product of arachidonate metabolism, relative to normal glucose. Because the lipoxygenase (LO) pathway has been associated with oxidant stress and the pathogenesis of atherosclerosis, we now examined 12-LO activation in vivo in a pig model of diabetes-induced accelerated atherosclerosis which displays human characteristics.

Methods. The animal model was developed in pigs who were fed a normal or high fat diet and given streptozotocin injections to produce normolipemic-normoglycaemic (NLNG), normolipemic-hyperglycaemic (NLHG), hyperlipemic-normoglycaemic (HLNG) and hyperlipemic-hyperglycaemic (HLHG) pigs. Tissue samples were obtained from key arterial beds to examine 12-LO expression at 20 weeks after the pigs began their diet.

Results. All HG pigs maintained threefold higher serum glucose concentrations. The HL groups developed atherosclerosis but diabetic HLHG pigs showed markedly accelerated atherosclerosis (twofold) relative to non-diabetic HLNG pigs. Immunostaining showed progressive increases in 12-LO in arteries in the order NLNG, NLHG, HLNG and HLHG. Leukocyte-type 12-LO protein (immuno-blotting) as well as mRNA expression (by competitive PCR) in abdominal and coronary arteries were significantly greater in HLHG pigs than in all the other three groups. Furthermore, increased oxidant stress was observed in monocytes from NLHG and HLNG pigs, and greatly potentiated in HLHG pigs.

Conclusions/interpretation. These results are consistent with the hypothesis that 12-LO activation plays a key role in accelerated atherosclerosis due to diabetes and hyperlipemia. [Diabetologia (2002) 45: 125–133]

Keywords Diabetes, atherosclerosis, lipoxygenase, oxidant stress, gene expression, lipid metabolism, hyperlipidemia, hyperglycaemia.

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Abbreviations: HG, High glucose; NG, normal glucose; NLNG, normolipemic-normoglycaemic; NLHG, normolipemic-hyperglycaemic; HLNG, hyperlipemic-normoglycaemic; HLHG, hyperlipemic-hyperglycaemic; 12-HETE, 12(S)-hydroxyeicosatetraenoic acid; 12-LO, 12-lipoxygenase; VSMC, vascular smooth muscle cells; PCR, polymerase chain reaction; RIA, radioimmunoassay; PDGF, platelet-derived growth factor; PMA, phorbol-12-myristate-13-acetate

Diabetes is associated with significantly accelerated rates of atherosclerotic and hypertensive cardiovascular diseases. The cellular and molecular mechanisms are still not fully understood, mainly due to the lack of animal models that mirror the changes in human beings. Atherosclerosis involves several cell types including monocytes, vascular smooth muscle cells and endothelial cells [1]. Increased oxidant stress in these cell types has been suggested to be one key factor in atherosclerosis [2].

We have recently shown that pig vascular smooth muscle cells (VSMC) cultured under high glucose (HG, 25 mmol/l) conditions to mimic the diabetic

state proliferated faster than those cultured under normal glucose (NG) conditions [3]. Furthermore, they also had increased activation and expression of the 12-lipoxygenase pathway of arachidonate metabolism [3, 4]. Growth factors such as angiotensin II and platelet-derived growth factor-BB (PDGF), as well as inflammatory cytokines such as interleukins-1, and -8 were potent inducers of 12-LO activity and expression in the VSMC [4–8]. Furthermore, inhibitors of 12-LO could block the growth-promoting effects of angiotensin II and high glucose as well as the chemotactic effects of PDGF in VSMC [5, 6, 8]. 12-LO products, such as 12-(S)-hydroxyeicosatetraenoic acid (12-HETE), have chemotactic and hypertrophic effects and also increase production of the matrix protein, fibronectin in VSMC [5, 8]. We also observed significantly more binding of monocytes to endothelial cells cultured under HG conditions [9]. Furthermore, these endothelial cells cultured under HG conditions produced larger amounts of the 12-LO product, 12-HETE, than those cultured under normal glucose conditions, and 12-HETE could directly induce monocyte binding [9, 10]. These results suggest that increased 12-LO activation could play a key role in the pathogenesis of accelerated vascular disease in diabetes. However, the *in vivo* relevance of these findings in diabetic atherosclerosis is not clear. Our objective was therefore to examine the role of 12-LO activation in an animal model of diabetes-induced accelerated atherosclerosis that shares several features with human beings.

We earlier established a diet-induced model of atherosclerosis in pigs [11]. In this study, we established a pig model of diabetes-induced accelerated atherosclerosis which shares several features with the human disease [12]. This model reflects the conditions in human Type II (non-insulin-dependent) diabetes mellitus, particularly increased triglycerides and vascular-wall calcification. In this model, pigs were fed a normal chow diet or a high lipid diet to produce normolipemic (NL) or hyperlipemic (HL) pigs respectively. Diabetes (high glucose, HG) was induced by injections of streptozotocin. This resulted in four groups of pigs: normolipemic-normoglycaemic (NLNG), normolipemic-hyperglycaemic (NLHG), hyperlipemic-normoglycaemic (HLNG), and hyperlipemic-hyperglycaemic (HLHG). All HL pigs developed atherosclerosis but diabetic HL pigs (HLHG) showed markedly accelerated atherosclerosis (two-fold) compared with non-diabetic pigs (HLNG). The lesions were also more severe in all the arterial beds examined. Occlusive (> 90%) calcified coronary artery lesions developed by 20 weeks in HLHG pigs, but were not seen in HLNG pigs even as late as 48 weeks.

In this study, we examined whether 12-LO activation correlates with the atherosclerotic-lesion formation in this pig model. In addition, because increased

lipid peroxidation and oxidant stress are thought to be involved in diabetic complications [13, 14], and also in the formation and action of LO products [15, 16], we examined whether the diabetic animals have increased oxidant stress by examining whether monocytes from the diabetic and hyperlipemic pigs increased respiratory burst as measured by amounts of oxidant hydrogen peroxide.

Materials and methods

Pig model of diabetes-induced atherosclerosis. Our investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Studies are also in accordance with the protocol approved by the Committee for Use of Animals in Education and Research at the Medical College of Georgia, and complied with those approved by the American Veterinary Medical Association Panel on Euthanasia. Male Yorkshire pigs (initially 8–10 weeks of age, 15–20 kg), were fed a normal chow diet or a diet containing 1.5% cholesterol and 15% lard to produce normolipemic (NL) or hyperlipemic (HL) pigs respectively. Diabetes was induced by streptozotocin (STZ) injection (50 mg/kg per day for three consecutive days) through the ear vein. The HL diet was initiated 5 days after the last STZ injection. This resulted in four groups of pigs: NLNG, NLHG, HLNG and HLHG. Urine samples were obtained by bladder puncture before pigs were killed 20 weeks after the initiation of the HL diet. Plasma glucose and lipid measurements were carried out as described previously [12]. Arterial samples were obtained when the pigs were killed from exactly the same site in each animal, rinsed in sterile PBS, and rapidly frozen in liquid nitrogen. Other samples from immediately adjacent sites were paraffin embedded for histology and immunohistochemistry.

Immunohistochemical detection of pig leukocyte 12-LO in arterial sections. The immunohistochemical methods were carried out using a modification [17] of the technique of Larsson [18]. We used a specific antibody to the pig leukocyte 12-LO raised to a peptide derived from the sequence of the pig leukocyte 12-LO [6, 17, 19].

Immunoblotting to detect 12-LO protein in tissue samples. Frozen tissues were thawed and cut into small pieces in the presence of ice-cold lysis buffer containing 150 mmol/l, NaCl, 20 mmol/l, HEPES, 1% Triton, 0.1% SDS, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l, sodium metabisulfite, leupeptin, 5 µg/ml aprotinin, 2 mmol/l, PMSF, pH 7.5. The tissues were then homogenized on ice using a polytron (3 × 30 s at 1 min intervals). The homogenate was vortexed, kept on ice for 20 min and centrifuged at 4°C at 3000 rpm for 10 min. The supernatant cytosolic proteins were stored in aliquots at –70°C before immunoblotting. Equal amounts of protein (20–30 µg) from each group were subjected to SDS-PAGE electrophoresis on 8% gels. They were then immunoblotted using a specific monoclonal antibody to the pig leukocyte 12-LO [6, 7]. Detection was by the Tropix chemiluminescence detection system [6, 7]. Band intensities were quantitated by computerized densitometry.

12-LO mRNA expression in pig tissues by competitive PCR. 12-LO has been cloned from pig leukocytes and has been detected in other porcine tissues [19]. We identified a leukocyte-type 12-

Table 1. Glucose, cholesterol and triglyceride levels in the four groups of pigs

Group	Glucose (mmol/l)	Cholesterol (mmol/l)	Triglycerides (mmol/l)
NLNG	5.9 ± 0.6	1.76 ± 0.23	0.22 ± 0.02
NLHG	17.1 ± 3.4 ^a	1.86 ± 0.47	0.25 ± 0.03
HLNG	5.7 ± 0.8	11.71 ± 3.23 ^b	0.27 ± 0.09
HLHG	18.0 ± 2.6 ^a	12.46 ± 3.13 ^b	0.58 ± 0.12 ^c

^a $p < 0.001$ vs the NG groups^b $p < 0.001$ vs the HL groups^c $p < 0.05$ vs all the other groups

LO in pig VSMC [4]. In the current study, we developed a reverse-transcriptase competitive PCR method to examine 12-LO expression in pig arterial segments obtained from the four groups of pig at death. Total RNA was first extracted from the tissues as follows: the artery fragments were homogenized in RNA-STAT 60 reagent (TelTest-B, Friendswood, Tex. USA) using a polytron. Total RNA was extracted using the single step method using RNA-STAT 60 according to manufacturer's instructions. The cDNA was prepared by reverse transcription from each of the samples using 1 µg total RNA, 200 U of reverse transcriptase (MMLV, Gibco-BRL, Gaithersburg, Md., USA) and 50 pmol of random hexamer primer (Perkin Elmer, Foster City, Calif., USA).

Competitive PCR. Pig leukocyte 12-LO competitor DNA was prepared by using a PCR-based mutagenesis procedure to introduce a 40 bp deleted porcine leukocyte 12-LO cDNA as described earlier [20, 21]. Template pig 12-LO cDNA-pUC19 plasmid was a gift from Dr. T. Yoshimoto (Tokushima, Japan). The primers used for preparing the competitor truncated 12-LO cDNA were: sense 5'-TTCAGTGTAGACGTGTCG-GAG (145–165) and antisense 5'-ATGTATGCCGGTGCTG-GCTATATTTAGCTTCTCTCTGCCAGC (451–477 and 495–509). The PCR product was subcloned into the pBlue-script II KS + plasmid. The sequence of the deletion fragment was confirmed in the positive clones. The competitor plasmid was then purified. The synthesized experimental 12-LO cDNA samples were co-amplified with a constant amount of the competitor in the presence of porcine 12-LO primers (2), α [³²P] dCTP and AmpliTaq Gold polymerase (Perkin Elmer, Foster City, Calif., USA) in a thermocycler (model 9600). The PCR conditions were 94°C for 11 min, 94°C for 30 s, 50°C for 30 s and 72°C for 30 s, for 32 cycles. The PCR products were separated by 5% page. Radioactivity in the gels was quantitated on a Phosphorimager using the Image Quant software (Both from Molecular Dynamics, Sunnyvale, Calif., USA).

Quantitation of urinary 12-HETE by radioimmunoassay (RIA). 10 ml urine samples from the pigs were spun down to remove particulates. The pH was adjusted to 3.0 with 1 mol/l HCl. Urine was then extracted on 3 cc Bond-Elut columns (Varian, Harbor City, Calif., USA) as follows: columns were primed with 10 ml methanol, 10 ml water, then urine was loaded. Columns were then sequentially washed with 2 ml 30% ethanol, 3 ml water, 2 ml pet ether. The 12-HETE was eluted with 4 ml ethyl acetate. The eluates were dried, reconstituted in ethanol, filtered and subjected to reverse phase HPLC to measure 12-HETE as described [4, 22]. For the RIA, 0.5 ml aliquots of the post HPLC fractions were dried down, reconstituted in assay buffer and then assayed by RIA as described [4, 22] using a specific antibody from Perseptive Diagnostics (Cambridge, Mass., USA).

Quantitation of hydrogen peroxide produced by swine monocytes. Peripheral blood monocytes were isolated from heparin-

ised pig blood taken by transcutaneous vena caval puncture or by jugular cannulization. Monocytes of up to 98% purity and numbering $200\text{--}400 \cdot 10^6$ [23, 24] were obtained from 200 to 400 ml blood by counterflow centrifugation in a Beckman elutriator rotor using methods described previously [25]. Monocytes were cultured at high density on plastic dishes for 90 min, washed to remove dead cells and any contaminating lymphocytes. Cells were then treated for 60 min with 10 ng/ml of the phorbol ester, PMA, and hydrogen peroxide concentrations measured as described [26].

Data analyses. Values from multiple experiments were expressed as means ± SEM. ANOVA with Dunnett's or Tukey Kramer post tests was used to compare data from the four groups of pigs. Prism Software (GraphPad Prism, San Diego, Calif., USA) was used. Western blots were quantitated densitometrically and competitive PCR data on a Phosphorimager using the Image Quant software (NIH). A p value of less than 0.05 was considered to be statistically significant. A semiquantitative score of 0–4+ was used to assess 12-LO immunohistochemical staining in aortic sections.

Results

Diabetes and hyperlipemia result in markedly accelerated atherosclerosis. Chronic streptozotocin-induced diabetes was produced and maintained in pigs for periods up to 48 weeks [12]. Based on quantitative immunohistochemistry, this resulted in a ninefold loss of pancreatic beta cells in pigs injected with STZ compared with normal pigs (221 ± 86 vs 23 ± 4 beta cells/standardized field) which was maintained throughout this period. Five days after the last STZ injection, half the pigs were placed on the high lipid diets to produce four groups of pigs: NLNG, NLHG, HLNG), and HLHG [12].

Diabetic pigs (HG) consistently showed a threefold to fourfold increase in serum glucose, regardless of lipemia status (Table 1). HG alone had little impact on plasma lipid profile and cholesterol levels, whereas both groups fed high fat chow (HLNG and HLHG) had markedly higher cholesterol values ($p < 0.05$, Table 1). However, even though HL alone did not alter triglyceride values, values of the latter were higher only in HLHG pigs ($p < 0.05$ vs other three groups) (Table 1). Only HL pigs developed atherosclerosis but the percentage of lesioned aortic-surface area was twofold greater in the diabetic HLHG group ($47.3 \pm 9.5\%$) than in non-diabetic HLNG

Fig. 1. Representative digital images of right coronary arteries from HLHG and HLNG pigs at 20 weeks of treatment. **A** A right coronary artery from HLHG pigs showing 96% occlusion. Plaque shows humanoid characteristics, including eccentric lumen (L), large necrotic lipid core (N), intra-plaque haemorrhage (H) and calcification (solid arrow), and extensive medial thinning and degeneration at base of plaque (open arrows). Compare extent of stenosis and lesion severity with lesion in **(B)**. Haematoxylin and Eosin stain $\times 18$. **B** – as in **(A)** but from a HLNG pig. A fatty streak lesion (arrow) is visible at one side of the lumen (L), and a more advanced plaque with organizing fibrous cap (F) and small lipid core (C) is approximately 25% stenotic. No haemorrhage or calcification is present. Compare with the diabetic lesion in **(A)**. Haematoxylin and Eosin stain $\times 18$

($24 \pm 4.4\%$) even as early as 12 weeks [12]. The lesions were also more severe in all the arterial beds examined. Occlusive ($> 90\%$) calcified coronary artery lesions developed by 20 weeks in HLHG (Fig. 1A), but were never seen in HLNG (Fig. 1B). Mean per-

centage stenosis in HLHG was $86 \pm 10\%$ compared with $48 \pm 8\%$ in HLNG pigs. Mean serum cholesterol levels were not significantly different between these two groups of pigs (Table 1) [12].

Immunohistochemical detection of porcine leukocyte-type 12-LO in paraffin-embedded sections obtained from the abdominal aortas of the four groups of swine.

We first examined the level of expression of leukocyte-type 12-LO in paraffin-embedded sections of abdominal aortic tissue specimens obtained from exactly the same site from each of the four pig groups 20 weeks after initiation of the HL diet when lesions were well established. Figure 2 shows the results of immunohistochemical staining using an antibody to the porcine leukocyte-type 12-LO [17]. Very little staining for 12-LO was seen in the smooth muscle cells and other cells in the sections obtained from NLNG pigs. However, there was distinct staining in the diabetic NLHG sections which progressively increased in HLNG to HLHG sections. The sections from the HLHG pigs showed intense staining in the smooth muscle cells and also in monocytes and macrophages, including the lipid-laden cells. Semiquantitative scoring of the staining on a scale of 0–4+ on two sets of tissue samples shows that HG alone increased staining by 1.5-fold, HL alone by 2.1-fold and HLHG combination by 4.6-fold relative to NLNG. These immunostaining results indicate that abdominal aortic sections from HL and HG pigs increased 12-LO expression, while the HLHG animals show markedly increased 12-LO expression relative to the other groups, particularly in the VSMC.

12-LO protein expression by Western blots in tissue specimens obtained from the same area of the abdominal aorta of the four pig groups. We examined the levels of leukocyte 12-LO protein expression by Western immunoblotting in the abdominal aortic tissue specimens from these four groups of pigs. Figure 3 shows a representative immunoblot using a peptide antibody to pig leukocyte 12-LO. Authentic pig leukocyte 12-LO protein was loaded in the first lane and appears as a band with a molecular weight of about 72 kDa. Equal amounts of cytosolic protein extracts from lysates obtained from abdominal aortic tissue from NLNG, NLHG, HLNG and HLHG pigs were loaded in the next four lanes. HG alone slightly, but not significantly, increased 12-LO protein by about 20% (NLHG vs NLNG), while HL alone (HLNG) increased 12-LO expression twofold over NLNG. Furthermore 12-LO expression in the HLHG was the highest of the four groups, being over fourfold greater than NLNG group and twofold greater than the HLNG pigs. A bar graph (Fig. 3) shows quantitation of data from three sets of tissue samples and indicates increase in LO expression ($p < 0.01$) in the HLNG and HLHG groups. Thus

Fig. 2. 12-LO Immunostaining in abdominal aortic sections from the four groups of pigs. Paraffin-embedded sections were used from same region of abdominal aortas which were removed on the death of the pigs 20 weeks after initiation of the diet. A polyclonal antibody raised to a peptide from the pig leukocyte 12-LO was used at 1:400 dilution for the immunostaining as described [17]. Results shown are representative of observations from two sets of samples

12-LO protein expression was markedly higher in the pigs displaying significant atherosclerotic lesions.

12-LO mRNA expression in abdominal and coronary arteries from the four pig groups. We next examined whether 12-LO mRNA expression was also up regulated in the pigs with atherosclerotic lesions. We evaluated tissue specimens from sites in abdominal and coronary arteries which are highly susceptible to lesion formation. Pig leukocyte 12-LO mRNA levels were examined by a specific competitive RT-PCR method. Figure 4 and Figure 5 show autoradiograms of PCR products from abdominal and coronary tissues, respectively, from the four groups of pigs. The upper band is the 12-LO mRNA PCR product while the lower band is the internal control for PCR amplification, a 12-LO competitor which produces a transcript of slightly smaller size than 12-LO mRNA. The bar graphs in Figure 4 and 5 show the measurements obtained by taking the ratio of the intensity of

the 12-LO band to the competitor band in multiple samples obtained from the NLNG and HLHG groups. The 12-LO mRNA was significantly higher in the abdominal and coronary arteries obtained only from the HLHG pigs compared with all other groups.

Because these tissue specimens could also contain areas that are not lesioned, we evaluated 12-LO mRNA expression in lesions alone and compared these with areas adjacent to lesions (non-lesion) in the atherosclerotic pigs, HLNG and HLHG. Lesions were stripped from the vessel at necropsy as described [3]. Of note, the lesions from these animals had significant, nearly sixfold greater 12-LO mRNA expression than the uninvolved non-lesion areas (Fig. 6).

Concentrations of the 12-LO product, 12(S)-HETE, in the urine obtained from the four pig groups. Concentrations of the 12-LO product 12-HETE, have been shown to be higher in urines of Type II diabetic patients relative to normal healthy control subjects [22]. In this study, we examined levels of 12-HETE in the urine of pigs collected by bladder puncture just before they were killed. The 12-HETE levels were quantitated by a specific radioimmunoassay after extraction and HPLC purification as described [4, 22]. Figure 7 shows urinary 12-HETE levels in three pigs for each group. HL and HG alone increased 12-HETE excretion but the HLHG pigs excreted significantly greater amounts of 12-HETE in the urine compared to all the other groups.

Fig. 3. Leukocyte-type 12-LO protein expression in abdominal aortic tissues from normal and atherosclerotic pigs. Artery segments excised from the same areas of abdominal aortas of the four groups of swine were lysed as described in the *Methods* section. Equal amounts of cytosolic protein fractions from these samples were electrophoresed and immunoblotted with a 1:400 dilution of a specific peptide antibody to porcine leukocyte 12-LO. Authentic porcine leukocyte 12-LO is seen in first lane. The bar graph below the figure shows the results of densitometric quantitation from 3 sets of samples (* $p < 0.01$ vs NLNG; ** $p < 0.01$ versus all other groups)

Levels of oxidant stress in these four groups of pigs. The production of the potent oxidant, hydrogen peroxide, after phorbol ester PMA stimulation was measured in monocytes isolated from the peripheral blood of the four groups of animals ($n = 4$ for each group) at 20 weeks to evaluate the effects of respiratory burst and oxidative potential of this cell type. Figure 8 shows that both diabetes NLHG (34 ± 4 $\mu\text{mol/l}/10^6$ cells) and hyperlipemia HLNG (34 ± 2 $\mu\text{mol/l}/10^6$ cells) alone increased hydrogen peroxide production relative to the normolipemic-normoglycaemic NLNG control (20 ± 1 $\mu\text{mol/l}$). Moreover, the HLHG combination resulted in a further three-fold increase or more (128 ± 9 $\mu\text{mol/l}/10^6$ cells) compared to HL and HG alone, suggesting a synergistic effect of these two risk factors on respiratory burst and oxidative stress.

Discussion

The pig model used in this study has several features associated with human diabetic atherosclerosis, including hypertriglyceridaemia and greatly accelerated formation of atherosclerotic plaques [12]. The extent of lesion coverage in the thoracic and abdominal aortas in diabetic pigs was double that seen in non-diabetic pigs, even as early as 12 weeks of treatment, and remained statistically greater up to 48 weeks. Of

Fig. 4. 12-LO mRNA expression in abdominal aortic tissues from normal, diabetic and atherosclerotic swine. Total RNA was prepared from tissue specimens excised from the same sections of abdominal arteries of the four groups of pigs at 20 weeks. Figure shows the representative results of competitive PCR from one set at 20 weeks. The upper band is the 12-LO mRNA PCR product band (333 bp), while the lower is the internal control competitor band (293 bp). Results are depicted as the ratio of phosphorimager counts in the 12-LO band to the competitor band. Analyses of tissues from four pigs for each group indicated a twofold increase in 12-LO mRNA expression in the HLHG group compared with the NLNG ($p < 0.02$ by paired t test)

Fig. 5. 12-LO mRNA expression in coronary artery tissues from normal, diabetic and atherosclerotic pigs. Total RNA prepared from tissue specimens excised from the same sections of coronary arteries of each of the four groups of pigs at 20 weeks after HL diet initiation. Representative RT-competitive PCR results are shown. The upper band is the 12-LO band (333 bp) and the lower is the competitor band (293 bp). Analyses of tissues from four pigs per group indicated an increase in 12-LO mRNA expression in the HLHG group relative to NLNG as seen in the bar graph below (21.2 ± 7.2 -fold over NLNG, $p < 0.01$ by paired t test)

particular interest to the human diabetic condition, is that the pigs, like human beings, develop highly stenotic coronary-artery plaques in the first 2–3 cm from the origin. In the diabetic or hyperlipemic state, pigs

Fig. 6. 12-LO mRNA levels in lesion compared with non-lesion areas of abdominal aorta by RT-competitive PCR. Abdominal aortic lesions were stripped from involved areas of HLNG and HLHG animals and non-lesion areas from the same pigs were also excised. Total RNA was prepared and subjected to RT-competitive PCR for quantitation of 12-LO mRNA as described in the *Methods* section. Non-lesion data shown ($n = 8$) were mRNA ratios from 5 HLNG and 3 HLHG pigs. Lesion data ($n = 4$) were mRNA ratios from 2 HLNG and 2 HLHG pigs. The analyses were performed on 12-LO to competitor ratios obtained from individual samples. Values are expressed as means $\pm$ SEM of 4–8 observations, * $p < 0.001$

developed highly stenotic lesions which were twice as occlusive as those seen in hyperlipemia alone by 20 weeks of treatment ($86 \pm 10\%$ vs. $48 \pm 8\%$). Not only was atherosclerosis accelerated in all arterial beds examined but plaque morphology closely resembled that seen in human complicated plaques, with necrotic lipid cores, intra-plaque haemorrhage and extensive calcification, particularly in coronary lesions. Thus, diabetic pigs had several close similarities with human diabetes-accelerated atherosclerosis.

In this study, we examined the *in vivo* expression of the 12-LO enzyme in the vascular tissue of this model. Our earlier *in vitro* studies indicated that the 12-LO pathway of arachidonate metabolism was induced in vascular smooth muscle cells by hyperglycaemia as well as by growth factors and cytokines [4–7]. Furthermore, the 12-LO pathway seemed to mediate the hypertrophic and chemotactic effects of high glucose, angiotensin II and platelet-derived growth factor [5, 6, 8]. The 12-LO products have been implicated in the increased adhesion of monocytes to endothelial cells cultured under high glucose conditions [9, 10]. The LO enzymes and their prod-

Fig. 7. Concentrations of 12-HETE excreted in the urines of normal compared with diabetic and hyperlipemic pigs. Urine was collected from the bladders of pigs when they were killed (20 weeks). Urinary 12-HETE was extracted and quantitated as described in the *Methods* section. Results shown are means $\pm$ SEM of 12-HETE data obtained from the urine of two sets of pigs run in triplicate (* $p < 0.01$ versus NLNG; ** $p < 0.01$ versus NLNG and NLHG and $p < 0.03$ versus HLNG)

Fig. 8. Hydrogen peroxide production by pig monocytes in culture. Peripheral blood monocytes were isolated from animals at 20 weeks ($n = 4$ per group) and cultured as described under *Methods*. Results shown are the levels of phorbol ester PMA-induced hydrogen peroxide production in the monocytes from the four groups. Results are expressed as means $\pm$ SEM of data from monocytes obtained from four pigs for each group (* $p < 0.05$ vs NLNG; ** $p < 0.001$ vs all other groups)

ucts are thought to play a role in the pathology of atherosclerosis and hypertension due to their inflammatory, growth and chemotactic properties [5, 27].

We examined 12-LO expression in arterial sections highly prone to atherosclerosis, such as the lower abdominal aorta and the first 2–3 cm of coronary arteries from the four groups of pigs: NLNG, NLHG, HLNG and HLHG. We found that 12-LO protein expression in the abdominal aortas, as determined by immuno-histochemistry, were increased by HL and HG alone relative to the NLNG group of pigs. Furthermore, there was a clear increase in the HLHG group compared with all the other three groups. Immunoblotting of lysates from these sections showed significant increases in the HLNG and

HLHG groups. The HLHG group again was much greater than all the other groups. Competitive PCR was used to detect pig leukocyte type –12-LO mRNA levels in whole tissues from abdominal as well as coronary arteries. Interestingly, 12-LO mRNA was significantly higher in abdominal and coronary arteries of only the HLHG group compared with all the other three groups, although HL alone had some increase. The reason for this discrepancy with the immunohistochemical data could be due to the fact that the mRNA and protein analyses were performed on heterogeneous/multicellular whole arterial segments which could mask the regulation in key component cells. This is strongly supported by our data showing clear up regulation of 12-LO mRNA in the lesion areas from the atherosclerotic pigs relative to the adjacent non-lesion areas from the same vessels. It could also mean that post-translational regulation mechanisms for 12-LO exist in vivo in the arteries.

We also observed that urinary excretion of 12-HETE was higher in the HG as well as HL groups, and remained greatest in the HLHG group. This 12-HETE could arise from renal or extra-renal sources as suggested by our data in human Type II diabetic patients where we showed significant increases in the urinary secretion of 12-HETE in diabetic patients compared with normal control subjects [22]. Overall, these results indicate a clear up regulation of the 12-LO pathway in this model of diabetes-induced accelerated atherosclerosis.

In addition, in this study, HL and HG alone led to significant increases in the capacity of peripheral blood monocytes to produce the potent oxidant, hydrogen peroxide, and this was greatly potentiated in the HLHG group. This indicated a marked synergistic up regulation of oxidant stress by the HL and HG combination. Oxidant stress is thought to be a pathogenic factor in diabetes-induced complications [13, 14]. This pig model provides evidence of synergistic effects of risk factors such as HG and HL on the development of oxidant stress. This could provide potential mechanisms for the observed synergistic effects of these factors on atherosclerosis. The 12-LO products are obtained through a lipid peroxidation step and increased oxidant stress induced by hyperglycaemia and hyperlipemia could lead to increased 12-LO activation, which in turn could initiate a series of pathological events in the blood vessel. The 12-LO could also be activated by oxidant stress and/or other mechanisms induced by advanced glycation end products (AGEs) [28].

Increased release of growth factors and cytokines in the initial lesions can also synergize to activate 12-LO as observed in vitro. This can be further aggravated by hyperlipidemia, an independent inducer of oxidant stress. Hyperlipidemia can increase amounts of subendothelial LDL in pig models [29, 30], the atherogenicity of LDL being augmented by oxidation [31].

Furthermore, the LO pathway is thought to be involved in LDL oxidation [32, 33]. 15-LO expression was associated with epitopes of oxidized LDL in macrophage-rich areas of atherosclerotic lesions [33]. Protein kinase C is also thought to be involved in the pathological effects of hyperglycaemia and diabetes [34]. Because 12-LO products can activate protein kinase C [5], this pig model could involve novel additive/synergistic effects between these two pathways. Thus a vicious cycle of events involving increased lipids, oxidant stress and LO activation could lead to the increase in atherosclerosis seen in this model.

These results do not fully address the question, whether 12-LO activation in this model can mediate, at least in part, the accelerated lesion development under hyperglycaemic and hyperlipemic conditions. However, our recent in vivo studies with LO inhibition in a rat model of restenosis indicate a role for 12-LO. Thus 12-LO mRNA and protein expression were markedly increased in the neointima of balloon-injured rat-carotid arteries [17]. Furthermore, treatment of these injured rat-carotid arteries with a pharmacological LO inhibitor [17, 35], or a specific molecular LO inhibitor, namely a 12-LO ribozyme [36, 37] could significantly reduce the extent of neointimal thickening in this model of restenosis. The direct in vivo significance of 12-LO activation in atherosclerosis was recently demonstrated by studies showing that cross-breeding of a leukocyte 12-LO knock-out mouse with the atherosclerosis-susceptible apoE-deficient mouse dramatically reduced lesion development in the latter [38]. This data, together with the observations that diabetes can up regulate 12-LO expression, supports the idea that 12-LO activation could play a role in accelerated atherosclerosis seen with the combination of high cholesterol and high glucose. This data fits with the results seen in epidemiologic studies of cardiac risk in people with diabetes. Hence, therapeutic strategies targeting the LO pathway could help treat diabetic vascular diseases.

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